Hematology Education

the education program for the annual congress of the European Hematology Association

ISSN 1872-5503

Education program for the 18th Congress of the European Hematology Association

Stockholm, Sweden
June 13-16, 2013

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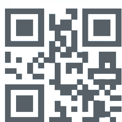

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STOCKHOLM

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Word of Welcome

On behalf of the EHA Board and the Scientific Program Committee we are proud to introduce to you the Education Program of the 18th Congress of EHA. The Education Program covers the whole spectrum of basic, translational and clinical research in the broad range of hematologic disorders. We have assembled a series of presentations from a distinguished cast of internationally recognized individuals. Their presentations form the basis for the manuscripts in this book.

To provide you, the congress delegate, with a program that better addresses your needs we asked the authors to prepare for each manuscript a set of learning goals. These goals you can find in this book as well as in the Final Program Book. We feel confident that these goals will provide more guidance for the selection of sessions of interest for hematologists early and later in their career.

The program will provide the state-of-the-art and most recent developments in the biology, clinical aspects and treatment of benign and neoplastic hematologic disorders, as well as in the field of hemostasis, thrombosis, blood transfusion, transplantation and cell therapy. The impact of recent genetic discoveries through next generation sequencing techniques; the research to identify cancer stem cells and their interaction with the marrow niche; the relevance of neoplastic subclones; the efforts for identifying novel biologic factors with impact on prognosis and the rationale and results of conventional and targeted therapies will be highlighted in the next chapters.

All authors, chairs and reviewers are required to disclose their affiliations with pharmaceutical companies. The overview of disclosures can be found in the back of the book.

We trust that, in addition to the spoken presentations, you find the peer-reviewed manuscripts in the Education Book a valuable source of information and references.

Jorge Sierra
Chair Scientific Program Committee

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The biology of T-cell acute lymphoblastic leukemia

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A B S T R A C T

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of immature thymocytes. Central to the pathogenesis of T-ALL is the acquisition of activating *NOTCH1* mutations and chromosomal rearrangements leading to the aberrant expression of TAL, TLX, HOXA or NKX transcription factor family members. Additional mutations in transcription factors and tyrosine kinase signaling pathways have been identified, and the ability to carry out next-generation sequencing in conjunction with functional screens has further increased our understanding of the genomic aberrations in T-ALL. In our recent exome sequencing study, we identified on average 8 protein altering mutations in pediatric T-ALL, while adult T-ALL cases harbored on average 21 mutations. A number of these mutations are likely drivers of T-ALL initiation, whilst others may either contribute to the evolution of the leukemic clone(s) or may just be passenger mutations. Here we provide an overview of some of the recent discoveries that help us to understand how T-ALL can develop and how this information may aid decisions on which novel treatment strategies could be explored for future therapeutic interventions.

Learning goals

On completion of this activity, participants should know that:
- *NOTCH1* mutations are drivers of T-ALL development;
- additional mutations accumulate, making T-ALL a genetically complex and heterogeneous malignancy;
- JAK kinase inhibitors should be explored for the treatment of T-ALL with JAK1, JAK3 or IL7R mutations or JAK2 fusion genes.

NOTCH1

The NOTCH1 signaling pathway plays a central role in normal T-cell development where it is involved in the specification of T cells and the proliferation and survival of committed T-cell progenitors.[1] NOTCH1 is a transmembrane receptor protein that, when bound by its ligand, undergoes a series of cleavage events that ultimately leads to the γ-secretase-mediated cleavage and release of the intracellular cytoplasmic domain (ICD). This ICD can then travel to the cell nucleus where it acts as a transcription factor to directly control the expression of a number of genes including HES1, MYC, IL7R, NFAT and NFκB subunits.[1] It is now well established that in over 60% of T-ALL cases, NOTCH1 harbors mutations that lead to either ligand independent activation (HD domain mutations), or increase its protein stability (PEST domain mutations), both of which result in the sustained activation of the NOTCH1 signaling pathway.[2] This ectopic activation of the NOTCH1 signaling pathway results in uncontrolled regulation of the downstream genes as listed above as well as the inactivation of tumor suppressor genes including p53 and PTEN. The importance of NOTCH1 in the pathogenesis of T-ALL is further demonstrated by the detection of mutations in genes that affect the NOTCH1 signaling pathway. An important example is the E3 ubiquitin ligase FBXW7. This protein normally acts as a negative regulator of the NOTCH1 pathway by targeting the ICD for ubiquitin-mediated degradation. Loss-of-function mutations in FBXW7 are present in 25% of T-ALL samples, and to some extent overlap with the presence of NOTCH1 mutations.[3,4] In addition to NOTCH1, FBXW7 also targets other important oncogenes for degradation including JUN, Cyclin E, MYC and MYB, indicating that its loss may have broad oncogenic effects.[5-8]

NOTCH1 has previously been described to be important for leukemia initiating cells (LICs) in T-ALL, but a clear link between NOTCH1 mutations and stem cell properties is still to be resolved.[9-13] Recently, two studies have investigated the role of NOTCH1 in leukemic stem cells. In the first, Giambra and colleagues identify NOTCH1 as a repressor of Protein Kinase C θ (PKC-θ) levels through a RUNX3-RUNX1 transcriptional network.[14] Here, low PKC-θ levels correlated with low levels of reactive oxygen species (ROS) in leukemia initiating T-ALL cells. This suggests one of the functions of NOTCH1 mutations in T-ALL is to maintain low ROS levels, thereby maintaining the leukemia stem cell pool. This

finding is in agreement with previous studies demonstrating an important role for NOTCH1 in T-ALL initiating cells, and with RUNX1 to be a tumor suppressor gene in T-ALL.[14] In another study, Chiang and co-workers showed that NOTCH1-induced LIC activity was enriched within immature T-cell populations, but even in that population leukemia initiating cells were uncommon (approx. 1 in 1000 cells).[15] The same study also demonstrated that expression of NOTCH1 gain-of-function alleles abolished long-term HSC activity by promoting T-cell differentiation, thereby adding to the debate on whether NOTCH1 signaling has either a positive or negative effect on HSC cells.

NOTCH1 may also aid cellular transformation by acting at the epigenetic level. In this instance, NOTCH1 activation was shown to antagonize the activity of the polycomb repressive complex 2 (PRC2) leading to the loss of the repressive Lysine27 trimethylation of histone 3 (H3K27me3).[16] Loss of PRC2 activity in T-ALL, by knockdown of its essential component EZH2 increased the *in vivo* tumorigenic potential of the leukemia cells, and also in other models a co-operation between NOTCH1 activation and loss of PRC2 was observed. Moreover, deletions and mutations of EZH2 and SUZ12, both crucial proteins of the PRC2 complex, were found in 25% of T-ALL samples, further demonstrating the tumor suppressor role of PRC2.

Taken together, these studies continue to reveal that activation of NOTCH1 results in the deregulation of several important functions and downstream target genes, some of which are themselves further modulated by mutations or deletions. In this way, T-ALL cells escape the normal control mechanisms that would otherwise protect and guide normal T-cell development.

Chromosomal rearrangements affecting transcription factor expression

Historically, the identification and characterization of chromosomal rearrangements has been extremely important as these chromosomal defects were among the first genomic lesions observed and characterized in T-ALL. The majority of the chromosomal translocations in T-ALL juxtapositions the promoter of T-cell receptor genes on one chromosome with a transcription factor on another chromosome (Table 1). Alternatively, chromosomal deletions and duplications can target transcription factors such as deletions generating the SIL-TAL1 fusion or duplications of MYB, respectively.[17-19] Collectively, these genomic aberrations lead to the overexpression of one particular transcription factor that can then drive an entire transcriptional program distinguished by gene expression profiling.[20-22] Clinically, the presence of these chromosomal aberrations and the associated ectopic expression of these transcription factors is used to classify T-ALL into specific subgroups that can also be associated with specific stages of T-cell differentiation arrest (Figure 1).

Work on the TLX1 and TLX3 transcription factors has revealed a clear link between the overexpression of these proteins and their effect on T-cell differentiation. Dadi and colleagues have shown that TLX proteins interact with ETS1 and suppress TCRα rearrangement.[23] Normally, TCRα rearrangement is a highly regulated process in which the TCRα enhancer (Eα), as well as the transcription factors LEF1, RUNX1 and ETS1, play an important role. The work by Dadi *et al.* demonstrated that TLX1 and TLX3 can bind ETS1 and thereby disturb Eα activity, preventing TCRα (but not TCRβ) rearrangement. In agree-

Table 1. T-cell receptor genes and their involvement in chromosomal aberrations in T-ALL.

	T-cell receptor genes		Partner gene	
Gene	Gene symbol	Chromosome location	Gene symbol	Chromosome location
T-cell receptor α	TRA@	14q11	TLX1	10q24
			TAL1	1p32
			LMO1	11p15
			LMO2	11p13
			NKX2-1	14q13
T-cell receptor β	TRB@	7q34-35	TLX1	10q24
			HOXA@ cluster	7p15
			LYL1	19p13
			TAL2	9q32
			LCK	1p34
			NOTCH1	9q34
			MYB	6q23
			NKX2-1	14q13
T-cell receptor γ	TRG@	7p15	No known chromosomal aberrations	
T-cell receptor δ	TRD@	14q11	TLX1	10q24
			TLX3	5q34
			TAL1	1p32
			LMO1	11p15
			LMO2	11p13
			NKX2-2	20p11

Note 1: TLX3 is implicated in a translocation with the T-cell receptor delta locus but more frequently also rearranged in translocations involving the BCL11B locus.Note 2: A complete list of all rearrangements involving T-cell receptor genes is available from: http://atlasgeneticsoncology.org/Anomalies/TALLID1374.html

ment with this, downregulation of TLX1 or TLX3 in T-ALL cell lines caused restoration of Vα-Jα rearrangement and massive apoptosis.[23] Other studies have also revealed that RUNX1 and LEF1 are frequently mutated or deleted in T-ALL cases.[24,25] Taken together, these data suggest that several mechanisms that interfere with proper TCRα rearrangement are implicated in the differentiation defects of T-ALL cells.

In addition to the well known TLX1, TLX3, TAL1 and HOXA subgroups, new molecular subgroups of T-ALL were recently defined through the use of gene expression profiling. In this way, Meijerink and colleagues have been able to identify two new subgroups based on transcription factor expression.[22] The first is based on NKX2-1 or NKX2-2 expression, and the second is based on MEF2C expression. In the majority of cases within these subgroups, the transcription factors were over-expressed as a consequence of chromosomal rearrangements that had been missed in the past due to the limitations of karyotyping and fluoresence *in situ* hybridization (FISH). The expression of both NKX and MEF2C transcription factors displayed oncogenic co-operation with RAS and MYC in transformation assays in fibroblasts, whereas the expression of MEF2C transcription factor was also shown to upregulate genes found to be expressed in immature T-ALL.[22] This study demonstrates that additional subgroups of T-ALL can be defined using combinations of gene expression profiling and molecular analyses. Furthermore, these additional subgroups will not only continue to be of interest to study at the functional and molecular level, but will also provide potential new prognostic markers or identify potential targets for therapy for those specific subgroups.

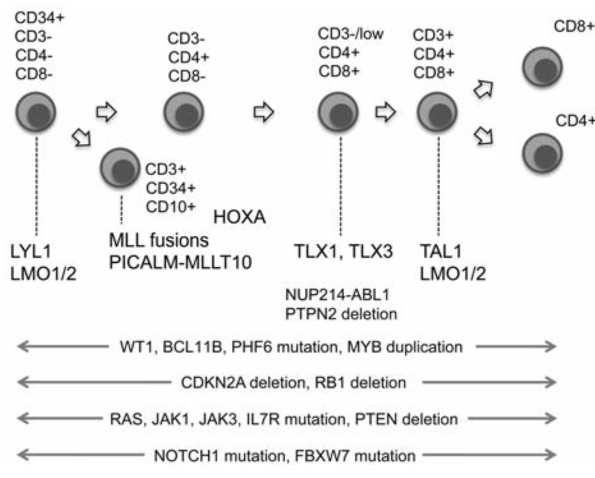

Figure 1. Overview of the major subgroups of T-ALL defined by the ectopic expression of the transcription factors. The transcription factors TLX1, TLX3, LMO1, LMO2, HOXA, TAL1 (including SIL-TAL1), chimeric MLL and PICALM-MLLT10 (CALM-AF10) are major oncogenic drivers of T-ALL. Additional oncogenes and tumor suppressor genes contributing to T-ALL development are also indicated.

Tyrosine kinase and cytokine receptor signaling

In 6% of T-ALL cases, there is expression of an NUP214-ABL1 fusion,[26] which is similar to the BCR-ABL1 fusion found so often in B-ALL but rarely found in T-ALL. In T-ALL, the NUP214-ABL1 fusion gene is the consequence of an unusual rearrangement that is often detectable as an episomal amplification. *In vitro* experiments confirmed that the NUP214-ABL1 fusion protein is an activated tyrosine kinase, but with weaker kinase activity and different substrate specificity compared to BCR-ABL1.[26-28] Nevertheless, both within cell-based models and within mouse models, expression of NUP214-ABL1 could transform hematopoietic cells to cytokine independent growth. In more recent work, we have observed that the NUP214-ABL1 protein is localized at the nuclear membrane where it interacts through the NUP214 moiety

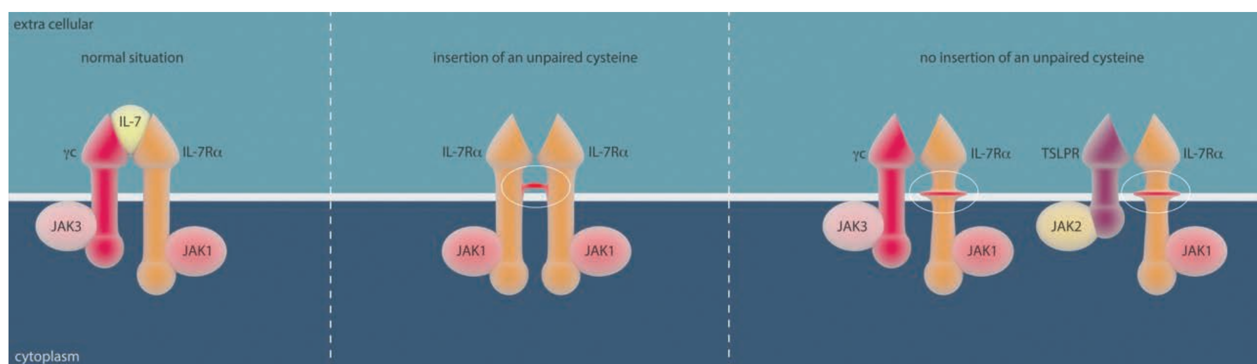

Figure 2. Interleukin-7 receptor and TSLP receptor complexes. The normal interleukin-7 receptor is a heterodimer of the IL7Rα and the common gamma chain (cγ; also named IL2Rγ). JAK1 and JAK3 are the cytosolic tyrosine kinases associated with this receptor. In the mutant cases, the IL7Rα is mutated so that additional amino acids are inserted close to or within its transmembrane region. In some cases, a cysteine residue can be inserted, which could lead to the formation of disulfide bridges between two adjacent IL7Rα proteins. In other cases, no such cysteine is present, but the insertion of additional amino acids is believed to change the conformation of the proteins allowing them to form heterodimers (and kinase activation) in the absence of the ligand.

with other nuclear pore proteins such as NUP88, NUP358 (RANBP2), and this interaction is required for the activation of the NUP214-ABL1 kinase.[27] The NUP214-ABL1 fusion has now also been identified in some cases of high-risk B-ALL. Finally, in addition to the NUP214-ABL1 fusion, other ABL1 fusions have also been identified in T-ALL, including EML1-ABL1.[29]

Recently, a variety of small insertion mutations have been identified in the alpha chain of the interleukin-7 receptor, encoded by the IL7R gene.[30-32] Most of these mutations lead to the introduction of a cysteine amino acid close to, or within, the transmembrane domain. These amino acid insertions close to the transmembrane domain are hypothesized to cause a conformational change in the receptor enabling dimerization of the receptors in the absence of ligand. The presence of the extra cysteine can then lead to the formation of stable cysteine bridges between two mutant IL7R proteins, resulting in the stable homodimerization and activation of the associated JAK1 kinases (Figure 2).[30,31] In a minority of cases, there are mutations within the transmembrane domain of the IL7R without cysteine insertion. It was hypothesized that such mutations may lead to ligand independent heterodimerization with the CRLF2 receptor or with the common gamma chain.[30] Again, this would lead to the constitutive activation of the JAK/STAT pathway through activation of JAK1 and JAK2/JAK3 (Figure 2). In addition to mutations in the IL7 receptors, activating mutations in the tyrosine kinase JAK1, JAK2, JAK3 have also been identified, as well as rare fusion genes involving JAK2.[32-36] Despite the fact that all individual mutations are rare, the entire group of T-ALL patients with either JAK kinase mutation or IL7R mutation is estimated to be 20%-30% of all T-ALL cases.

Interestingly, several JAK kinase inhibitors are currently under development for the treatment of myeloproliferative neoplasms and auto-immune diseases.[37-39] Ruxolitinib has been approved by the US Food and Drug Administration (FDA) for the treatment of myelofibrosis, and tofacitinib has received approval for the treatment of rheumatoid arthritis. Most of these inhibitors are not very specific, and JAK2 inhibitors usually also target JAK1 and JAK3, and so-called 'selective JAK3 inhibitors' often also target JAK1. In addition to the JAK2 V617F mutation, ruxolitinib was shown to have potent activity against JAK1 mutants *in vitro*.[40] Similarly, tofacitinib was shown to inhibit leukemia-specific JAK3 mutants at low nanomolar concentrations.[41] These findings suggest these and other JAK inhibitors that are being developed for myeloproliferative neoplasms and autoimmune diseases may also be of value for the treatment of ALL with JAK1, JAK3 or IL7R mutations. It will be of interest to test the efficacy of these inhibitors in T-ALL models, and to set up exploratory trials, for example, for relapsed T-ALL patients.

The genomics (r)evolution and its implications for T-ALL

It is now possible to perform unbiased genome wide searches for novel oncogenes and tumor suppressors in T-ALL with the availability of novel genomics technologies. High-resolution array comparative hybridization (arrayCGH) has allowed the identification of copy number alterations in the genome with an unprecedented resolution. Application of this technology in T-ALL has resulted in the detection of previously unrecognized deletions in tumor suppressors, such as *PHF6,*[42] *WT1,*[43] *PTPN2,*[44] *LEF1*[45] and *BCL11B,*[46] or in the detection of cryptic deletions resulting in LMO2 expression[47] or the generation of the SET-NUP214 fusion.[48] The research group of Jules Meijerink, in collaboration with the research group of Wouter de Laat, complemented arrayCGH approaches with analysis of T-ALL gene expression profiles and with the Chromosome Conformation Capture on Chip (4C) technique[49] to characterize chromosomal rearrangements, allowing them to identify NKX2-1, NKX2-2 and MEF2C as novel oncogenes in T-ALL.[22,49]

The recent introduction of massively parallel sequencing technologies (also referred to as 'next generation sequencing') has further improved our capacity to characterize the mutational landscape of T-ALL. Pieter Van Vlierberghe and colleagues used this technology for the resequencing of the entire X-chromosome in T-ALL patients, and in this way, identified mutations in PHF6.[42] Similarly, the team of Charles Mullighan performed whole genome sequencing of 12 T-ALL patients of the immature early T-cell precursor (ETP) T-ALL subtype. This study underscored the high incidence of cytokine and RAS signaling mutations, and lesions in regulators of hematopoietic development and in epigenetic regulators in respectively 67%, 58% and 48% of ETP T-ALLs. In addition, and in agreement with the observations made by Adolfo Ferrando and colleagues,[50] the immature ETP leukemias have a transcriptional profile that resembles that of myeloid leukemias and hematopoietic stem cells. Moreover, the mutational profile of these immature T-ALL tumors is highly enriched for defects in typical myeloid leukemia oncogenes and tumor suppressors, such as *IDH1*, *IDH2*, *DNMT3A*, *FLT3*, *NRAS* and *ETV6.* It remains to be determined if these findings will have clinical implications, and whether patients with immature T-lineage leukemias could benefit from therapies developed for myeloid malignancies.

We used the power of next generation sequencing to further characterize the genetics of both pediatric and adult T-ALL. We performed exome sequencing on 67 T-ALL cases representing all different molecular subgroups and age groups.[35] Interestingly, we found that the number of somatic mutations in T-ALL samples increases with age. This positive correlation between patient age and mutation number has also been found previously in AML using whole genome sequencing approaches.[51] These observations are likely caused by the accumulation of random, benign passenger mutations during the normal aging process and underscore the need for thorough filtering methods and functional follow-up experiments to distinguish random passenger mutations from cancer driving mutations. Our study also identified *CNOT3* as a novel tumor suppressor gene that is mutated specifically in adult T-ALL patients. The CNOT3 protein is part of the CCR4-NOT complex regulating gene expression transcriptionally and post-transcriptionally.[52] In addition, CNOT3 mediates self-renewal in mouse embryonic stem cells, where CNOT3 shares many target genes with MYC,[53] a known oncogene in T-ALL. Another intriguing observation in our study was the finding that 10% of pediatric T-ALL patients carry mutations in *RPL10* or *RPL5*, 2 genes encoding pro-

teins of the large 60S ribosomal subunit. The exact role of the RPL5 and RPL10 defects in leukemogenesis are still unknown, but mutations in other genes encoding proteins of the 60S ribosomal subunit have been confirmed by other groups, including Rao and colleagues who independently reported that *RPL22* is deleted or mutated in 10% of T-ALL cases.[54] In this study, they also found that loss of *RPL22* leads to the upregulation of the stemness factor Lin28B in T-ALL and can accelerate tumor development in an established T-ALL mouse cancer model.[54]

Additional layers of complexity: co-occurrence of mutations and clonal evolution

The genomics studies described above illustrate the complexity of mutations in T-ALL. However, yet another layer of complexity is added by the multiplicity of genetically distinct leukemic subclones. Indeed, careful molecular analyses such as the mapping of genetic alterations by multiplex FISH in individual cells of diagnostic ALL samples showed genetic heterogeneity in such samples with the presence of multiple related leukemic subpopulations carrying subclone specific lesions in addition to lesions that are shared between subclones.[55] In addition, studies in which careful genetic characterization was performed on paired diagnosis-relapse samples illustrated that the clonal architecture of these leukemias is dynamic and is subject to continuous changes based on Darwinian natural selection.[55,56]

Interestingly, evolution of leukemia cell clones with selection and expansion of more aggressive malignant cells also occurs during expansion of diagnostic leukemia samples in immunodeficient mice (xenograft models). This makes xenograft models very attractive as they recapitulate the development of relapse clones as observed in patients, thereby allowing the study of the Darwinian clonal evolution process in several independent animals. This led to interesting observations: the same diagnostic leukemia sample, when injected in parallel into multiple animals, could give rise to leukemias that showed distinct genetic lesions targeting the same gene. One example was the appearance of distinct deletions in the *CDKN2A* locus in the different xenografts originating from injection of a diagnostic tumor sample in which the bulk of the tumor cells had an intact *CDKN2A* locus, indicating that the distinct *CDKN2A* lesions had been acquired independently more than once.[56] These observations suggest that the presence of a particular lesion in a (pre-)leukemic cell can put a high selective pressure on that cell to acquire a very specific genomic lesion. The presence of such selective pressure is likely to result from a synergistic co-operation between the initiating lesion and the lesions that are acquired later on.

In the context of T-ALL, it is known that particular lesions tend to co-occur together. For example, deletions of the phosphatase gene PTPN2 are frequently found in T-ALL cases with expression of either NUP214-ABL1 or with JAK1 mutation, both of which are substrates of PTPN2.[44,57] In these cases, loss of the negative regulator PTPN2 leads to increased activation of the JAK1 or NUP214-ABL1 signaling pathways. However, for other combinations, the mechanism of co-operation between co-occurring lesions is currently unknown. These include various observations in T-ALL with RAS and JAK mutations occurring frequently in ETP-ALL,[32] WT1 mutations in TLX1 and TLX3 T-ALL,[43] IL7R mutations in TLX3, TLX1 and HOXA T-ALL,[31] and a higher incidence of PTEN/AKT mutations in TAL/LMO positive T-ALL.[58] Another example is the frequent co-occurrence of apparently totally different lesions such as overexpression of the TLX1 or TLX3 transcription factors and of NUP214-ABL1.[26]

In addition to the use of xenograft models, the study of clonal complexity and clonal evolution of T-ALL is also obtaining benefit from massive parallel deep sequencing approaches. Ideally, one would perform whole genome sequencing on a significant number of single cells isolated from different samples collected over time to determine clonal composition and evolution of the tumor. Although such analyses are for the moment expensive and technically challenging, the first examples of such studies have been reported in the context of JAK2-negative myeloproliferative neoplasms.[59] At this point, deep sequencing of entire tumor cell populations followed by targeted deep sequencing represents a more straightforward approach to study clonal architecture.[60] Similar analyses in the context of T-ALL will provide more insight into the clonal evolution of T-ALL.

Conclusions

T-ALL is a genetically complex leukemia, not only because so many different lesions contribute to the development of this type of leukemia, but also because T-ALL at diagnosis is a mixture of multiple leukemia clones with slightly different genomes that are under constant evolution. Therefore, it is difficult to identify all the changes that are important to transform a normal thymocyte to a leukemic cell. Similarly, despite our increasing knowledge of the various oncogenic drivers in T-ALL, it remains extremely difficult to predict the development of resistance mechanisms that allow leukemic cells to escape targeted therapies. Nonetheless, improvements in chemotherapy regimens over the past 30 years have steadily increased the cure rates of childhood T-ALL and these are now over 70%. However, the treatment of adult T-ALL remains more difficult, and this, therefore, continues to be an area of intensive research in order to provide improved and targeted treatments, and, ultimately, a cure.

References

1. Koch U, Radtke F. Notch in T-ALL: new players in a complex disease. Trends in Immunology. 2011;32(9):434-42.
2. Weng AP, Ferrando AA, Lee W, Morris JP, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306(5694):269-71.
3. Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G, et al. The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. J Exp Med. 2007;204(8):1825-35.
4. O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C, et al. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. J Exp Med. 2007;204(8):1813-24.
5. Kanei-Ishii C, Nomura T, Takagi T, Watanabe N, Nakayama KI, Ishii S. Fbxw7 acts as an E3 ubiquitin ligase that targets c-Myb for nemo-like kinase (NLK)-induced degradation. J Biol Chem. 2008;283(45):30540-8.

6. Onoyama I, Tsunematsu R, Matsumoto A, Kimura T, de Alboran IM, Nakayama K, et al. Conditional inactivation of Fbxw7 impairs cell-cycle exit during T cell differentiation and results in lymphomatogenesis. J Exp Med. 2007;204 (12):2875-88.
7. Kitagawa K, Hiramatsu Y, Uchida C, Isobe T, Hattori T, Oda T, et al. Fbw7 promotes ubiquitin-dependent degradation of c-Myb: involvement of GSK3-mediated phosphorylation of Thr-572 in mouse c-Myb. Oncogene. 2009;28(25):2393-405.
8. Crusio KM, King B, Reavie LB, Aifantis I. The ubiquitous nature of cancer: the role of the SCFFbw7 complex in development and transformation. Oncogene. 2010;29(35):4865-73.
9. Tatarek J, Cullion K, Ashworth T, Gerstein R, Aster JC, Kelliher MA. Notch1 inhibition targets the leukemia-initiating cells in a Tal1/Lmo2 mouse model of T-ALL. Blood. 2011;118(6):1579-90.
10. Ma W, Gutierrez A, Goff DJ, Geron I, Sadarangani A, Jamieson CAM, et al. NOTCH1 signaling promotes human T-cell acute lymphoblastic leukemia initiating cell regeneration in supportive niches. PLoS ONE. 2012;7(6):e39725.
11. Panepucci RA, Oliveira LHB, Zanette DL, Viu Carrara R de C, Araujo AG, Orellana MD, et al. Increased levels of NOTCH1, NF-kappaB, and other interconnected transcription factors characterize primitive sets of hematopoietic stem cells. Stem Cells Dev. 2010;19(3):321-32.
12. Armstrong F, Brunet de la Grange P, Gerby B, Rouyez M-C, Calvo J, Fontenay M, et al. NOTCH is a key regulator of human T-cell acute leukemia initiating cell activity. Blood. 2009;113(8):1730-40.
13. Cullion K, Draheim KM, Hermance N, Tammam J, Sharma VM, Ware C, et al. Targeting the Notch1 and mTOR pathways in a mouse T-ALL model. Blood. 2009;113(24):6172-81.
14. Giambra V, Jenkins CR, Wang H, Lam SH, Shevchuk OO, Nemirovsky O, et al. NOTCH1 promotes T cell leukemia-initiating activity by RUNX-mediated regulation of PKC-θ and reactive oxygen species. Nat Med. 2012;18(11):1693-8.
15. Chiang MY, Shestova O, Xu L, Aster JC, Pear WS. Divergent effects of supraphysiologic Notch signals on leukemia stem cells and hematopoietic stem cells. Blood. 2013;121(6):905-17.
16. Ntziachristos P, Tsirigos A, Van Vlierberghe P, Nedjic J, Trimarchi T, Sol Flaherty M, et al. Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. Nat Med. 2012;18(2):298-303.
17. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. Science. 1990;250(4986):1426-9.
18. Lahortiga I, De Keersmaecker K, Van Vlierberghe P, Graux C, Cauwelier B, Lambert F, et al. Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. Nat Genet. 2007;39(5):593-5.
19. Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, Dik WA, et al. The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. Blood. 2007;110(4):1251-61.
20. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. Cancer Cell. 2002;1(1):75-87.
21. Soulier J, Clappier E, Cayuela JM, Regnault A, García-Peydró M, Dombret H, et al. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). Blood. 2005;106(1):274-86.
22. Homminga I, Pieters R, Langerak AW, de Rooi JJ, Stubbs A, Verstegen M, et al. Integrated transcript and genome analyses reveal NKX2-1 and MEF2C as potential oncogenes in T cell acute lymphoblastic leukemia. Cancer Cell. 2011;19(4):484-97.
23. Dadi S, Le Noir S, Payet-Bornet D, Lhermitte L, Zacarias-Cabeza J, Bergeron J, et al. TLX Homeodomain Oncogenes Mediate T Cell Maturation Arrest in T-ALL via Interaction with ETS1 and Suppression of TCRα Gene Expression. Cancer Cell. 2012;21(4):563-76.
24. Gatta Della G, Palomero T, Perez-Garcia A, Ambesi-Impiombato A, Bansal M, Carpenter ZW, et al. Reverse engineering of TLX oncogenic transcriptional networks identifies RUNX1 as tumor suppressor in T-ALL. Nat Med. 2012;18(3):436-40.
25. Gutierrez A, Sanda T, Ma W, Zhang J, Grebliunaite R, Dahlberg S, et al. Inactivation of LEF1 in T-cell acute lymphoblastic leukemia. Blood. 2010;115(14):2845-51.
26. Graux C, Cools J, Melotte C, Quentmeier H, Ferrando A, Levine R, et al. Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukemia. Nat Genet. 2004;36(10):1084-9.
27. De Keersmaecker K, Rocnik JL, Bernad R, Lee BH, Leeman D, Gielen O, et al. Kinase activation and transformation by NUP214-ABL1 is dependent on the context of the nuclear pore. Mol Cell. 2008;31(1):134-42.
28. De Keersmaecker K, Versele M, Cools J, Superti-Furga G, Hantschel O. Intrinsic differences between the catalytic properties of the oncogenic NUP214-ABL1 and BCR-ABL1 fusion protein kinases. Leukemia. 2008;22(12):2208-16.
29. De Keersmaecker K, Graux C, Odero MD, Mentens N, Somers R, Maertens J, et al. Fusion of EML1 to ABL1 in T-cell acute lymphoblastic leukemia with cryptic t(9;14)(q34;q32). Blood. 2005;105(12):4849-52.
30. Shochat C, Tal N, Bandapalli OR, Palmi C, Ganmore I, Kronnie Te G, et al. Gain-of-function mutations in interleukin-7 receptor-α (IL7R) in childhood acute lymphoblastic leukemias. Journal of Experimental Medicine. 2011;208(5):901-8.
31. Zenatti PP, Ribeiro D, Li W, Zuurbier L, Silva MC, Paganin M, et al. Oncogenic IL7R gain-of-function mutations in childhood T-cell acute lymphoblastic leukemia. Nat Genet. 2011;43(10):932-9.
32. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012;481(7380):157-63.
33. Flex E, Petrangeli V, Stella L, Chiaretti S, Hornakova T, Knoops L, et al. Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia. J Exp Med. 2008;205(4):751-8.
34. Elliott NE, Cleveland SM, Grann V, Janik J, Waldmann TA, Davé UP. FERM domain mutations induce gain of function in JAK3 in adult T-cell leukemia/lymphoma. Blood. 2011;118(14):3911-21.
35. De Keersmaecker K, Atak ZK, Li N, Vicente C, Patchett S, Girardi T, et al. Exome sequencing identifies mutation in CNOT3 and ribosomal genes RPL5 and RPL10 in T-cell acute lymphoblastic leukemia. Nat Genet. 2013;45(2):186-90.
36. Peeters P, Raynaud SD, Cools J, Wlodarska I, Grosgeorge J, Philip P, et al. Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. Blood. 1997;90(7):2535-40.
37. Burmester GR, Blanco R, Charles-Schoeman C, Wollenhaupt J, Zerbini C, Benda B, Gruben D, Wallenstein G, Krishnaswami S, Zwillich SH, Koncz T, Soma K, Bradley J, Mebus C; ORAL Step investigators. Tofacitinib (CP-690,550) in combination with methotrexate in patients with active rheumatoid arthritis with an inadequate response to tumour necrosis factor inhibitors: a randomised phase 3 trial. Lancet. 2013;381(9865):451-60.
38. Lierman E, Selleslag D, Smits S, Billiet J, Vandenberghe P. Ruxolitinib inhibits transforming JAK2 fusion proteins in vitro and induces complete cytogenetic remission in t(8;9)(p22;p24)/PCM1-JAK2-positive chronic eosinophilic leukemia. Blood. 2012;120(7):1529-31.
39. Harrison C, Kiladjian J-J, Al-Ali HK, Gisslinger H, Waltzman R, Stalbovskaya V, et al. JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. New England Journal of Medicine. 2012;366(9):787-98.
40. Hornakova T, Springuel L, Devreux J, Dusa A, Constantinescu SN, Knoops L, et al. Oncogenic JAK1 and JAK2-activating mutations resistant to ATP-competitive inhibitors. Haematologica. 2011;96(6):845-53.
41. Koo GC, Tan SY, Tang T, Poon SL, Allen GE, Tan L, et al. Janus kinase 3-activating mutations identified in natural killer/T-cell lymphoma. Cancer Discovery. 2012;2(7):591-7.
42. Van Vlierberghe P, Palomero T, Khiabanian H, Van der Meulen J, Castillo M, Van Roy N, et al. PHF6 mutations in T-cell acute lymphoblastic leukemia. Nat Genet. 2010;42(4):338-42.
43. Tosello V, Mansour MR, Barnes K, Paganin M, Sulis ML, Jenkinson S, et al. WT1 mutations in T-ALL. Blood. 2009;114(5):1038-45.
44. Kleppe M, Lahortiga I, Chaar El T, De Keersmaecker K, Mentens N, Graux C, et al. Deletion of the protein tyrosine phosphatase gene PTPN2 in T-cell acute lymphoblastic leukemia. Nat Genet. 2010;42(6):530-5.
45. Gutierrez A, Sanda T, Ma W, Zhang J, Grebliunaite R, Dahlberg S, et al. Inactivation of LEF1 in T-cell acute lymphoblastic leukemia. Blood. 2010;115(14):2845-51.
46. De Keersmaecker K, Real PJ, Gatta GD, Palomero T, Sulis

ML, Tosello V, et al. The TLX1 oncogene drives aneuploidy in T cell transformation. Nat Med. 2010;16(11):1321-7.
47. Van Vlierberghe P, van Grotel M, Beverloo HB, Lee C, Helgason T, Buijs-Gladdines J, et al. The cryptic chromosomal deletion del(11)(p12p13) as a new activation mechanism of LMO2 in pediatric T-cell acute lymphoblastic leukemia. Blood. 2006;108(10):3520-9.
48. Van Vlierberghe P, van Grotel M, Tchinda J, Lee C, Beverloo HB, van der Spek PJ, et al. The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia. Blood. 2008;111(9):4668-80.
49. Simonis M, Klous P, Homminga I, Galjaard R-J, Rijkers E-J, Grosveld F, et al. High-resolution identification of balanced and complex chromosomal rearrangements by 4C technology. Nat Methods. 2009;6(11):837-42.
50. Van Vlierberghe P, Ambesi-Impiombato A, Perez-Garcia A, Haydu JE, Rigo I, Hadler M, et al. ETV6 mutations in early immature human T cell leukemias. J Exp Med. 2011;208(13):2571-9.
51. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, et al. The origin and evolution of mutations in acute myeloid leukemia. 2012;150(2):264-78.
52. Collart MA, Panasenko OO. The Ccr4–Not complex. Gene. Elsevier B.V; 2012;492(1):42-53.
53. Hu G, Kim J, Xu Q, Leng Y, Orkin SH, Elledge SJ. A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal. Genes Dev. 2009;23(7):837-48.
54. Rao S, Lee S-Y, Gutierrez A, Perrigoue J, Thapa RJ, Tu Z, et al. Inactivation of ribosomal protein L22 promotes transformation by induction of the stemness factor, Lin28B. Blood. 2012;120(18):3764-73.
55. Anderson K, Lutz C, van Delft FW, Bateman CM, Guo Y, Colman SM, et al. Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature. 2011;469(7330):356-61.
56. Clappier E, Gerby B, Sigaux F, Delord M, Touzri F, Hernández L, et al. Clonal selection in xenografted human T cell acute lymphoblastic leukemia recapitulates gain of malignancy at relapse. Journal of Experimental Medicine. 2011;208(4):653-61.
57. Kleppe M, Soulier J, Asnafi V, Mentens N, Hornakova T, Knoops L, et al. PTPN2 negatively regulates oncogenic JAK1 in T-cell acute lymphoblastic leukemia. Blood. 2011;117(26):7090-8.
58. Zuurbier L, Petricoin EF, Vuerhard MJ, Calvert V, Kooi C, Buijs-Gladdines JGCAM, et al. The significance of PTEN and AKT aberrations in pediatric T-cell acute lymphoblastic leukemia. Haematologica. 2012;97(9):1405-13.
59. Hou Y, Song L, Zhu P, Zhang B, Tao Y, Xu X, et al. Single-cell exome sequencing and monoclonal evolution of a JAK2-negative myeloproliferative neoplasm. Cell. 2012;148(5):873-85.
60. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-9.

The challenges of incorporating novel biomarkers in acute lymphoblastic leukemia

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A B S T R A C T

An effective treatment of acute lymphoblastic leukemia (ALL) starts with risk-stratification guided by well-established risk factors and rationally designed and well-controlled treatment protocols. To further improve clinical outcome, both the failure rate (approx. 20% in children and approx. 70% in adults) as well as the side effects should be reduced. The currently applied chemotherapeutic drugs were largely discovered decades ago (1950-1970s) and have improved the clinical outcome tremendously. However, the event-free survival has reached a plateau in recent years whereas, with a few exceptions, contemporary therapies still use the same drugs as decades ago. Meanwhile, the molecular knowledge has exploded in this last decade mainly driven by deciphering the human genome, characterization of the epigenetic landscape of gene regulation, and the acceleration in the development of new and often high-throughput molecular biological techniques. Our knowledge of the biology of ALL is now beyond its infancy, and more and more studies are emerging that discover new (genetic) abnormalities in leukemic cells. Some of these features may serve as new diagnostic and/or prognostic markers and some as a target for new drugs. The challenge is to identify, validate and functionally prove the importance of new (genetic) lesions in the pathobiology of ALL in order to guide personalized medicine by more optimized risk stratification and targeted drugs.

Learning goals

At the conclusion of this activity, participants should have learnt about:
- the clinical need for informative biomarkers in ALL;
- the different types of biomarkers;
- the challenges to discover and implement new biomarkers in the treatment of ALL.

The clinical need for informative biomarkers in ALL

The prognosis of ALL heavily relies on the effective stratification of patients into risk-adapted treatment regimens. This risk stratification is based on clinical features such as presenting white blood cell count and age, as well as pathobiological features such as immunophenotype and genotype of the leukemic cells. Monitoring the response to treatment revealed that the presence of residual cells at given time points (e.g. Day 33 and Day 79 in children with newly diagnosed ALL) was a strong and independent predictor for unfavorable outcome in both children and adults with ALL, and therefore minimal residual disease (MRD) status is used as risk factor in contemporary treatment protocols.[1-2] The current 5-year event-free survival estimates for patients who received risk-stratified treatment are 80% for children (<18 years) and 30% for adults with newly diagnosed ALL.[3-4]

These event-free survival rates reflect the average of the total group of patients. However, ALL is a hetereogeneous disease that comprises different genetic abnormalities contributing to the leukemogenic process and/or to maintenance of the leukemia. Identical genetic lesions are found in children and adults, although the frequency of these abnormalities widely differs. Adult ALL is characterized by a higher frequency of *BCR-ABL1*-positive B-cell precursor (BCP) ALL whereas in children, *ETV6-RUNX1* (formerly known as *TEL-AML1*)-positive and hyperdiploid (>50 chromosomes or a DNA-index ≥1.16) BCP-ALL are most prevalent (Figure 1).[4-6] *BCR-ABL1*-positive ALL is linked to an unfavorable prognosis and the higher incidence in adults may, therefore, be one of the explanations for the observed poorer clinical outcome in adults compared to children with ALL. Many relapses occur in the 'apparent' favorable risk groups like those with *ETV6-RUNX1*-positive or hyperdiploid ALL, or those with undefined genetic lesion (B-other). For example, *ETV6-RUNX1*-positive and hyperdiploid ALL accounts for approximately 50% of all pediatric ALL cases and approximately 10% of these cases relapse. Since both types of ALL represent a large population in size, the absolute number of patients who relapse is high for this apparent good prognosis group (Table 1). Moreover, the highest number of relapses occurs in the group of BCP-ALL cases negative for *BCR-ABL1* and *ETV6-RUNX1*, being non-hyperdiploid as well

as *MLL*- and *TCF3*-wild type (defined here as B-other). This exemplifies the need for more discriminative factors, i.e. biomarkers, in the diagnosis and treatment of ALL.

Definition of a biomarker

A biomarker in the context of leukemia is a feature that reflects a biological process that is predictive for the susceptibility to develop leukemia, the clinical manifestation of disease (diagnosis), the subtype of disease, the re-occurrence of disease (relapse), and the response to a given treatment in an individual patient. Most 'leukemic' biomarkers are measured in blood, bone marrow and cerebrospinal fluid samples taken from patients and can be studied using a variety of sources such as specific cells, DNA, protein-coding mRNAs, non-coding RNAs, (phospho)proteins, lipids, hormones and other molecules (e.g. drug metabolites). In medicine, both physiological biomarkers (e.g. blood pressure, body temperature and white blood cell count) and molecular biomarkers (e.g. gene mutations, gene fusion products, change in phosphorylation state of proteins) are being used. In this educational review, the emphasis will be on the challenge to incorporate *molecular* biomarkers in personalized medicine and tailored therapies of ALL. Types of biomarkers commonly used in leukemia are shown in Table 2**.**

Biomarkers related to the diagnosis and prognosis of the disease in individual patients

Diagnostic biomarkers: are used to determine the presence and/or the subtype of leukemia. For example, *ETV6-RUNX1*-positive ALL can be identified by fluorescence *in situ* hybridization (FISH) assays with a green-labeled ETV6 and a red-labeled RUNX1 probe using leukemic cells captured on microscopic slides. Presence of a yellow fusion signal indicates that the patient suffers from an *ETV6-RUNX1*-positive leukemia. The information of this diagnostic biomarker is taken forward to assign the patient to the appropriate risk arm of a treatment protocol for ALL, i.e. non-high risk treatment for an *ETV6-RUNX1*-positive patient.

Prognostic biomarkers: are indicative for the risk of patients to fail to respond to treatment (e.g. an induction failure) or to develop a relapse. Prognostic biomarkers are not 100% predictive for the occurrence of an event but give an estimate of the risk for an event. Deletions in the Ikaros gene (*IKZF1*) are predictive for an unfavorable outcome in both childhood and adult ALL, but not all *IKZF1*-deleted cases will suffer from a relapse, nor do 100% of the wild-type cases survive without an event.

In ALL clinical practice, there is not a big difference between diagnostic and prognostic biomarkers and most often a lesion can be both diagnostic and prognostic. Examples are *BCR-ABL1* and *ETV6-RUNX1* gene fusions and genes affecting the biology of leukemic cells such as *IKZF1* deletions. In combination, these biomarkers may further fine-tune the prediction for clinical outcome of patients. Examples are the additive value of the *IKZF1* deletion status to disseminate cases with highly favorable and unfavorable prognosis among patients with *BCR-ABL1*-positive ALL or the fact that IKZF1 deletions combined with MRD status can identify more patients at high risk of relapse than each of these features alone.[9-10]

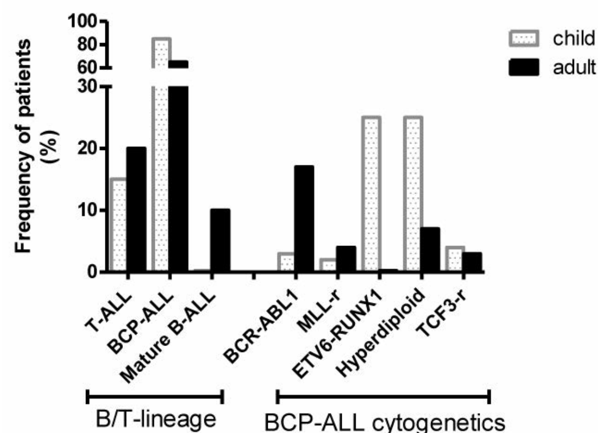

Figure 1. Distribution of (cytogenetic) subtypes in children and adults with newly diagnosed ALL. Estimated frequencies were based on Kamps *et al.*,[3] Labar *et al.*,[4] Moorman *et al.*,[6] Pui *et al.*,[7] and Moricke *et al.*[8] MLL-r: *MLL*-rearranged; TCF3-r: *TCF3*-rearranged.

Table 1. Frequency of events in (cytogenetic) subtypes of ALL in children.

ALL subtype	Frequency in children (1-18 years)	5-year event-free survival estimates in children	Estimated absolute number of events in 1000 patients
BCR-ABL1	~3%	25-30%*	20
MLL-rearranged	~2%	20-40%	15
ETV6-RUNX1	~25%	90%	25
Hyperdiploid	~25%	90%	25
TCF3-rearranged	~4%	85%	5
B-other	~25%	70%	75
T-lineage	~15%	75%	35
ALL total	100%	80%	200

**Event-free survival estimate of BCR-ABL1-positive ALL in the pre-tyrosine kinase inhibitor era (e.g. imatinib, dasatinib). Events are defined as relapse, non-response, death due to leukemia.[3,7-8]*

Biomarkers used to prioritize drugs and new drug development

Predictive biomarkers: are used to *predict* the response to a particular drug or treatment in a patient. Positivity for a predictive biomarker results in the use of a drug targeting this feature to optimize treatment results for individual patients (personalized medicine). Positivity for the *BCR-ABL1* gene fusion predicts that the patient may benefit from ABL1-tyrosine kinase inhibitors like imatinib (Gleevec) or dasatinib (Sprycel). Therefore, the *BCR-ABL1* fusion can, besides being diagnostic and prognostic, also serve as predictive biomarker. In addition to *BCR-ABL1* positivity, the mutation status of this fusion gene is indicative for the actual clinical response to ABL1-tyrosine kinase inhibitors. *BCR-ABL1*-positive cases harboring an *ABL1* T315I mutation are resistant to imatinib and dasatinib, but are still sensitive to ponatinib.[11] Well-defined predictive biomarkers are, therefore, essential for prioritizing the most optimal type of drugs used to treat the patient.

Pharmacodynamic biomarkers: are used to study what a drug does to the leukemic cells. Pharmacodynamic studies determine which proteins and signaling pathways are affected by a drug (proof-of-mechanism) and determine the phenotypic effect of exposure to this drug in leukemic cells (proof-of-concept). Changes in expression levels or activation status (e.g. phosphorylation status of kinases) of targeted proteins are often dose-dependent and provide a tool to optimize drug dosages in clinical studies including phase I/II early clinical trials.

Pharmacokinetic biomarkers: are used to monitor the kinetics of a drug in the human body. The active drug level in plasma depends on the type of drug and the (genetic) make up of individual patients, indicating the clinical need for discriminative biomarkers. Genomic markers can be used to identify patients who need pharmacokinetic monitoring of achieved drug levels in plasma to enable dose-reduction or increment. A classical example is the genetic variation in thiopurine S-methyltransferase (*TPMT*) which increases the bio-availability of thiopurine drugs (e.g. 6-mercaptopurine), drugs frequently used in the treatment of ALL. Polymorphisms in *TPMT* identify patients who may benefit from a dose-reduction in order to avoid side-effects caused by a prolonged presence of active thiopurine metabolites in the plasma.[12]

Surrogate response biomarkers: are dynamic biomarkers used to *monitor* the effect of a given treatment. Surrogate response biomarkers are used as alternative to a primary end point of treatment that is undesired (death) or to avoid repetitive invasive bone marrow punctures. A biomarker can only serve as surrogate response marker if a change in the biomarker also predicts the true clinical response to the given therapy. A biomarker that only predicts prognosis at the start of treatment without being dynamically affected by the given treatment is not a surrogate response biomarker but a prognostic biomarker. An example of a surrogate response marker in ALL is the monitoring of minimal residual disease by patients' unique T-cell receptor and immunoglobulin-rearrangement signatures of leukemic cells.[13,14]

Biomarkers can be both diagnostic, prognostic, predictive, pharmacodynamic, pharmacokinetic and/or a surrogate marker for response to one or to a cocktail of drugs. Most importantly for personalized medicine, biomarkers can be used to identify the presence of drug targets and/or drug metabolizing enzymes to tailor treatment in individual patients. The challenge is to pick the winner(s)!

Table 2. Examples of molecular biomarkers in ALL.

Type of molecular biomarker	Examples in ALL
Diagnostic and prognostic	*BCR-ABL1* *ETV6-RUNX1* *MLL*-rearrangement *TCF3*-rearrangement *IKZF1* deletions *BCR-ABL1*-like gene expression signature *JAK2* mutations and translocations deregulated CRLF2 expression
Surrogate response	Minimal residual disease
Predictive (for selecting drugs)	*BCR-ABL1* and mutation status (imatinib, dasatinib, nilotinib, ponatinib) *FLT3* expression levels and mutation status (midostaurin, lestaurtinib, sunitinib) *JAK2* mutations and translocations (ruxolitinib) RAS-MEK pathway activating mutations (selumetinib, trametinib)
Pharmacodynamic (for monitoring response)	pABL1, pCRKL (ABL1 tyrosine kinase inhibitors) pFLT3 (FLT3 inhibitors) pSTAT5 (JAK inhibitors) pERK (MEK inhibitors)
Pharmacokinetic	*TPMT*

P: phosphorylated.

Recently discovered molecular biomarkers

In essence, the features that are currently being used for risk-stratification of patients – immunophenotype and genotype, as well as white blood cell count and age – are all diagnostic and/or prognostic biomarkers in ALL. As discussed above, these clinical and biological features fail to predict the majority of relapses in pediatric ALL and similar observations are found in adult ALL. This means that the prognosis of a substantial part of apparent non-high risk patients may increase by more intensified or more targeted therapy whereas the prognosis of other patients may remain similar using a reduction in the given treatment but with the benefit of less treatment-related toxicity (e.g. by leaving out hematopoietic stem cell transplantation). In the next part we will discuss the identification of molecular features that are of high interest to use as biomarkers to guide new clinical trials in ALL.

The pathobiology of ALL and the discovery of new biomarkers

BCR-ABL1-positive and BCR-ABL1-like ALL

The *BCR-ABL1* fusion is a key example of a biomarker having diagnostic, prognostic and predictive value in both childhood and adult ALL. The introduction of the tyrosine kinase inhibitor imatinib (Gleevec/Glivec, STI571) in

combination with conventional chemotherapy has improved the event-free survival of *BCR-ABL1*-positive patients significantly from 25%-30% in the pre-inhibitor era to 50%-70% for children and from 10% to 35%-50% for adults in contemporary protocols.[15-18] However, prolonged exposure to imatinib resulted in resistance to this inhibitor, often caused by acquired mutations in *ABL1*. A key mutation is the T315I-mutation resulting in a conformational change of the ABL1 kinase domain that results in loss of effective binding of the inhibitor, but also other inactivating mutations have been identified (e.g. Y253H and F317L).[19] The unfavorable long-term prognosis despite the use of tyrosine kinase inhibitors, as well as the acquired resistance to imatinib, indicate that other *BCR-ABL1*-driven features and targeted drugs need to be explored to cure these patients. New ABL1 kinase domain directed drugs have been developed like dasatinib (BMS-354825), nilotinib (AMN-107) and bosutinib (SKI-606). These 2nd generation drugs face the same drawback of acquired resistance of leukemic cells or outgrowth of resistant subclones in time and, moreover, these drugs also do not overcome resistance associated with the *ABL1* T315I mutation.[20-21] Recent studies show that ponatinib (AP24534), a 3rd generation tyrosine kinase inhibitor, is able to overcome resistance to many ABL1-kinase domain mutations, including *ABL1* T315I.[22] This inhibitor may, therefore, be used to salvage *BCR-ABL1*-positive patients who are resistant to 1st and 2nd generation tyrosine kinase inhibitors as recently shown for chronic myeloid leukemia and ALL.[11]

In addition to drugs targeting BCR-ABL1 and its tyrosine kinase domain, downstream activated genes may also serve as candidates for new drugs. Intriguingly, *BCR-ABL1* activates divergent signaling pathways in ALL and chronic myeloid leukemia, stressing the importance of studying the pathobiology in the correct cellular context. *BCR-ABL1* in chronic myeloid leukemia triggers growth factor-independent RAS-mediated proliferation and phosphoinositide 3-kinase (PI3K)/AKT-mediated survival pathways.[23] In contrast, *BCR-ABL1* in ALL activates the JAK/STAT pathway thereby deregulating the transcription of genes involved in many different cellular processes. This JAK/STAT pathway includes Janus kinase-family members (JAKs) and signal transducer and activator of transcription family members (STATs).[24-26] Activating mutations in JAK-family members (mainly *JAK2*) have rarely been found in *BCR-ABL1*-positive ALL which opposes the relative high frequency of approximately 10% seen in other high-risk BCP-ALL patients.[27-28] STAT5 activation seems important in both the initiation and prolongation of *BCR-ABL1*-positive ALL, whereas STAT3 mainly contributes to the initiation of *BCR-ABL1*-positive ALL.[25] As shown in Figure 2, phosphorylated (and hence activated) but not total STAT5 protein levels were significantly raised in *BCR-ABL1*-positive ALL compared to other precursor B-ALL cells taken from children with newly diagnosed ALL. It is evident that STAT5 and its associated JAK/STAT pathway need further exploration as alternative strategy to circumvent resistance to tyrosine kinase domain-directed inhibitors in ALL.

As mentioned in the introduction, there is especially a need to improve outcome for those patients who are not recognized as high-risk patients because their leukemic cells do not harbor *BCR-ABL1* or *MLL*-fusion genes. Recent genomic studies have identified an unfavorable prognostic subtype of precursor B-ALL with a gene expression signature resembling that of *BCR-ABL1*-positive ALL. These so-called *BCR-ABL1*-like ALL cases are negative for the *BCR-ABL1*-translocation but have, like *BCR-ABL1*-positive ALL, a high frequency (>80%) of genomic lesions in genes involved in B-cell commitment (*PAX5*), B-cell differentiation and immunoglobulin rearrangements (*EBF1*, *TCF3*, *IKZF1*) and pre-B cell receptor formation (*VPREB1*).[29-30] Intriguingly, RNA and whole genome sequencing of 15 cases with *BCR-ABL1*-like ALL revealed genomic lesions in cytokine receptors genes (*PDGFRB*, *EPOR*, *CRLF2*, *IL7R*, *FLT3*) and non-receptor effector genes (*ABL1*, *JAK2*, *LNK*) with deregu-

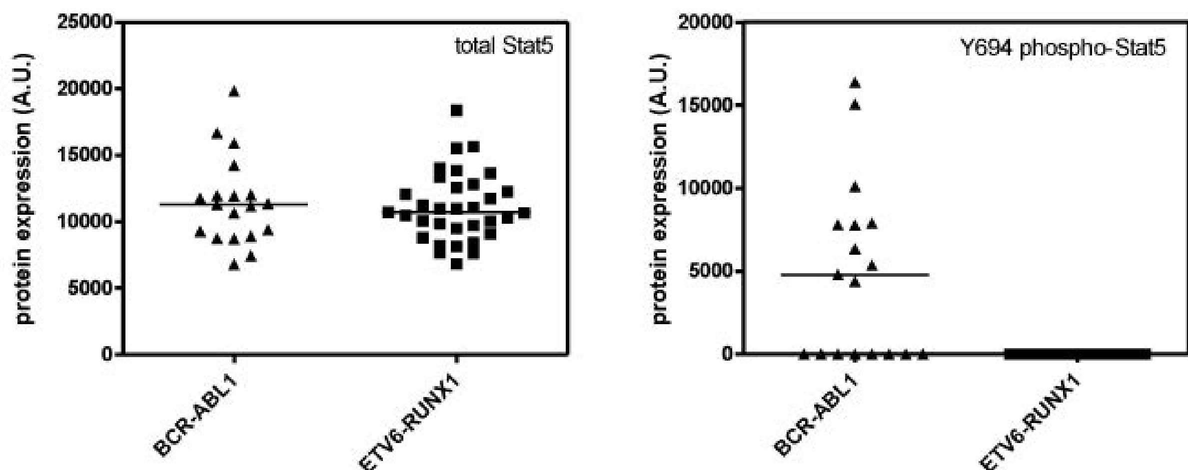

Figure 2. Phosphorylated STAT5, but not total STAT5 protein levels, are higher in *BCR-ABL1*-positive leukemic cells compared to a reference group of *ETV6-RUNX1* positive cases (Den Boer *et al.*, unpublished results, 2012)

lated *CRLF2*, *ABL1*- and *JAK2*-translocations being most recurrent.[31] In an independent validation cohort, deregulated *CRLF2* and the *EBF1-PDGFRB* translocation were found in 50% and 8% of *BCR-ABL1*-like ALL cases, respectively. The other lesions as reported in the discovery cohort were not or only in single cases present.[31] Deregulated *CRLF2* and concomitant *JAK2* mutations were reported in 50%-60% of *BCR-ABL1*-like cases (R8 cluster).[32-33] *JAK2* mutations and the *EBF1-PDGFRB* fusion gene both induced interleukin 3 independent proliferation of Ba/F3 cells, which illustrates their oncogenic potential.[28,31] In addition, the growth of leukemic cells with *JAK2* activity affected by mutations or translocations as well as that of *NUP214-ABL1*-positive ALL could be inhibited by ruxolitinib and dasatinib, respectively, in xenograft models of ALL.[31,34] Irrespective of the frequency of these individual genomic lesions, these findings show the potential of these lesions as predictive biomarkers to identify *BCR-ABL1*-negative patients who may benefit from inhibitors directed against JAK2 and ABL1.

Interestingly, lesions affecting JAK/STAT signaling not only associate with *BCR-ABL1*-positive and *BCR-ABL1*-like ALL, but are also found in other types of BCP-ALL, most often together with deregulated expression of the cytokine receptor-like factor 2 (*CRLF2*). The high expression level is mediated by the translocation of *CRLF2* to the *IGH@* enhancer or by an interstitial deletion which positions *CRLF2* next to the *P2RY8* promoter, and, albeit infrequently, by activating mutations.[35-39] Deregulated *CRLF2* expression might serve as prognostic biomarker predictive for an unfavorable outcome in pediatric and adult BCP-ALL although controversy remains as to whether deregulated *CRLF2* is an independent prognostic feature.[27,40-42] Deregulated *CRLF2* often co-occurs with *JAK*-family gene mutations (primarily J*AK2*), *IKZF1* deletions and a *BCR-ABL1*-like gene expression signature.[27,37,42-44] The inconsistent reports on the prognostic value of deregulated *CRLF2* may, therefore, largely rely on the type(s) of genomic lesions affecting *CRLF2* expression levels (*P2RY8-CRLF2*, *IGH@-CRLF2*, others) that were included and differences in the composition of patients with the aforementioned adverse features. In addition, deregulated *CRLF2* and mutations in *JAK*-family genes were more frequently found in patients of Hispanic and Latino ethnicity, suggesting that demographical differences affect the prognostic value ascribed to deregulated *CRLF2*.[37] Another confounder in the discussion of deregulated *CRLF2* as prognostic biomarker are Down syndrome patients with ALL who often have *CRLF2*-rearrangements with concomitant *JAK* activating mutations (primarily in *JAK2*) and deletions in *IKZF1*.[27,36,45-47] Down syndrome ALL patients are at high risk of treatment-related toxicity and, as such, inclusion/exclusion of these patients will affect the prognostic value of deregulated *CRLF2*.[48] Leukemic cells of patients with deregulated CRLF2 expression were sensitive to the JAK1/2 inhibitor ruxolitinib.[34,49] This finding implies that JAK-inhibitors may be effective in patients with deregulated *CRLF2*, which would especially be of benefit to Down syndrome patients to reduce the high morbidity caused by current chemotherapeutic drugs.

IKZF1 deletions in precursor B-ALL

Deletions in the B-cell transcription factor Ikaros (*IKZF1*) is one of the most frequently found genomic lesion in children (approx.15%) and adults (approx. 50%) with BCP-ALL, and is associated with a highly unfavorable prognosis across all ages.[29,37,50-54] Deletions in *IKZF1* were frequently found in newly diagnosed children with BCP-ALL at high risk for relapse based on unfavorable age at presentation (≥10 years), gender (male), high white blood cell count at presentation ($\geq 50 \times 10^9$/L), and presence of extramedullary disease.[29,37] The frequency of *IKZF1* deletions in the unfavorable prognostic group of pediatric *MLL*-rearranged ALL was rather low. In contrast, *IKZF1* deletions were detected in 60%-80% of children and adult *BCR-ABL1*-positive ALL cases.[52,54-57] Neonatal blood spot analysis from *BCR-ABL1*-positive twins demonstrated that deletions in *IKZF1* are not the primary leukemogenic event, but facilitate the outgrowth of a pre-leukemic clone.[58] In correspondence, *IKZF1* deletions were shown to trigger SRC kinase mediated proliferation at the expense of cell cycle exit mediated by a normal activation of pre-B cell receptors.[59]

The prognosis of *BCR-ABL1*-positive cases with concomitant deletions in *IKZF1* is highly unfavorable compared to those with unaffected *IKZF1*, even upon treatment with imatinib.[9] Given the fact that deletions in *IKZF1* trigger SRC kinases, also in the context of *BCR-ABL1*-positive ALL, one may propose including dual SRC/ABL1 kinase inhibitors and more specific SRC kinase family inhibitors, e.g. those targeting LYN, HCK or FGR.[60] However, to guide treatment more specifically, the biology of *IKZF1*-deleted leukemic cells needs to be explored for drugable genes downstream of an *IKZF1* deletion. Wild-type Ikaros has a pleiotrophic function in B-cell development since studies in mice revealed a role for Ikaros in both pre B-cell receptor signaling, cell cycle arrest/progression and immunoglobulin V(D)J recombination processes.[61,62] Recent gene expression studies revealed many genes with increased expression levels in *IKZF1*-deleted BCP-ALL patients (e.g. *ETV6*, *YES1* and *MCL1*) that need further functional studies to determine which may be suitable to interfere with drugs in clinical practice.[63]

Mutations affecting MAPK/ERK-pathway in ALL

Activation of the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathway induces proliferation and reduces apoptosis of cells. Leukemia-specific mutations that constitutively activate this MAPK/ERK (MEK) pathway have been reported in the membrane-bound FLT3-receptor and downstream effector genes including *KRAS*, *NRAS*, *PTPN11, NF11*, *BRAF* and *CBL* in 10%-35% of BCP-ALL cases.[64-67] Mutations in these genes were most frequently found in *MLL*-rearranged, hyperdiploid (>50 chromosomes) and hypodiploid (<44 chromosomes) BCP-ALL.[33,67-70] Mutations in RAS pathway genes were less frequently found in *BCR-ABL1*-like ALL.[33]

RAS mutations itself are presumably not leukemogenic and this is also underscored by the lack of *RAS* mutations in neonatal blood spots and loss of some *RAS* mutations at

relapse; but they do facilitate growth factor and cytokine-independent proliferation.[71,72] Intriguingly, mutated *RAS* can down-regulate the signaling from a tyrosine kinase receptor like the epidermal growth factor receptor (*EGFR*). Inhibition of mutated *RAS* (by RNA interference) abolished this negative feedback loop and activated the EGFR and wild-type RAS signaling pathway which resulted in proliferation of cancer cells.[73] This important finding indicates that inhibition of activating mutations in the RAS pathway needs to be combined with inhibition of more upstream receptor tyrosine kinases to be effective in clinical trials. An *in vitro* study nicely demonstrated this proof-of-concept for chronic myeloid leukemia in which a SRC/ABL1 kinase inhibitor (dasatinib) worked synergistically with a RAS-pathway MEK1/2 inhibitor (PD184352) in inducing death of chronic myeloid leukemia cells.[74] In contrast to studies showing effective *in vitro* cell death induced by MEK-inhibitors in chronic myeloid leukemia[74] and diploid/hyperdiploid BCP-ALL,[65,66] hypodiploid BCP-ALL cells seem to be resistant to MEK inhibition but are relatively sensitive to PI3K inhibitors.[67]

The aforementioned mutations in RAS/MAPK/ERK pathway genes may serve as predictive biomarker to identify patients who may benefit from targeted drugs such as the MEK inhibitors selumetinib and trametinib or the RAF-kinase inhibitor sorafenib. The challenge, however, is to choose the right drug given the fact that the pathobiological effect of such mutations may depend on the cell type and/or co-occurrence of other deregulated genes.

The challenge in biomarker discovery and clinical implementation

An informative biomarker is preferably a genomic lesion because DNA is more stable and less vulnerable to breakdown by wrong shipment conditions than RNA and proteins. These lesions include gene mutations, gene fusions, and copy number alterations (losses and gains) that directly affect the activity of their corresponding proteins, and are a driving force for the altered more downstream signaling cascade. In practice, biomarkers with diagnostic, prognostic, predictive (for choice of targeted drugs) and pharmacodynamic (for target-specificity and efficacy testing of a drug) potency are hard to find in ALL, and are currently limited to the *BCR-ABL1* gene fusion. To optimize treatment results for individual patients, predictive biomarkers are needed to identify patients with a high likelihood of responding to a selected drug. To predict prognosis and/or which drugs would be beneficial to a patient, the technical procedure to detect biomarker-positive cells needs to be highly sensitive. Moreover, the clonality of the (genetic) lesion in the patient needs to be known. For example, are all leukemic cells affected by the same lesion, or is the mutation only present in a subclone of the leukemic cell population? In the latter case, do we first eradicate the bulk of leukemic cells and then target the subclone more specifically with a targeted drug, or should we perform an all-at-once strategy? The mutational landscape changes between initial diagnosis and relapse, some mutations disappear (e.g. *NRAS* and *NF1* mutations) whereas others become more prominent at the time of relapse (e.g. *CREBBP* and *ERG* mutations).[72] The application of mutated genes as biomarker, therefore, also largely depends on the sensitivity of techniques to detect subclones at presentation of disease. The development of molecular techniques such as next generation sequencing with a high number of reads per amplicon (high read depth) allows mutations in small subclones to be detected. Sequencing of a selected panel of genes in high-risk pediatric BCP-ALL revealed a high frequency of mutations in genes representing four signaling pathways, i.e. B-cell development (68%), TP53/RB (54%), RAS (50%) and JAK (11%) pathways.[33] Despite the fact that these mutations can be brought back to mutations affecting a few signaling pathways, the identity of single genes that were affected, as well as the site and functional consequence of these mutations, varied per patient and per subtype of ALL, illustrating the complexity of genomic lesions underlying the pathobiology of ALL. The high frequency of single gene mutations highlights another important issue of biomarker discovery and drug development, i.e. the fact that we need many well-established biomarkers for the increasing number of genomic lesions that are identified by genomics studies. The less frequently patients are found positive for a given predictive biomarker, the more patients need to be screened for that biomarker, and the longer the accrual time before a study has sufficient power to detect a difference in efficacy of a new drug. Unfortunately, the speed with which new genomic lesions are identified in the present "-omics" era, is not reflected by an increased number of proof-of-concept pre-clinical studies and subsequent early clinical trials. This is mainly caused by the fact that the development of targeted drugs lags behind and, moreover, by the limited number of relapsed and refractory cases of the specific subtypes that can be included in phase I/II clinical studies. High-quality pre-clinical data of the pathobiology of leukemia subtypes and identification of molecular biomarkers that predict subtype, prognosis and/or responsiveness to (targeted) drugs are, therefore, essential to re-design and optimize treatment protocols for ALL. The challenge is to incorporate these biomarkers and (targeted) drugs not only at relapse/refractory disease but also more upfront in therapies for newly diagnosed patients.

References

1. Conter V, Bartram CR, Valsecchi MG, Schrauder A, Panzer-Grumayer R, Moricke A, et al. Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. Blood. 2010;115:3206-14.
2. Gokbuget N, Kneba M, Raff T, Trautmann H, Bartram CR, Arnold R, et al. Adult patients with acute lymphoblastic leukemia and molecular failure display a poor prognosis and are candidates for stem cell transplantation and targeted therapies. Blood. 2012;120:1868-76.
3. Kamps WA, van der Pal-de Bruin KM, Veerman AJ, Fiocco M, Bierings M, Pieters R. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. Leukemia. 2010;24:309-19.
4. Labar B, Suciu S, Willemze R, Muus P, Marie JP, Fillet G, et al. Dexamethasone compared to prednisolone for adults with acute lymphoblastic leukemia or lymphoblastic lymphoma: final results of the ALL-4 randomized, phase III trial of the EORTC Leukemia Group. Haematologica. 2010;95:1489-95.
5. Harrison CJ. Acute lymphoblastic leukemia. Clin Lab Med. 2011;31:631-47.
6. Moorman AV, Chilton L, Wilkinson J, Ensor HM, Bown N, Proctor SJ. A population-based cytogenetic study of adults with acute lymphoblastic leukemia. Blood. 2010;115:206-14.

7. Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol. 2011;29:551-65.
8. Moricke A, Zimmermann M, Reiter A, Henze G, Schrauder A, Gadner H, et al. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia. 2010;24:265-84.
9. Van der Veer A, Willemse ME, De Haas V, Veerman AJP, Kamps W, Pieters R, et al. Unfavorable prognostic value of IKAROS but not CRLF2 in children with BCRABL1-positive acute lymphoblastic leukemia. ASH Annual Meeting Abstracts. Blood. 2011;118:1504-5.
10. Waanders E, van der Velden VH, van der Schoot CE, van Leeuwen FN, van Reijmersdal SV, de Haas V, et al. Integrated use of minimal residual disease classification and IKZF1 alteration status accurately predicts 79% of relapses in pediatric acute lymphoblastic leukemia. Leukemia. 2011;25:254-8.
11. Cortes JE, Kantarjian H, Shah NP, Bixby D, Mauro MJ, Flinn I, et al. Ponatinib in refractory Philadelphia chromosome-positive leukemias. N Engl J Med. 2012;367:2075-88.
12. Crews KR, Hicks JK, Pui CH, Relling MV, Evans WE. Pharmacogenomics and individualized medicine: translating science into practice. Clin Pharmacol Ther. 2012;92:467-75.
13. Bruggemann M, van der Velden VH, Raff T, Droese J, Ritgen M, Pott C, et al. Rearranged T-cell receptor beta genes represent powerful targets for quantification of minimal residual disease in childhood and adult T-cell acute lymphoblastic leukemia. Leukemia. 2004;18:709-19.
14. Van der Velden VH, de Bie M, van Wering ER, van Dongen JJ. Immunoglobulin light chain gene rearrangements in precursor-B-acute lymphoblastic leukemia: characteristics and applicability for the detection of minimal residual disease. Haematologica. 2006;91:679-82.
15. Aricò M, Schrappe M, Hunger SP, Carroll WL, Conter V, Galimberti S, et al. Clinical outcome of children with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia treated between 1995 and 2005. J Clin Oncol. 2010;28:4755-61.
16. Biondi A, Schrappe M, De Lorenzo P, Castor A, Lucchini G, Gandemer V, et al. Imatinib after induction for treatment of children and adolescents with Philadelphia-chromosome-positive acute lymphoblastic leukaemia (EsPhALL): a randomised, open-label, intergroup study. Lancet Oncol. 2012;13:936-45.
17. Gleißner B, Gökbuget N, Bartram CR, Janssen B, Rieder H, Janssen JWG, et al. Leading prognostic relevance of the BCR-ABL translocation in adult acute B-lineage lymphoblastic leukemia: a prospective study of the German Multicenter Trial Group and confirmed polymerase chain reaction analysis. Blood. 2002;99:1536-43.
18. Gruber F, Mustjoki S, Porkka K. Impact of tyrosine kinase inhibitors on patient outcomes in Philadelphia chromosome-positive acute lymphoblastic leukaemia. Br J Haematol. 2009;145:581-97.
19. Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, et al. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood. 2002;99:3472-5.
20. O'Hare T, Walters DK, Stoffregen EP, Jia T, Manley PW, Mestan J, et al. In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. Cancer Res. 2005;65:4500-5.
21. Khoury HJ, Cortes JE, Kantarjian HM, Gambacorti-Passerini C, Baccarani M, Kim DW, et al. Bosutinib is active in chronic phase chronic myeloid leukemia after imatinib and dasatinib and/or nilotinib therapy failure. Blood. 2012;119:3403-12.
22. O'Hare T, Shakespeare WC, Zhu X, Eide CA, Rivera VM, Wang F, et al. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell-2009;16:401-12.
23. Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. Nat Rev Cancer-2005;5:172-83.
24. Malin S, McManus S, Cobaleda C, Novatchkova M, Delogu A, Bouillet P, et al. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. Nat Immunol. 2010;11:171-9.
25. Hoelbl A, Schuster C, Kovacic B, Zhu B, Wickre M, Hoelzl MA, et al. Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. EMBO Mol Med. 2010;2:98-110.
26. Xie S, Wang Y, Liu J, Sun T, Wilson MB, Smithgall TE, et al. Involvement of Jak2 tyrosine phosphorylation in Bcr-Abl transformation. Oncogene. 2001;20:6188-95.
27. Ensor HM, Schwab C, Russell LJ, Richards SM, Morrison H, Masic D, et al. Demographic, clinical, and outcome features of children with acute lymphoblastic leukemia and CRLF2 deregulation: results from the MRC ALL97 clinical trial. Blood. 2011;117:2129-36.
28. Mullighan CG, Zhang J, Harvey RC, Collins-Underwood JR, Schulman BA, Phillips LA, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci USA. 2009;106:9414-8.
29. Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med. 2009;360:470-80.
30. Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JG, Peters ST, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. Lancet Oncol. 2009;10:125-34.
31. Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell. 2012;22:153-66.
32. Harvey RC, Mullighan CG, Wang X, Dobbin KK, Davidson GS, Bedrick EJ, et al. Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. Blood. 2010;116:4874-84.
33. Zhang J, Mullighan CG, Harvey RC, Wu G, Chen X, Edmonson M, et al. Key pathways are frequently mutated in high-risk childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. Blood. 2011;118:3080-7.
34. Maude SL, Tasian SK, Vincent T, Hall JW, Sheen C, Roberts KG, et al. Targeting JAK1/2 and mTOR in murine xenograft models of Ph-like acute lymphoblastic leukemia. Blood. 2012;120:3510-8.
35. Russell LJ, Capasso M, Vater I, Akasaka T, Bernard OA, Calasanz MJ, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. Blood. 2009;114:2688-98.
36. Mullighan CG, Collins-Underwood JR, Phillips LA, Loudin MG, Liu W, Zhang J, et al. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet. 2009;41:1243-6.
37. Harvey RC, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood. 2010;115:5312-21.
38. Chapiro E, Russell L, Lainey E, Kaltenbach S, Ragu C, Della-Valle V, et al. Activating mutation in the TSLPR gene in B-cell precursor lymphoblastic leukemia. Leukemia. 2010;24:642-5.
39. Shochat C, Tal N, Bandapalli OR, Palmi C, Ganmore I, te Kronnie G, et al. Gain-of-function mutations in interleukin-7 receptor-alpha (IL7R) in childhood acute lymphoblastic leukemias. J Exp Med. 2011;208:901-8.
40. Moorman AV, Schwab C, Ensor HM, Russell LJ, Morrison H, Jones L, et al. IGH@ translocations, CRLF2 deregulation, and microdeletions in adolescents and adults with acute lymphoblastic leukemia. J Clin Oncol. 2012;30:3100-8.
41. Cario G, Zimmermann M, Romey R, Gesk S, Vater I, Harbott J, et al. Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood. 2010;115:5393-7.
42. Chen IM, Harvey RC, Mullighan CG, Gastier-Foster J, Wharton W, Kang H, et al. Outcome modeling with CRLF2, IKZF1, JAK, and minimal residual disease in pediatric acute lymphoblastic leukemia: a Children's Oncology Group study. Blood. 2012;119:3512-22.
43. Attarbaschi A, Morak M, Cario G, Cazzaniga G, Ensor HM, te Kronnie T, et al. Treatment outcome of CRLF2-rearranged childhood acute lymphoblastic leukaemia: a comparative analysis of the AIEOP-BFM and UK NCRI-CCLG study groups. Br J Haematol. 2012;158:772-7.
44. Van der Veer A, Waanders E, Pieters R, Willemse ME, Van Reijmersdal SV, Russell LJ, et al. Leukemia cells with a BCR-ABL1-like signature and/or IKZF1 deletions, but not high CRLF2 expression, are predictive of an unfavorable prognosis

in childhood B cell precursor acute lymphoblastic leukemia. ASH Annual Meeting Abstracts. Blood. 2012;120.

45. Hertzberg L, Vendramini E, Ganmore I, Cazzaniga G, Schmitz M, Chalker J, et al. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: a report from the International BFM Study Group. Blood. 2010;115:1006-17.
46. Blink M, Buitenkamp TD, van den Heuvel-Eibrink MM, Danen-van Oorschot AA, de Haas V, Reinhardt D, et al. Frequency and prognostic implications of JAK 1-3 aberrations in Down syndrome acute lymphoblastic and myeloid leukemia. Leukemia. 2011;25:1365-8.
47. Buitenkamp TD, Pieters R, Gallimore NE, van der Veer A, Meijerink JP, Beverloo HB, et al. Outcome in children with Down's syndrome and acute lymphoblastic leukemia: role of IKZF1 deletions and CRLF2 aberrations. Leukemia. 2012;26:2204-11.
48. Maloney KW. Acute lymphoblastic leukaemia in children with Down syndrome: an updated review. Br J Haematol. 2011;155:420-5.
49. Tasian SK, Doral MY, Borowitz MJ, Wood BL, Chen IM, Harvey RC, et al. Aberrant STAT5 and PI3K/mTOR pathway signaling occurs in human CRLF2-rearranged B-precursor acute lymphoblastic leukemia. Blood. 2012;120:833-42.
50. Kuiper RP, Waanders E, van der Velden VH, van Reijmersdal SV, Venkatachalam R, Scheijen B, et al. IKZF1 deletions predict relapse in uniformly treated pediatric precursor B-ALL. Leukemia. 2010;24:1258-64.
51. Dorge P, Meissner B, Zimmermann M, Moericke A, Schrauder A, Bourquin JP, et al. IKZF1 deletion is an independent predictor of outcome in pediatric acute lymphoblastic leukemia treated according to the ALL-BFM 2000 protocol. Haematologica. 2013;98:428-32.
52. Caye A, Beldjord K, Mass Malo K, Drunat S, Soulier J, Gandemer V, et al. Breakpoint-specific multiplex PCR allows the detection of IKZF1 intragenic deletions and minimal residual disease monitoring in B-cell precursor acute lymphoblastic leukemia. Haematologica. 2013;98:428-32.
53. Mi JQ, Wang X, Yao Y, Lu HJ, Jiang XX, Zhou JF, et al. Newly diagnosed acute lymphoblastic leukemia in China (II): prognosis related to genetic abnormalities in a series of 1091 cases. Leukemia. 2012;26:1507-16.
54. Dupuis A, Gaub MP, Legrain M, Drenou B, Mauvieux L, Lutz P, et al. Biclonal and biallelic deletions occur in 20% of B-ALL cases with IKZF1 mutations. Leukemia. 2013;27:503-7.
55. Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J, et al. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature. 2008;453:110-4.
56. Iacobucci I, Storlazzi CT, Cilloni D, Lonetti A, Ottaviani E, Soverini S, et al. Identification and molecular characterization of recurrent genomic deletions on 7p12 in the IKZF1 gene in a large cohort of BCR-ABL1-positive acute lymphoblastic leukemia patients: on behalf of Gruppo Italiano Malattie Ematologiche dell'Adulto Acute Leukemia Working Party (GIMEMA AL WP). Blood. 2009;114:2159-67.
57. Martinelli G, Iacobucci I, Storlazzi CT, Vignetti M, Paoloni F, Cilloni D, et al. IKZF1 (Ikaros) deletions in BCR-ABL1-positive acute lymphoblastic leukemia are associated with short disease-free survival and high rate of cumulative incidence of relapse: a GIMEMA AL WP report. J Clin Oncol. 2009;27:5202-7.
58. Cazzaniga G, van Delft FW, Lo Nigro L, Ford AM, Score J, Iacobucci I, et al. Developmental origins and impact of BCR-ABL1 fusion and IKZF1 deletions in monozygotic twins with Ph+ acute lymphoblastic leukemia. Blood. 2011;118:5559-64.
59. Trageser D, Iacobucci I, Nahar R, Duy C, von Levetzow G, Klemm L, et al. Pre-B cell receptor-mediated cell cycle arrest in Philadelphia chromosome-positive acute lymphoblastic leukemia requires IKAROS function. J Exp Med. 2009;206:1739-53.
60. Hu Y, Liu Y, Pelletier S, Buchdunger E, Warmuth M, Fabbro D, et al. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. Nat Genet. 2004;36:453-61.
61. Merkenschlager M. Ikaros in immune receptor signaling, lymphocyte differentiation, and function. FEBS Lett. 2010;584:4910-4.
62. Ferreiros Vidal I, Carroll T, Taylor B, Terry A, Liang Z, Bruno L, et al. Genome-wide identification of Ikaros targets elucidates its contribution to mouse B cell lineage specification and pre-B cell differentiation. Blood. 2013;121:1769-82.
63. Iacobucci I, Iraci N, Messina M, Lonetti A, Chiaretti S, Valli E, et al. IKAROS deletions dictate a unique gene expression signature in patients with adult B-cell acute lymphoblastic leukemia. PLoS One. 2012;7:e40934.
64. Gustafsson B, Angelini S, Sander B, Christensson B, Hemminki K, Kumar R. Mutations in the BRAF and N-ras genes in childhood acute lymphoblastic leukaemia. Leukemia. 2005;19:310-2.
65. Case M, Matheson E, Minto L, Hassan R, Harrison CJ, Bown N, et al. Mutation of genes affecting the RAS pathway is common in childhood acute lymphoblastic leukemia. Cancer Res. 2008;68:6803-9.
66. Nicholson L, Knight T, Matheson E, Minto L, Case M, Sanichar M, et al. Casitas B lymphoma mutations in childhood acute lymphoblastic leukemia. Genes Chromosomes Cancer. 2012;51:250-6.
67. Holmfeldt L, Wei L, Diaz-Flores E, Walsh M, Zhang J, Ding L, et al. The genomic landscape of hypodiploid acute lymphoblastic leukemia. Nat Genet. 2013;45:242-52.
68. Paulsson K, Horvat A, Strömbeck B, Nilsson F, Heldrup J, Behrendtz M, et al. Mutations in FLT3, NRAS, KRAS and PTPN11 are frequent and possibly mutually exclusive in high hyperdiploid childhood acute lymphoblastic leukemia. Genes Chromosomes Cancer. 2008;47:26-33.
69. Stam RW, Schneider P, De Lorenzo P, Valsecchi MG, Den Boer ML, Pieters R. Prognostic significance of high-level FLT3 expression in MLL-rearranged infant acute lymphoblastic leukemia. Blood. 2007;110:2774-5.
70. Driessen EM, van Roon EH, Spijkers-Hagelstein JA, Schneider P, de Lorenzo P, Valsecchi MG, et al. Frequencies and prognostic impact of RAS mutations in MLL-rearranged acute lymphoblastic leukemia in infants. Haematologica. 2013 Feb 12 [Epub ahead of print].
71. Wiemels JL, Kang M, Chang JS, Zheng L, Kouyoumji C, Zhang L, et al. Backtracking RAS mutations in high hyperdiploid childhood acute lymphoblastic leukemia. Blood Cells Mol Dis. 2010;45:186-91.
72. Mullighan CG, Zhang J, Kasper LH, Lerach S, Payne-Turner D, Phillips LA, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. Nature. 2011;471:235-9.
73. Young A, Lou D, McCormick F. Oncogenic and Wild-type Ras Play Divergent Roles in the Regulation of Mitogen-Activated Protein Kinase Signaling. Cancer Discov. 2013;3:112-23.
74. Nguyen TK, Rahmani M, Harada H, Dent P, Grant S. MEK1/2 inhibitors sensitize Bcr/Abl+ human leukemia cells to the dual Abl/Src inhibitor BMS-354/825. Blood. 2007;109:4006-15.

Novel treatment approaches for acute lymphoblastic leukemia in childhood and adolescence

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A B S T R A C T

Systematic enrollment of children and adolescents with acute lymphoblastic leukemia (ALL) into clinical trials has greatly widened our knowledge of clinical and biological prognostic parameters. Clinical trials have significantly reduced the risk of both disease recurrence and also that of acute and late side effects. Some distinct unfavorable subgroups were identified in which treatment success is less prevalent while others have been shown to benefit from novel strategies. High tumor load (white blood cell count, WBC), lack of response, age under one year, or over ten years (more pronounced over 15 years), and (rare) cytogenetic subtypes, such as t(9;22), t(4;11), or presence of *IKZF1* may characterize a significant proportion of children and adolescents with high risk (HR-) ALL. However, these will miss the patients in the intermediate-risk group who will eventually relapse as they lack specific risk parameters. Recently, genetic signatures were developed which may characterize these new high relapse risk patients. Careful response assessment, preferably by detection of minimal residual disease (MRD), is mandatory to identify patients at risk for relapse but also those who can be spared intensive therapy. MRD monitoring may also facilitate the evaluation of novel therapies, such as functionally targeted or immunotherapeutic strategies, and allogeneic hematopoietic stem cell transplantation.

Learning goals

At completion of this activity, participants should know about:
- the clinical relevance of prognostic parameters to define risk-adapted treatment groups in childhood ALL;
- response to treatment. Exact and reproducible analysis is essential to define individual relapse risk;
- lack of treatment response or failure and possible alternative strategies.

Prognostic factors in acute lymphoblastic leukemia of childhood and adolescence: evolution and consequences for risk-adapted treatment stratification

Risk-adapted stratification in acute lymphoblastic leukemia (ALL) is first of all an issue that is in permanent evolution towards new systems. This may be heralded as progress, but obviously the complexity of new stratification systems will seriously hamper the comparability of clinical trials. Any risk-adapted stratification will depend heavily on the availability of diagnostic tools (which in turn depends on availability of resources for health care),[1,2] on the precise characterization of all clinical data including the analysis of treatment response and outcome, treatment realisation, treatment-related toxicity, and on a comprehensive data platform on which all these findings are registered and appropriately stored. Most importantly, any risk-adapted stratification depends heavily on appropriate application of chemotherapy resulting in at least 75% overall 5-year event-free survival for unselected cohorts of patients with ALL (up to 18 years of age) which has been achieved in the last two decades by most contemporary pediatric ALL study groups.[3]

Standard risk ALL (and its exceptions)

Most study groups would consider the following pediatric ALL patient groups as standard risk even though there is probably no consensus if patients with T-ALL should be excluded *per se*:

- WBC at diagnosis below 50×10^9/L
- age <10 years but > 1 year
- no central nervous system (CNS) involvement
- *ETV6/RUNX1* positivity
- MRD at Day 15 of induction therapy < 0.1%
- MRD at end of induction negative (if sensitivity reaches at least 10^{-4})

There is some debate as to whether hyperdiploid ALL can also be considered standard risk as the exact definition of hyperdiploidy varies between study groups. A large British series underlined that patients with high-hyperdiploidy (51-65 chromosomes) comprise low-relapse risk and can be considered standard risk.[4] A large prospective trial of the AIEOP and BFM Study groups implemented

MRD screening for all patients; it revealed that there are high-risk patients (defined by high levels of MRD at end of induction-consolidation which is approx. 12 weeks from diagnosis) even among hyperdiploid cases as much as among patients with *ETV6/RUNX1* positivity due to their slow response to treatment. They are characterized by residual disease at a level of 10^{-3} or higher at 12 weeks from diagnosis if treated on this regimen.[5] The authors concluded that patients harboring these genetic aberrations should be considered high-risk if a slow MRD response has been diagnosed.

Intermediate-risk ALL

The following subgroups *as such* (or any combination of them) may be considered intermediate-risk as they do not comprise standard risk features but also lack the prognostically unfavorable high-risk features:

- T-precursor cell ALL[6,7]
- t(1;19)[4,8]
- iAMP21[9,10]
- CNS involvement and/or traumatic lumbar puncture[11,12]
- WBC ≥50×10^9/L[13]
- age ≥10 years
- age <1 year

Obviously, the prognostic relevance of high WBC as much as for the two age groups listed here depends largely on the response to treatment as measured early by the prednisone response in peripheral blood,[14,15] or in bone marrow Day 15,[16] and the presence of MRD at the end of induction and later at the end of induction-consolidation.[17-19] Even in patients with intrachromosomal amplification of chromosome 21 (iAMP21) the response to treatment determines the prognostic relevance of this genetic finding.[20] The prognosis in T-ALL depends on the subtype, additional mutations, and the response to therapy.[7,21,22] In particular, the early T-precursor cell ALL subtype (ETP-ALL) may comprise a poor risk subtype[23] with very specific genetic lesions which may provide a rationale for modified treatment approaches.[24] It can be argued that those ETP-ALL patients at high risk to relapse will be picked up by measuring MRD at later time points.[7]

High-risk ALL

There is a rather large heterogeneity between study groups on how to define high-risk patients. Some rely on the NCI criteria (WBC ≥ 50×10^9/L, or age ≥10 years), others use combinations, in particular with response to treatment, or focus on genetic aberrations to define this important subgroup. In 2010, major study groups re-analyzed their results using the same risk criteria.[5,25-32] This type of comparative analysis has its limitations but it facilitates direct outcome comparisons with regard to major but also minor patient subsets.[3]

The comprehensive description of new genetic aberrations in the past few years make the general consensus on who should be considered high-risk even more challenging. Usually, any subgroup with an expected event-free survival (EFS) of less than 50% (without hematopoietic stem cell transplantation, hSCT) would qualify, given that appropriate intensive chemotherapy has been applied. The lack of general agreement is partly due to the fact that the transition from basic research (detection of new genetic lesions) to clinical application (diagnostics) is demanding, particularly in large multi-institutional study groups. In addition, the approach to assess treatment response differs between groups which implies that certain subsets of slow responders (which are usually characterized by poor prognosis) may be missed.[33-36, 7,19]

There is wide agreement that the following subgroups qualify as high-risk ALL despite some remarkable heterogeneity in some, and large improvements in others:

- t(9;22) or *BCR/ABL1* present[37-40]
- t(4;11) or *MLL* rearrangement present[4,41,42]
- hypodiploidy (modal chromosome number below 45 chromosomes)[43]
- induction failure[44]
- inadequate early response:
  - 'prednisone poor response' >1000 blasts in peripheral blood at Day 8 of therapy[26]
  - M3 marrow at Day 7 or Day 14 of induction therapy[16,45]
  - by MRD detection on Day 8, Day 15, and Day 28-33 of induction therapy (this applies in particular for pcB-ALL)[19,33,35,36,46,47]
- slow response: persisting high levels of MRD at the end of induction-consolidation (week 12) or even later [7,17]

Intrinsically refractory ALL, as defined by the lack of complete remission at the end of induction therapy, has recently been found to be a very heterogeneous subgroup of ALL. It comprises, on the one hand, patients with an overall survival (OS) at ten years of 71%±6% but also patients with an OS of less than 15%. The first group comprised patients with high-hyperdiploid ALL, whereas the latter subgroup comprised patients who had *MLL* aberrations or *BCR/ABL1*.[44] Obviously, the classical risk features such as age and WBC have been partly overcome by our growing knowledge about genetic subtypes. One example is infant ALL, in which the large proportion of patients with MLL rearrangements is the main reason for the frequent treatment failure in this age group.[41] In an earlier analysis of ALL patients with chromosomal 11q23 aberrations, age and type of translocation were leading risk factors, but no clear benefit of treatment by allogeneic hSCT could be demonstrated.[42] In the context of the clinical trial Interfant-99, the benefit of allogeneic hSCT for infants with MLL positive ALL was shown.[48]

In Philadelphia chromosome positive (Ph^+) ALL, two findings were remarkable. First, this subtype of ALL in childhood has been shown to be very heterogeneous with regard to treatment response in the era before tyrosine kinase inhibitors (TKIs) were used on a larger scale.[19,37,49] The use of MRD monitoring in the AIEOP-BFM ALL 2000 trial revealed that there are Ph^+ ALL patients who are fast responders, and so have already cleared residual disease at the end of the 5-week induction therapy, and they have an excellent outcome. Clinically, this may imply that, apart from therapy with a TKI, such patients would not qualify for allogeneic SCT in first complete remission.[19] Secondly, a COG study demonstrated large improvement in outcome by the intensive use of imatinib on top of very intensive chemotherapy which was the first time that this subgroup showed major improvement.[39] A study by the ESPhALL group used a different approach while introducing imatinib into the BFM-derived chemotherapy. It was the first clinical trial in which a randomized evaluation of

imatinib in pediatric ALL has been performed successfully.[40] More recent approaches to identify additional critical subsets in pediatric ALL have produced several interesting insights. In precursor B-cell ALL, a large number of genes involved in lymphoid development, cell-cycle control and tumor suppression, signaling pathways, or transcriptional regulation are affected by deletions, translocations, and sequence mutations.[50-53] This research may decipher important mechanisms of disease development but it will also prove clinically useful as until recently it has been difficult to characterize the significant number of disease recurrences which mostly occur in the large group of so-called intermediate-risk patients.[15,19,54,55]

Gene expression profiles in precursor B-cell ALL have been described which are reminiscent of that in Ph+ ALL and are associated with a poor prognosis.[51,56-58] As it appears that no single gene mutation or specific translocation is typical for this subset, however, it may be attractive to define a robust genetic signature which is able to reproducibly identify these patients. In a few of these so-called Ph-like ALL genetic alterations activating kinase or cytokine receptor signaling have been identified.[59] Among them are rearrangements involving *ABL1*, *JAK2, PDGFRB, CRLF2, EPOR*, and mutations of *IL7R* and *FLT3*, as well as deletions of *SH2B3*, a negative regulator of *JAK2*. As shown *in vitro*, tyrosine phosphorylation was reduced by treating leukemic cells with *ABL1*, *PDGFRB,* and *JAK2* rearrangements with tyrosine kinase or JAK2 inhibitors, respectively. Similar efficacy was demonstrated *in vivo* in a xenograft model of *BCR-JAK2* rearranged ALL treated with the JAK2 inhibitor ruxolitinib, and in a xenograft model of *NUP214-ABL1* ALL treated with dasatinib. A patient with *EBF1-PDGFRB* rearranged ALL who was refractory to induction therapy entered remission after exposure to imatinib.[59]

Deletions and mutations of the transcription factor *IKZF1,* and gene rearrangements involving *CRLF2* are recurrent alterations in pcB-ALL.[51,60,61] Alterations of *CRLF2* are often associated with activating mutations in the Janus kinase genes *JAK1* and *JAK2*.[53,60] The prognostic impact may depend on some co-factors and the patient population.[57] Thus, the prognostic significance of *CRLF2* rearrangements is being assessed differently.[61-65] The finding that the presence of the fusion gene *P2RY8/CRLF2* is associated with late relapses of intermediate risk pcB-ALL (as determined by MRD) was the most striking finding.[63] This appeared to prove that such late disease recurrences cannot be predicted by analysis of MRD, or that the driving leukemic clone escapes such disease monitoring. A direct comparison of 114 patients with *CRLF2*-rearrangements treated in two European trials (AIEOP-BFM ALL 2000 and MRC ALL97) indicated that differences in treatment may modulate the prognostic impact as shown for the fusion gene *IGH@-CRLF2*: None of the 9 patients with this rearrangement treated in the AIEOP-BFM series relapsed, but 5 of 6 with the identical aberration treated in the MRC ALL-97 trial. Remarkably, while the adverse prognostic impact of *P2RY8/CRLF2* on EFS was confirmed, it was shown that the overall survival at six years in the two cohorts (81% and 83%, respectively) was surprisingly favorable.[66]

The presence of *IKZF1* alterations has been described to be associated with poor prognosis.[51] This is most evident in *BCR/ABL1* positive patients, where genetic alterations of *IKZF1* are found in approximately 70% of the cases.[67] In unselected groups of patients, *IKZF1* deletions are found in 12% of cases, and the adverse prognostic impact was less pronounced but still significant (5y-EFS 69% *vs.* 86%, respectively).[68]

The Ph-like ALL subgroup may comprise approximately 15% of all pcB-ALL cases, among these nearly 10% present overexpression of *CRLF2* and approximately 12% present *IL7R* mutations.[59] The authors investigated an unselected cohort of high-risk pcB-ALL patients for rearrangements involving *ABL1*, *JAK2, PDGFRB,* genetic alterations that are primary targets for multikinase inhibitors such as dasatinib, or for JAK2 inhibitors like ruxolitinib. Unfortunately, they were found in less than 3% of the patients. While these genetic alterations are rare, the therapeutic efforts for this unfavorable subgroup must reach beyond these first identified targets.[58]

The clinical need for novel therapies

Most study groups that perform population-based clinical trials for *de novo* ALL focus on the following general aspects:

i) to improve risk stratification for better adaptation of treatment intensity;
ii) to investigate if the previously established system of early *in vivo* response analysis (by MRD detection) can be further refined through panels of molecular markers at time of diagnosis;
iii) to improve outcome by additional potentially more targeted interventions in selected subgroups while reducing the risk of long-term side effects;
iv) to evaluate the therapeutic benefit of alternative approaches such as immunotherapy and/or allogeneic hematopoietic stem cell transplantation in patients refractory to conventional treatment.

The first target group for new therapeutic interventions is obviously high-risk ALL. If agents are used which have not shown unique activity in other settings, it is most likely that the first patients to be treated will be patients with otherwise dismal prognosis, usually due to refractory or relapsed ALL.[69] If agents are available which have truly selective activity and do not impose additional toxicity it is attractive to introduce them in those patient subsets in which current treatment cannot achieve EFS rates comparable to those of the overall ALL population in childhood and adolescence. This has been the case for Ph+ ALL.[39,40] Unfortunately, there has been no attempt to replace toxic chemotherapy elements with more targeted agents, as has been shown for Ph+ ALL in elderly patients.[70]

A wide range of known agents in new applications (e.g. vorinostat as histone deacetylase inhibitor) or new agents in better characterized subsets of ALL patients (obatoclax for BCL-2 positive ALL; ruxolitinib for ALL with *JAK2* rearrangements or mutations; bortezomib as proteasome inhibitor) may open new therapeutic opportunities. Induction of autophagy-dependent necroptosis, in particular in glucocorticoid resistant ALL with the use of rapamycin and obatoclax *in vitro* and *in vivo*, was an important finding due to the fact that resistance to glucocorticoids is a strong indicator of high relapse risk.[71] Activated Janus kinases as potential targets for ruxolitinib have been described above and have been used in *in vivo*

models of ALL as well.[59,72] Development of bortezomib for use in pediatric ALL has been ongoing for several years, and the clinical activity in combination with cytotoxic agents is remarkable.[73,74] Derivatives of nucleosid analogs (clofabarine, nelarabine) have been successfully used in refractory patients.[69]

Considering the heterogeneity of disease, the complexity of genetic alterations (see above), and the numerous mechanisms of treatment resistance, it is very likely, however, that only combination therapies will result in substantial improvement in outcome. Refractory and maybe even the slow responding leukemias may also be approached by novel immunotherapeutic strategies. The success story of the anti-CD20 antibody rituximab opened a whole new area of activity.[75] The efficacy in pediatric non-Hodgkin's lymphoma was also remarkable.[76] The anti-CD22 antibody epratuzumab showed activity in pediatric ALL which triggered interest by several study groups.[77] The activation of patients' T cells for anti-leukemic response towards CD19 positive ALL cells by using the bi-specific chimeric tool blinatumomab showed remarkable clinical activity in adult and pediatric ALL patients.[78-80] While this approach was first planned to bridge the time towards allogeneic hSCT in patients refractory to chemotherapy (persistently MRD positive), it was surprising to see that patients have also achieved long-term remissions without hSCT.[81] This may open up interesting studies in which immunotherapy in addition to allogeneic hSCT will fill the gap left by all patients who are resistant to chemotherapy, or may even allow toxic elements to be replaced by agents which utilize different therapeutic mechanisms. Certainly, all novel approaches must be monitored for long-term activity towards disease recurrence in controlled prospective clinical trials. Importantly, these new strategies must also be carefully monitored towards any type of toxicity, which is a special responsibility for anybody in charge for pediatric patients.[82]

Conclusion

Conventional methods of risk classification in childhood ALL including standard MRD analyses provide excellent tools for clinical treatment stratification of childhood ALL. Both comprehensive molecular characterization and early identification of these patients will be essential in future clinical trials to utilize the optimal therapy in the first treatment cycles and, for those in need of it, to secure the timely introduction of potential targeted treatment based on individual molecular characteristics of leukemic cells, and for allogeneic hematopoietic stem cell transplantation. It is important that all future approaches should be evaluated in the context of classical risk-adapted treatment strategies and molecular monitoring of treatment response.

References

1. Pui CH, Schrappe M, Masera G, Nachman J, Gadner H, Eden OB, et al. Ponte di Legno Working Group: statement on the right of children with leukemia to have full access to essential treatment and report on the Sixth International Childhood Acute Lymphoblastic Leukemia Workshop. Leukemia. 2004;18(6):1043-53.
2. Stanulla M, Schrauder A. Bridging the gap between the north and south of the world: the case of treatment response in childhood acute lymphoblastic leukemia. Haematologica. 2009;94 (6):748-52.
3. Schrappe M, Nachman J, Hunger S, Schmiegelow K, Conter V, Masera G, et al. 'Educational symposium on long-term results of large prospective clinical trials for childhood acute lymphoblastic leukemia (1985-2000)'. Leukemia. 2010; 24(2):253-4.
4. Moorman AV, Ensor HM, Richards SM, Chilton L, Schwab C, Kinsey SE, et al. Prognostic effect of chromosomal abnormalities in childhood B-cell precursor acute lymphoblastic leukaemia: results from the UK Medical Research Council ALL97/99 randomised trial. Lancet Oncol. 2010;11(5):429-38.
5. Conter V, Arico M, Basso G, Biondi A, Barisone E, Messina C, et al. Long-term results of the Italian Association of Pediatric Hematology and Oncology (AIEOP) Studies 82, 87, 88, 91 and 95 for childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2):255-64.
6. Reiter A, Schrappe M, Ludwig WD, Hiddemann W, Sauter S, Henze G, et al. Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood. 1994;84(9):3122-33.
7. Schrappe M, Valsecchi MG, Bartram CR, Schrauder A, Panzer-Grumayer R, Moricke A, et al. Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. Blood. 2011;118(8):2077-84.
8. Kager L, Lion T, Attarbaschi A, Koenig M, Strehl S, Haas OA, et al. Incidence and outcome of TCF3-PBX1-positive acute lymphoblastic leukemia in Austrian children. Haematologica. 2007;92(11):1561-4.
9. Harewood L, Robinson H, Harris R, Al-Obaidi MJ, Jalali GR, Martineau M, et al. Amplification of AML1 on a duplicated chromosome 21 in acute lymphoblastic leukemia: a study of 20 cases. Leukemia. 2003;17(3):547-53.
10. Rand V, Parker H, Russell LJ, Schwab C, Ensor H, Irving J, et al. Genomic characterization implicates iAMP21 as a likely primary genetic event in childhood B-cell precursor acute lymphoblastic leukemia. Blood. 2011;117(25):6848-55.
11. Gajjar A, Harrison PL, Sandlund JT, Rivera GK, Ribeiro RC, Rubnitz JE, et al. Traumatic lumbar puncture at diagnosis adversely affects outcome in childhood acute lymphoblastic leukemia. Blood. 2000;96(10):3381-4.
12. Bürger B, Zimmermann M, Mann G, Kühl J, Löning L, Riehm H, et al. Diagnostic cerebrospinal fluid examination in children with acute lymphoblastic leukemia: significance of low leukocyte counts with blasts or traumatic lumbar puncture. J Clin Oncol. 2003;21(2):184-8.
13. Smith M, Arthur D, Camitta B, Carroll AJ, Crist W, Gaynon P, et al. Uniform approach to risk classification and treatment assignment for children with acute lymphoblastic leukemia. J Clin Oncol. 1996;14(1):18-24.
14. Riehm H, Reiter A, Schrappe M, Berthold F, Dopfer R, Gerein V, et al. [Corticosteroid-dependent reduction of leukocyte count in blood as a prognostic factor in acute lymphoblastic leukemia in childhood (therapy study ALL-BFM 83)]. Klin Padiatr. 1987;199(3):151-60.
15. Möricke A, Reiter A, Zimmermann M, Gadner H, Stanulla M, Dördelmann M, et al. Risk-adjusted therapy of acute lymphoblastic leukemia can decrease treatment burden and improve survival: treatment results of 2169 unselected pediatric and adolescent patients enrolled in the trial ALL-BFM 95. Blood. 2008;111(9):4477-89.
16. Lauten M, Moricke A, Beier R, Zimmermann M, Stanulla M, Meissner B, et al. Prediction of outcome by early bone marrow response in childhood acute lymphoblastic leukemia treated in the ALL-BFM 95 trial: differential effects in precursor B-cell and T-cell leukemia. Haematologica. 2012;97(7):1048-56.
17. van Dongen JJ, Seriu T, Panzer-Grümayer ER, Biondi A, Pongers-Willemse MJ, Corral L, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet. 1998;352(9142):1731-8.
18. van der Velden VH, Corral L, Valsecchi MG, Jansen MW, de Lorenzo P, Cazzaniga G, et al. Prognostic significance of minimal residual disease in infants with acute lymphoblastic leukemia treated within the Interfant-99 protocol. Leukemia. 2009;23(6):1073-9.
19. Conter V, Bartram CR, Valsecchi MG, Schrauder A, Panzer-Grumayer R, Moricke A, et al. Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study.

Blood. 2010;115(16):3206-14.
20. Attarbaschi A, Mann G, Panzer-Grumayer R, Rottgers S, Steiner M, Konig M, et al. Minimal residual disease values discriminate between low and high relapse risk in children with B-cell precursor acute lymphoblastic leukemia and an intrachromosomal amplification of chromosome 21: the Austrian and German acute lymphoblastic leukemia Berlin-Frankfurt-Munster (ALL-BFM) trials. J Clin Oncol. 2008;26(18):3046-50.
21. Breit S, Stanulla M, Flohr T, Schrappe M, Ludwig WD, Tolle G, et al. Activating NOTCH1 mutations predict favorable early treatment response and long-term outcome in childhood precursor T-cell lymphoblastic leukemia. Blood. 2006;108(4):1151-7.
22. Bandapalli OR, Zimmermann M, Kox C, Stanulla M, Schrappe M, Ludwig WD, et al. NOTCH1 activation clinically antagonizes the unfavorable effect of PTEN inactivation in BFM-treated children with precursor T-cell acute lymphoblastic leukemia. Haematologica. 2013 April 12. [Epub ahead of print.]
23. Coustan-Smith E, Mullighan CG, Onciu M, Behm FG, Raimondi SC, Pei D, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. Lancet Oncol. 2009;10(2):147-56.
24. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012;481(7380):157-63.
25. Gaynon PS, Angiolillo AL, Carroll WL, Nachman JB, Trigg ME, Sather HN, et al. Long-term results of the children's cancer group studies for childhood acute lymphoblastic leukemia 1983-2002: a Children's Oncology Group Report. Leukemia. 2010;24(2):285-97. 26. Moricke A, Zimmermann M, Reiter A, Henze G, Schrauder A, Gadner H, et al. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia. 2010;24(2):265-84.
27. Silverman LB, Stevenson KE, O'Brien JE, Asselin BL, Barr RD, Clavell L, et al. Long-term results of Dana-Farber Cancer Institute ALL Consortium protocols for children with newly diagnosed acute lymphoblastic leukemia (1985-2000). Leukemia. 2010;24(2):320-34.
28. Schmiegelow K, Forestier E, Hellebostad M, Heyman M, Kristinsson J, Soderhall S, et al. Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2):345-54.
29. Mitchell C, Richards S, Harrison CJ, Eden T. Long-term follow-up of the United Kingdom medical research council protocols for childhood acute lymphoblastic leukaemia, 1980-2001. Leukemia. 2010;24(2):406-18.
30. Tsuchida M, Ohara A, Manabe A, Kumagai M, Shimada H, Kikuchi A, et al. Long-term results of Tokyo Children's Cancer Study Group trials for childhood acute lymphoblastic leukemia, 1984-1999. Leukemia. 2010;24(2):383-96.
31. Salzer WL, Devidas M, Carroll WL, Winick N, Pullen J, Hunger SP, et al. Long-term results of the pediatric oncology group studies for childhood acute lymphoblastic leukemia 1984-2001: a report from the children's oncology group. Leukemia. 2010;24(2):355-70.
32. Pui CH, Pei D, Sandlund JT, Ribeiro RC, Rubnitz JE, Raimondi SC, et al. Long-term results of St Jude Total Therapy Studies 11, 12, 13A, 13B, and 14 for childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2):371-82.
33. Borowitz MJ, Devidas M, Hunger SP, Bowman WP, Carroll AJ, Carroll WL, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. Blood. 2008;111(12):5477-85.
34. Schultz KR, Pullen DJ, Sather HN, Shuster JJ, Devidas M, Borowitz MJ, et al. Risk- and response-based classification of childhood B-precursor acute lymphoblastic leukemia: a combined analysis of prognostic markers from the Pediatric Oncology Group (POG) and Children's Cancer Group (CCG). Blood. 2007;109(3):926-35.
35. Basso G, Veltroni M, Valsecchi MG, Dworzak MN, Ratei R, Silvestri D, et al. Risk of relapse of childhood acute lymphoblastic leukemia is predicted by flow cytometric measurement of residual disease on day 15 bone marrow. J Clin Oncol. 2009;27(31):5168-74.
36. Ratei R, Basso G, Dworzak M, Gaipa G, Veltroni M, Rhein P, et al. Monitoring treatment response of childhood precursor B-cell acute lymphoblastic leukemia in the AIEOP-BFM-ALL 2000 protocol with multiparameter flow cytometry: predictive impact of early blast reduction on the remission status after induction. Leukemia. 2009;23(3):528-34.
37. Arico M, Valsecchi MG, Camitta B, Schrappe M, Chessells J, Baruchel A, et al. Outcome of treatment in children with Philadelphia chromosome-positive acute lymphoblastic leukemia. N Engl J Med. 2000;342(14):998-1006.
38. Arico M, Schrappe M, Hunger SP, Carroll WL, Conter V, Galimberti S, et al. Clinical outcome of children with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia treated between 1995 and 2005. J Clin Oncol. 2010;28(31):4755-61.
39. Schultz KR, Bowman WP, Aledo A, Slayton WB, Sather H, Devidas M, et al. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. J Clin Oncol. 2009;27(31):5175-81.
40. Biondi A, Schrappe M, De Lorenzo P, Castor A, Lucchini G, Gandemer V, et al. Imatinib after induction for treatment of children and adolescents with Philadelphia-chromosome-positive acute lymphoblastic leukaemia (EsPhALL): a randomised, open-label, intergroup study. Lancet Oncol. 2012;13(9):936-45.
41. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, et al. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. Lancet. 2007;370(9583):240-50.
42. Pui CH, Gaynon PS, Boyett JM, Chessells JM, Baruchel A, Kamps W, et al. Outcome of treatment in childhood acute lymphoblastic leukaemia with rearrangements of the 11q23 chromosomal region. Lancet. 2002;359(9321):1909-15.
43. Nachman JB, Heerema NA, Sather H, Camitta B, Forestier E, Harrison CJ, et al. Outcome of treatment in children with hypodiploid acute lymphoblastic leukemia. Blood. 2007;110(4):1112-5.
44. Schrappe M, Hunger SP, Pui CH, Saha V, Gaynon PS, Baruchel A, et al. Outcomes after induction failure in childhood acute lymphoblastic leukemia. N Engl J Med. 2012;366(15):1371-81.
45. Gaynon PS, Desai AA, Bostrom BC, Hutchinson RJ, Lange BJ, Nachman JB, et al. Early response to therapy and outcome in childhood acute lymphoblastic leukemia: a review. Cancer. 1997;80(9):1717-26.
46. Dworzak MN, Froschl G, Printz D, Mann G, Potschger U, Muhlegger N, et al. Prognostic significance and modalities of flow cytometric minimal residual disease detection in childhood acute lymphoblastic leukemia. Blood. 2002;99(6):1952-8.
47. Gaipa G, Cazzaniga G, Valsecchi MG, Panzer-Grumayer R, Buldini B, Silvestri D, et al. Time point-dependent concordance of flow cytometry and real-time quantitative polymerase chain reaction for minimal residual disease detection in childhood acute lymphoblastic leukemia. Haematologica. 2012;97(10):1582-93.
48. Mann G, Attarbaschi A, Schrappe M, De Lorenzo P, Peters C, Hann I, et al. Improved outcome with hematopoietic stem cell transplantation in a poor prognostic subgroup of infants with mixed-lineage-leukemia (MLL)-rearranged acute lymphoblastic leukemia: results from the Interfant-99 Study. Blood. 2010;116(15):2644-50.
49. Schrappe M, Arico M, Harbott J, Biondi A, Zimmermann M, Conter V, et al. Philadelphia chromosome-positive (Ph+) childhood acute lymphoblastic leukemia: good initial steroid response allows early prediction of a favorable treatment outcome. Blood. 1998;92(8):2730-41.
50. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446(7137):758-64.
51. Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med. 2009;360(5):470-80.
52. Mullighan CG, Zhang J, Harvey RC, Collins-Underwood JR, Schulman BA, Phillips LA, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci USA. 2009;106(23):9414-8.
53. Hertzberg L, Vendramini E, Ganmore I, Cazzaniga G, Schmitz M, Chalker J, et al. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: a report from the International BFM Study Group. Blood. 2010;115(5):1006-17.
54. Mullighan CG, Downing JR. Global genomic characterization of acute lymphoblastic leukemia. Semin Hematol. 2009;46(1):3-15.

55. Hunger SP, Raetz EA, Loh ML, Mullighan CG. Improving outcomes for high-risk ALL: translating new discoveries into clinical care. Pediatr Blood Cancer. 2011;56(6):984-93.
56. Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JG, Peters ST, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. Lancet Oncol. 2009;10(2):125-34.
57. Harvey RC, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood. 2010;115(26):5312-21.
58. Loh ML, Zhang J, Harvey RC, Roberts K, Payne-Turner D, Kang H, et al. Tyrosine kinome sequencing of pediatric acute lymphoblastic leukemia: a report from the Children's Oncology Group TARGET Project. Blood. 2013;121(3):485-8.
59. Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell. 2012;22(2):153-66.
60. Mullighan CG, Collins-Underwood JR, Phillips LA, Loudin MG, Liu W, Zhang J, et al. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. Nat Genet. 2009;41(11):1243-6.
61. Chen IM, Harvey RC, Mullighan CG, Gastier-Foster J, Wharton W, Kang H, et al. Outcome modeling with CRLF2, IKZF1, JAK, and minimal residual disease in pediatric acute lymphoblastic leukemia: a Children's Oncology Group study. Blood. 2012;119(15):3512-22.
62. Harvey RC, Mullighan CG, Wang X, Dobbin KK, Davidson GS, Bedrick EJ, et al. Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. Blood. 2010;116(23):4874-84.
63. Cario G, Zimmermann M, Romey R, Gesk S, Vater I, Harbott J, et al. Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood. 2010;115(26):5393-7.
64. Ensor HM, Schwab C, Russell LJ, Richards SM, Morrison H, Masic D, et al. Demographic, clinical, and outcome features of children with acute lymphoblastic leukemia and CRLF2 deregulation: results from the MRC ALL97 clinical trial. Blood. 2011;117(7):2129-36.
65. Palmi C, Vendramini E, Silvestri D, Longinotti G, Frison D, Cario G, et al. Poor prognosis for P2RY8-CRLF2 fusion but not for CRLF2 over-expression in children with intermediate risk B-cell precursor acute lymphoblastic leukemia. Leukemia. 2012;26(10):2245-53.
66. Attarbaschi A, Morak M, Cario G, Cazzaniga G, Ensor HM, te Kronnie T, et al. Treatment outcome of CRLF2-rearranged childhood acute lymphoblastic leukaemia: a comparative analysis of the AIEOP-BFM and UK NCRI-CCLG study groups. Br J Haematol. 2012;158(6):772-7.
67. Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J, et al. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature. 2008;453(7191):110-4.
68. Dorge P, Meissner B, Zimmermann M, Moericke A, Schrauder A, Bourquin JP, et al. IKZF1 deletion is an independent predictor of outcome in pediatric acute lymphoblastic leukemia treated according to the ALL-BFM 2000 protocol. Haematologica. 2012 Aug 8. [Epub ahead of print.]
69. Locatelli F, Schrappe M, Bernardo ME, Rutella S. How I treat relapsed childhood acute lymphoblastic leukemia. Blood. 2012;120(14):2807-16.
70. Wassmann B, Gokbuget N, Scheuring UJ, Binckebanck A, Reutzel R, Gschaidmeier H, et al. A randomized multicenter open label phase II study to determine the safety and efficacy of induction therapy with imatinib (Glivec, formerly STI571) in comparison with standard induction chemotherapy in elderly (>55 years) patients with Philadelphia chromosome-positive (Ph+/BCR-ABL+) acute lymphoblastic leukemia (ALL) (CSTI571ADE 10). Ann Hematol. 2003;82(11):716-20.
71. Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK, et al. Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. J Clin Invest. 2010;120(4):1310-23.
72. Maude SL, Tasian SK, Vincent T, Hall JW, Sheen C, Roberts KG, et al. Targeting JAK1/2 and mTOR in murine xenograft models of Ph-like acute lymphoblastic leukemia. Blood. 2012;120(17):3510-8.
73. Horton TM, Pati D, Plon SE, Thompson PA, Bomgaars LR, Adamson PC, et al. A phase 1 study of the proteasome inhibitor bortezomib in pediatric patients with refractory leukemia: a Children's Oncology Group study. Clin Cancer Res. 2007;13(5):1516-22.
74. Messinger YH, Gaynon PS, Sposto R, van der Giessen J, Eckroth E, Malvar J, et al. Bortezomib with chemotherapy is highly active in advanced B-precursor acute lymphoblastic leukemia: Therapeutic Advances in Childhood Leukemia & Lymphoma (TACL) Study. Blood. 2012;120(2):285-90.
75. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002;346(4):235-42.
76. Meinhardt A, Burkhardt B, Zimmermann M, Borkhardt A, Kontny U, Klingebiel T, et al. Phase II window study on rituximab in newly diagnosed pediatric mature B-cell non-Hodgkin's lymphoma and Burkitt leukemia. J Clin Oncol. 2010;28(19):3115-21.
77. Raetz EA, Cairo MS, Borowitz MJ, Blaney SM, Krailo MD, Leil TA, et al. Chemoimmunotherapy reinduction with epratuzumab in children with acute lymphoblastic leukemia in marrow relapse: a Children's Oncology Group Pilot Study. J Clin Oncol. 2008;26(22):3756-62.
78. Topp MS, Kufer P, Gokbuget N, Goebeler M, Klinger M, Neumann S, et al. Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. J Clin Oncol. 2011;29(18):2493-8.
79. Klinger M, Brandl C, Zugmaier G, Hijazi Y, Bargou RC, Topp MS, et al. Immunopharmacologic response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab. Blood. 2012;119(26):6226-33.
80. Handgretinger R, Zugmaier G, Henze G, Kreyenberg H, Lang P, von Stackelberg A. Complete remission after blinatumomab-induced donor T-cell activation in three pediatric patients with post-transplant relapsed acute lymphoblastic leukemia. Leukemia. 2011;25(1):181-4.
81. Topp MS, Gokbuget N, Zugmaier G, Degenhard E, Goebeler ME, Klinger M, et al. Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. Blood. 2012;120(26):5185-7.
82. Horton TM, Sposto R, Brown P, Reynolds CP, Hunger SP, Winick NJ, et al. Toxicity assessment of molecularly targeted drugs incorporated into multiagent chemotherapy regimens for pediatric acute lymphocytic leukemia (ALL): review from an international consensus conference. Pediatr Blood Cancer. 2010;54(7):872-8.

Subclonal architecture in acute myeloid leukemia

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A B S T R A C T

Increasing evidence suggests that acute myeloid leukemia (AML) develops through a process of branching evolution. Using single cell analysis, quantitative sequencing, and temporal analysis it is possible to identify the leukemic evolutionary architecture of specific mutations in individual patients: founding mutations exist in all leukemic cells, while subclonal mutations exist in only a fraction of the leukemia. Review of the current literature suggests that the balanced translocations (t(15;17), t(8;21), inv(16), and *MLL* rearrangements) and nucleotide variants in *DNMT3A* and *TET2* most commonly occur in the founding clone at diagnosis, and are neither gained nor lost at relapse. In contrast, +8, +22, -X, -Y, and nucleotide variants in *FLT3, NRAS/KRAS, WT1* and *KIT* frequently occur in subclones that either emerge or are lost at relapse. Thus, understanding the subclonal architecture of individual patients will be critical to predict individual response to therapy; drugs that target mutations that exist within a subclone are unlikely to eliminate the founding clone, and will leave the patient at high risk of relapse.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe the role of branching evolution in the acquisition of AML-associated mutations;
- describe the most common AML mutations that exist in founding clones *versus* in subclones;
- predict response to targeted therapy based on an understanding of a patient's subclonal architecture.

Introduction

The last decade of acute myeloid leukemia (AML) study has resulted in an increased ability to detect pathogenic mutations and an expanding pharmacopeia of agents that specifically target many of these mutations. At the same time, there is increased awareness that cancer mutations are situated within a larger genomic structure of branching evolution (reviewed in[1]).

Branching evolution was originally proposed as a common oncogenic mechanism by Peter Nowell, based on karyotypic evaluation of diverse cancers.[2] Testa *et al.*[3] demonstrated that metaphase karyotype could also identify complex, branching evolution in select AML cases.

The application of technologies with increasing resolution has demonstrated that most, if not all, cases of AML emerge through a process of branching evolution. Southern blot, spectral karyotyping (SKY), fluorescence *in situ* hybridization (FISH), comparative genomic hybridization/single nucleotide polymorphism (CGH/SNP) arrays, polymerase chain reaction (PCR), and now next-generation sequencing have all improved our ability to detect subclones and to integrate mutations into a clonal hierarchy.[4-10]

In order to fully integrate patient-specific mutations and targeted agents into clinical care, it will be imperative to understand each-mutation on three axes: 1) is it a 'driver' *versus* 'passenger'; 2) is it an 'initiation' *versus* 'progression' event; and 3) is it situated in a 'founding clone' *versus* a 'subclone'. Figure 1 models the relationship of leukemia-associated mutations and evolution following selection pressure applied by chemotherapy: initiation mutations exist within the founding clone and are found in all AML cells. Progression mutations emerge later in leukemic evolution, can be found in subclones, and exist in only a fraction of AML cells. Thus, selection pressure in the form of chemotherapy can favor the elimination or outgrowth of different branches within the AML evolutionary tree; mutations within the founding clones will be present in all branches, while mutations found in sub-clones can emerge or be eliminate at progression.

Mutation evolutionary hierarchy can be either directly measured or can be inferred based on temporal changes. Mutations that were present at diagnosis and lost at relapse must have existed in a subclone that was eliminated by therapy (Figure 1A). Mutations that are absent at diagnosis and present at relapse must have existed in a resistant subclone not detected at diagnosis or have been acquired by a cell that randomly escaped chemotherapy (the latter cannot be distinguished from the former if the subclone exists below the level of detection at diagnosis) (Figure 1B). Therapy that eradicates all leukemia cells must target a

population of cells that share a set of susceptible mutations (*e.g.* founding clone mutations) (Figure 1C). Thus, interpreting the outcomes of targeted therapies will require an understanding of sequential mutations, evolution dynamics, and selection pressures. This paper will review the current understanding of AML mutations in the context of subclonal architecture and of dynamic change at relapse, with a focus on which mutations most frequently occur in subclones *versus* in a founding clone.

Subclonal architecture assessed by karyotype

Subclonal architecture can be determined through a variety of mechanisms. Karyotype analysis provides a single cell, low-resolution analysis of genome-wide structural variants. Because karyotype analysis routinely assesses only a small number of metaphases (usually 20), this approach is inadequate to determine if a variant exists in 'all' leukemia cells. However, when two variants co-occur, the subclonal architecture (as related to these two genetic changes) can be determined as both mutations are simultaneously evaluated in single cells.

The translocation t(15;17) occurs concurrently with +8 in 25-40% of cases.[11-14] Combined data from t(15;17)-positive APL patients assessed in 6 separate studies identified 36 cases with concurrent +8 and information regarding the frequency of these two mutations within the leukemia cells.[14-19] Of these, 16 cases presented with +8 in a subclone (44%); in each case additional t(15;17) cells were present that lacked +8, while all +8 positive cells carried t(15;17). Loss of chromosome 7 co-occurs less frequently with t(15;17), although this too is frequently observed in a subclone, again suggesting the t(15;17) may be the founding event.[17] Consistent with these findings, APL patients with additional cytogenetic abnormalities had similar outcomes compared with patients presenting with isolated t(15;17).[11-13,19-22] Collectively, these data suggest that t(15;17) is likely to be the founding mutation in APL and that +8 and -7 are more likely to occur in subclones. This may explain why chemosensitivity in APL is predominantly determined by the presence of t(15;17).

Trisomy 8 also co-occurs frequently with the core binding factor (CBF) translocations t(8;21), t(16;16), and inv(16).[23,24] Within the CBF leukemias, co-occurring structural variants show distinct patterns: +13, and +22 co-occur with inv(16), while -X and -Y co-occur almost exclusively with t(8;21).[24,25] Regardless of this, all of these additional structural variants are typically observed in subclones by karyotype.[24,25] Also, the presence of additional karyotypic abnormalities generally does not affect clinical outcome[26,27] (the exceptions being *KIT* mutations and secondary CBF AML),[28-30] suggesting that the CBF translocation may be a critical sensitizer to high-dose cytarabine.

Paired analysis at diagnosis and relapse permits identification of structural variants that are stable over time (likely founding variants) and variants that are gained or lost (subclonal variants). Four studies evaluating pair-wise samples by metaphase karyotype found that half of all patients demonstrated genomic evolution at relapse, and half of the patients who presented initially with a normal karyotype retained a normal karyotype.[3,31-33] This would suggest that AML is fundamentally not a disease associated with an unstable genome, but it is rather a disease that emerges through constrained clonal evolution.

Few cases have been reported that show balanced translocations gained at relapse.[34-36] Some authors suggest that gains at relapse were likely cases of false negatives, as many were associated with M2 or M3 morphology at both diagnosis and relapse, in t(8;21) and t(15;17)-positive

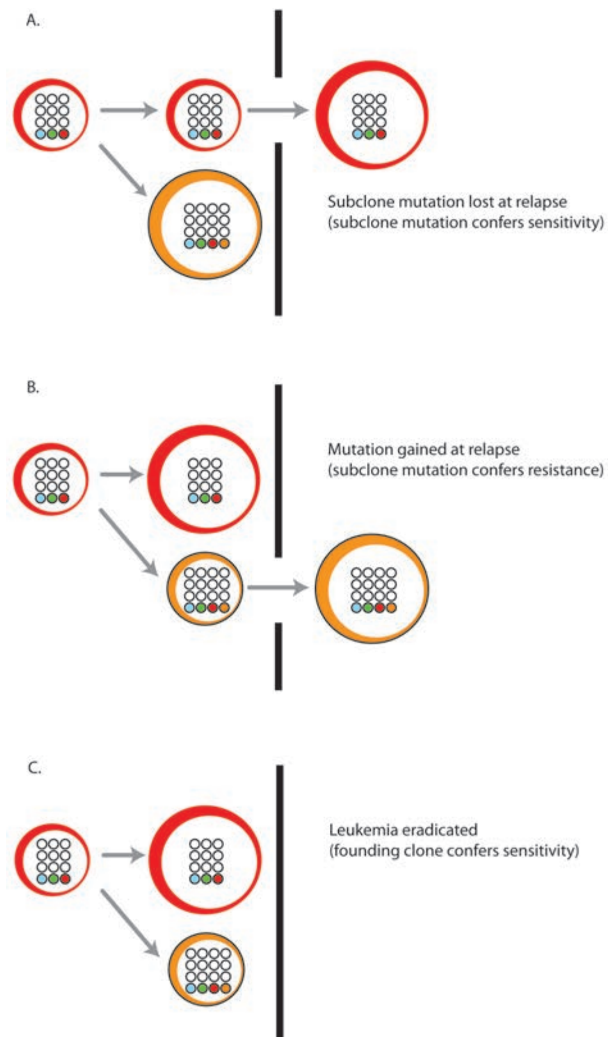

Figure 1. Model of AML mutational evolution. AML cells contain both 'driver' (colored circles), and 'passenger' (open circles) mutations. There are many more 'passenger' mutations than 'driver' mutations. 'Initiation' mutations occur within the founding clone and are found in all AML cells. Branching evolution occurs as 'progression' mutations give rise to new subclones with growth advantages. The extrinsic pressure of chemotherapy results in different outcomes depending on the sensitivities of mutations within the founding *versus* subclones. (A) Subclone lost following chemotherapy: consistent with a mutation in the subclone conferring sensitivity to the therapy. At relapse, mutations in the subclone will no longer be identified. (B) Subclone gained following chemotherapy: consistent with a mutation in the subclone conferring resistance to chemotherapy. At relapse, new mutations will be identified. These mutations may have been detected at low levels at diagnosis, or may have existed below the level of detection at diagnosis. (C) Clearance of all AML cells, consistent with sensitivity of mutations in the founding clone. No cells relapse because the initiation mutation was in all AML cells and was sensitive to therapy.

cases, respectively.[31,32] Likewise, losses of the balanced translocations are only rarely reported, but can recur at relapse despite long remission intervals;[37] those cases with loss of a balanced translocation may include new *RUNX1* and *MLL* translocations at diagnosis, suggesting that these relapses were treatment-related malignancies that may have emerged independently from the initial leukemia.[38] Furthermore, in pediatric leukemias, these translocations can occasionally be detected in Guthrie card smears (although not as commonly as acute lymphocytic leukemia balanced translocations), while other variants (*e.g. FLT3* mutations) are not detected.[39-43]

Other cytogenetic abnormalities are frequently gained or lost at relapse. Trisomy 8 is the most frequently observed chromosomal gain at relapse (22 of 236 separate cases).[3,32,33] This suggests that +8 is likely to be a co-operating event that tends to occur in subclones, rather than a founding event. Likewise, the secondary structural variants that are observed in core binding factor (CBF) leukemias are also more prevalent at relapse (+13, +22, -X, and –Y).[24,25] Additional variants associated with karyotypic evolution at relapse include gains of 11q, and 17q, although the number of assessable cases was not always stated.[44-46] Interestingly, studies focused on leukemic evolution following stem cell transplantation have observed recurrent losses in immunologically active regions including the HLA-locus (6p), as well as recurrent losses at 5q, 9q, 12p13, 13q12.2, and 17p13, and gains at 15q.[44,47]

Although it is possible for two independent karyotypic clones to co-exist within the same patient, this has only rarely been reported.[8,31,46,48] The addition of spectral karyotyping was sufficient to identify a shared variant in what otherwise appeared to be two independent leukemic clones, suggesting that higher resolution analysis may be able to identify shared founding variants in many such cases, and that most of these cases are likely to represent subclones of a founding clone associated with variants not detected by standard karyotype, rather than two truly independent clones.[8]

Subclonal architecture determined by nucleotide variants

Nucleotide variants also have been assessed at diagnosis and relapse. In addition to temporal analysis, two alternative approaches have been employed to determine the subclonal architecture of individual cases at single time points. First, digital sequencing quantifies the variant allele frequency (VAF: how commonly a mutation occurs within a population of cells); clusters of VAFs can then identify mutations that occur in subclones *versus* in the founding clone. However, mutations that occur in less than 5% of sampled cells are likely to be missed, and subclones with overlapping average VAFs are indistinguishable. Second, single cell analysis can be performed by FISH, and recently by PCR.[4,49] However, FISH studies are limited to structural abnormalities, and single cell multiplexed PCR remains technically challenging. To increase DNA yield, colonies can be grown from single AML cells, which are subsequently analyzed. However, this approach may be biased toward subclones with augmented *ex vivo* growth potential.[10,50]

Temporal analysis of nucleotide variants has been performed by many different groups, most of whom assessed a single gene for gains and losses in paired samples at diagnosis and at relapse.[25,50-62] Meta-analysis of these results is summarized in Figure 2. The mutations most commonly gained at relapse were: *FLT3*, *KIT, NRAS/KRAS, WT1* and *CEBPA* (Figure 2A). In contrast, several other genes do not appear to gain mutations at relapse: *NPM1, DNMT3A, IDH1/2* and *TET2.* Similarly, loss of a mutation at relapse has been observed in *FLT3,*

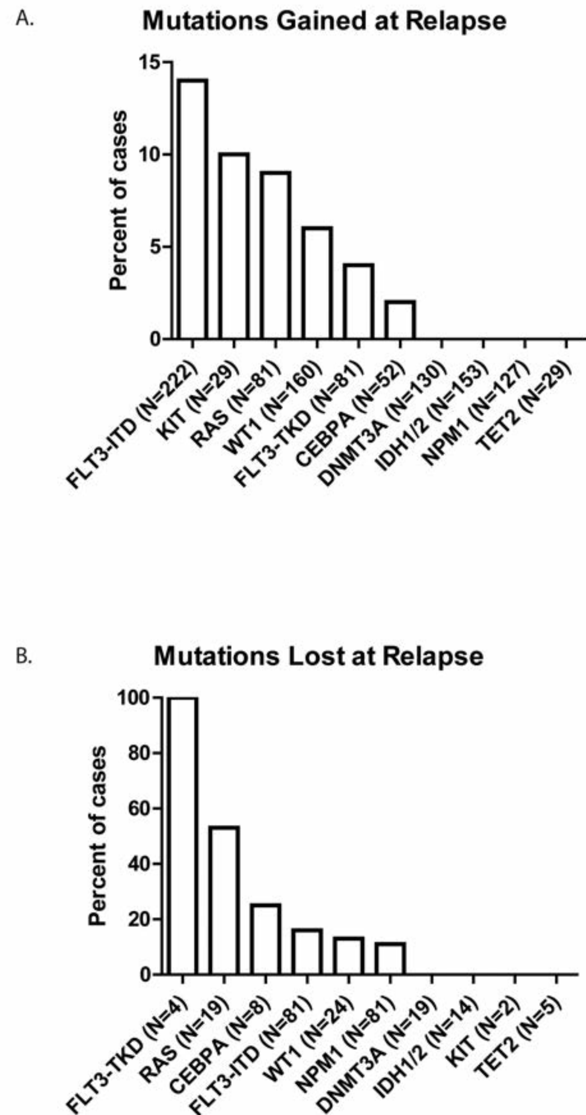

Figure 2. Meta-analysis of nucleotide variants gained at relapse. Pubmed was searched for reported cases of paired diagnosis and relapse samples that were analyzed for AML mutations. (A) Frequency of mutations that are gained at relapse. These mutations are absent in the diagnosis sample and present in the relapse sample and are consistent with being in an evolving subclone. (B) Frequency of mutations that are lost at relapse. These mutations are present in the diagnosis sample and absent in the relapse sample, and are consistent with being in a subclone that was eliminated.

NRAS/KRAS, CEBPA, WT1 and *NPM1* (Figure 2B). Collectively, these data suggest that *FLT3, KIT, NRAS/KRAS* and *WT1* mutations frequently occur in subclones (*e.g.* they are co-operating events) that may emerge or disappear at relapse. These results are consistent with the recent results of 440 paired AML cases, except that loss of *IDH1/2* was reported in 13 of 42 patients.[36]

Due to sampling limitations, it is impossible to know whether a mutation that is gained at relapse existed below the level of detection in a minor subclone at diagnosis or whether the mutation was gained after therapy in a cell that randomly survived chemotherapy (Figure 1B). Higher sensitivity PCR-based platforms have detected *KIT* mutations that were missed by Sanger sequencing. Interestingly, in this analysis, Wakita *et al.* identified 3 patients with *KIT* mutations that were not detected at diagnosis by Sanger sequencing, but were detected at relapse with this method. In all 3 cases, the mutation could be detected at diagnosis by high sensitivity PCR methodologies, suggesting that these mutations pre-existed in rare cells at diagnosis (*e.g.* minor subclones).[25] Likewise, patients with new *FLT3* variants at relapse tend to relapse more quickly than patients without new *FLT3* mutations (6.6 *vs.* 13.5 months).[63] This short window of time from treatment to relapse suggests that *FLT3* mutations that are 'gained' at relapse are likely to have pre-existed in an undetected subclone, rather than to have been acquired later in a cell from the founding clone that survived. Similar analysis of other mutations that are gained at relapse has not yet been performed.

Our group has recently used deep-digital read-counts to quantify somatic mutations in individual AML patients identified during whole genome sequencing. We found that half of the 24 cases of M1 and M3 AML had one or more subclones, in addition to a founding clone.[9] We identified cases with *NRAS, FLT3*, *ETV6* and *EWSR1* mutations clustering within distinct subclones, while *NPM1, IDH1* and *SMC1A* variants were observed within the founding clone of individual cases (Figure 3). This approach remains expensive, and only a limited number of cases have been studied. Subclonal architecture requires multiple variants per subclone to accurately define the subclone. Thus, exome sequencing is typically inadequate in AML cases due to the small number of exome variants per genome (typically 10-20). Furthermore, it remains technically challenging to quantify the subclonal identity of structural variants and indels (small insertions and deletions) using this approach.

Paired whole genome sequencing at diagnosis and relapse followed by deep digital sequencing improves identification of variants within subclones. This approach was applied to 7 cases of AML, and identified *FLT3, IDH1* and *ETV6* variants within leukemic subclones.[64] Furthermore, variants in *NPM1, DNMT3A, SMC3, WT1, RUNX1* and *IDH2* were identified in individual founding clones at diagnosis and relapse. Similarly, paired analysis of myelodysplastic syndromes (MDS) and subsequent secondary AML identified *WT1, PTPN11, RUNX1* and *SMC3*

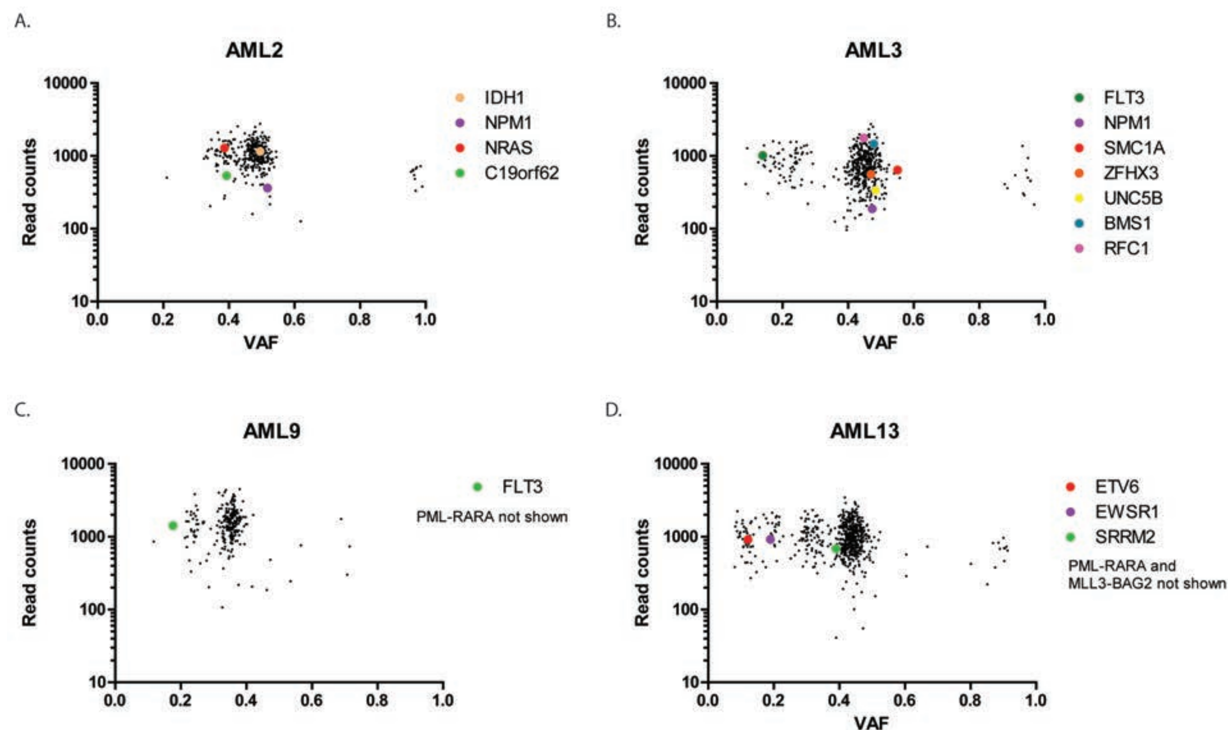

Figure 3. Subclonal architecture in 4 AML cases determined by whole-genome sequencing. The variant allele frequency (VAF: the number of reads with a mutation divided by the total number of reads at that nucleotide position) and total read counts for each validated variant in the 4 cases are indicated. Variants that are likely to be 'driver' mutations are indicated in color. Mutations in these genes are either recurrently observed in AML or in the Cosmic database. Note the subclonal occurrence of the mutations in *NRAS, FLT3* and *ETV6*.

in subclones gained in at least one secondary AML case, but absent in corresponding MDS sample.[65] Mutations in *STAG2, TP53* and *U2AF1* were observed in nearly all cells in both the MDS cells and in the secondary AML cells in 3 separate patients, respectively, suggesting these mutations existed in the founding MDS clone that gave rise to the subsequent secondary AML.

Jan *et al.* recently combined exome sequencing of leukemic cells with flow sorting of residual 'normal' hematopoietic stem cells (HSCs) to identify evolutionarily early *versus* late variants.[10] Five *FLT3*-positive cases were assessed; in all 5 cases, the *FLT3* variant was identified in the leukemic sample but not in the HSC samples. In contrast, variants in *NPM1, TET2* and *SMC1A* were identified in both the leukemic and HSC samples of at least one case each, suggesting that these variants occurred early during leukemic evolution and are likely to be founding events.

Two groups have looked at genetic changes associated with mosaic hematopoiesis. X-inactivation ratios in females have been known to develop age-associated skewing, especially in the hematopoietic compartment.[66,67] Furthermore, this phenomenon tends to be myeloid biased.[67] Laurie *et al.* retrospectively analyzed SNP arrays obtained for non-hematologic genome-wide association study (GWAS); since peripheral blood was used as the source of genetic information in these cases, they could be assessed for acquired, hematologic structural alterations if these occurred in more that 5% of blood cells.[68] They identified mosaic hematopoiesis in multiple cases, and noted an increasing incidence that was proportional to age. Furthermore, they observed recurrent deletions involving *DNMT3A, TET2* and *RB1.* Likewise, Busque *et al.* sequenced *TET2* in patients with X-inactivation skewing and asymptomatic mosaic hematopoiesis.[69] They identified 10 of 182 cases with clonal mutations in *TET2.*

Finally, single cells can be grown *ex vivo* in clonogenic assays, and individual colonies can be assessed for mutation combinations. Because each colony is derived from a single cell, this permits effective clustering of co-occurring variants. Price *et al.* derived 26 colonies from a single patient and evaluated these for trisomy 8 and for an *NRAS* mutation. They found +8 in 25 of 26 colonies and an *NRAS* variant in only 19 of 26 colonies, consistent with sequential acquisition of the *NRAS* variant in a subclone that already carried +8.[70]

Further data involving more cases will be required to better understand the frequency with which each variant occurs in subclones *versus* the founding clone. However, at this time variants in *FLT3, NRAS/KRAS, WT1* and *KIT* appear to be the most commonly occurring subclonal variants, with variants in *NPM1, IDH1/2* and *CEBPA* occurring less frequently, while mutations in *DNMT3A, TET2* and cohesin genes rarely appear in subclones, and are nearly always associated with the founding clone.

Mouse models

Diverse AML mutations and fusion genes have been studied in mouse models using retroviral expression, transgenic, and knock-in strategies (reviewed in[71-73]). Naturally, these experiments are limited in their ability to predict the common subclonal architecture of AML mutations. However, it is worth noting that, consistent with many of the findings in patients described above, several models of t(15;17)/*PML-RARA,* t(8;21)/*AML1-ETO,* inv(16)/*CBF -SMMHC,* and t(11;19)/*MLL-AF9* have resulted in leukemia that phenocopies human AML. Many of these have a long latency period (~8-15 months) which can be decreased with N-ethyl-N-nitrosourea (ENU) treatment, radiation treatment, or overexpression of additional mutations, all consistent with the possibility that these fusion genes act as founding mutations that require a second hit. In contrast, models of *FLT3*-ITD, *FLT3*-TKD, *KIT* and *KRAS* lead to myeloproliferation without overt maturation arrest and leukemia, consistent with the possibility that these mutations, typically commonly observed in subclones, are more likely to be progression events rather than founding events.

Subclonal architecture and therapy implications

As modeled in Figure 1A, application of a targeted drug to a patient whose mutation of interest exists in a subclone is unlikely to eradicate the founding leukemic clone. In contrast, the most successful targeted drugs must affect an initiating event for that tumor, which will always be in the founding clone. Acute promyelocytic leukemia is an example of such a strategy. As described above, t(15;17) is likely the initiating event for this disease, and is almost universally observed in the founding clone; all-trans retinoic acid (ATRA) and arsenic both target the resultant fusion oncoprotein *PML-RARA.* The efficacy of these agents probably relates to the fact that they abrogate the initiating event, which defines the founding clone (Figure 1C). Based on this model, one would predict that cases with low *FLT3* mutant allelic burden (*e.g.* cases where the mutation is likely in a subclone) would be less susceptible to *FLT3* inhibitors, and that resistance would emerge through the selection of the founding clone (or alternative subclones). Preliminary evidence for both of these outcomes has been observed. First, Pratz *et al.*[74] correlated *ex vivo* cytotoxicity to 6 different *FLT3* inhibitors and observed that samples with low allelic burden (which suggests that the mutation is in a subclone) were less sensitive to these inhibitors than were cases with high allelic burden (which suggests that mutations are in founding clones). Second, all 9 of the patients clinically treated with AC220 who relapsed after achieving a complete response had acquired AC220-resistant *FLT3* D835 or F691 mutations within the pre-existing *FLT3*-ITD allele, and one-third of the patients who discontinued therapy for any reason also had such mutations.[75,76] Because concurrent *FLT3*-ITD and *FLT3*-TKD mutations have been observed spontaneously in 1-2% of patients,[77-79] it is possible that these variants pre-existed in a minor (undetected) subclone that was then selected by cytotoxic pressure against sensitive subclones.

Conclusions

Multiple lines of evidence now suggest that leukemogenesis involves a process of branching evolution, and that these branch points can be delineated based on shared genomic mutations within each subclone. To date, the bal-

anced translocations (*e.g.* t(15;17), t(8;21), t(16;16), inv(16), and *MLL* rearrangements) and nucleotide variants in *DNMT3A,* and *TET2* appear almost universally in the founding clone, and are likely to be initiation events. In contrast, +8, +22, -X, -Y, and variants in *FLT3, NRAS/KRAS, WT1* and *KIT* appear frequently in subclones and are, therefore, likely to be progression events. This model of leukemogenesis, and the position of these mutations within the AML subclonal architecture, has important implications for the administration and interpretation of response to targeted agents, especially because many of the most promising small molecules in development target mutations that may present in subclones rather than in the founding clone.

References

1. Greaves M, Maley CC. Clonal evolution in cancer. Nature. 2012;481:306-13.
2. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194:23-8.
3. Testa JR, Mintz U, Rowley JD, Vardiman JW, Golomb HM. Evolution of karyotypes in acute nonlymphocytic leukemia. Cancer Res. 1979;39:3619-27.
4. Anderson K, Lutz C, van Delft FW, et al. Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature. 2011;469:356-61.
5. Schmetzer HM, Gerhartz HH. Acute myeloid leukemia (AML) can be oligoclonal. Leukemia. 1993;7:1965-70.
6. Gale RE. Evaluation of clonality in myeloid stem-cell disorders. Semin Hematol. 1999;36:361-72.
7. Lo Coco F, Saglio G. Single or multistep origin of hemopoietic tumors: the contribution of clonality studies. Leukemia. 1995;9:1586-9.
8. Stark B, Jeison M, Gobuzov R, et al. Apparently unrelated clones shown by spectral karyotyping to represent clonal evolution of cryptic t(10;11)(p13;q23) in a patient with acute monoblastic leukemia. Cancer Genet Cytogen. 2000;120:105-10.
9. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in Acute Myeloid Leukemia. Cell. 2012;150:1-15.
10. Jan M, Snyder TM, Corces-Zimmerman MR, et al. Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. Sci Transl Med. 2012;4(149):149ra118.
11. De Botton S, Chevret S, Sanz M, et al. Additional chromosomal abnormalities in patients with acute promyelocytic leukaemia (APL) do not confer poor prognosis: results of APL 93 trial. Br J Haematol. 2000;111:801-6.
12. Schoch C, Haase D, Haferlach T, et al. Incidence and implication of additional chromosome aberrations in acute promyelocytic leukaemia with translocation t(15;17)(q22;q21): a report on 50 patients. Br J Haematol. 1996;94:493-500.
13. Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood. 1998;92:2322-33.
14. Berger R, Le Coniat M, Derre J, Vecchione D, Jonveaux P. Cytogenetic studies in acute promyelocytic leukemia: a survey of secondary chromosomal abnormalities. Genes Chromosomes Cancer. 1991;3:332-7.
15. Wan TS, Ma SK, Au WY, Liu HS, Chan JC, Chan LC. Trisomy 21 and other chromosomal abnormalities in acute promyelocytic leukemia. Cancer Genet Cytogen. 2003; 140:170-3.
16. Sessarego M, Fugazza G, Balleari E, Bruzzone R, Ballestrero A, Patrone F. High frequency of trisomy 8 in acute promyelocytic leukemia: a fluorescence in situ hybridization study. Cancer Genet Cytogen. 1997;97:161-4.
17. Pantic M, Novak A, Marisavljevic D, et al. Additional chromosome aberrations in acute promyelocytic leukemia: characteristics and prognostic influence. Med Oncol. 2000;17:307-13.
18. Kwong YL, Wong KF, Chan TK. Trisomy 8 in acute promyelocytic leukaemia: an interphase study by fluorescence in situ hybridization. Br J Haematol. 1995;90:697-700.
19. Hiorns LR, Swansbury GJ, Mehta J, et al. Additional chromosome abnormalities confer worse prognosis in acute promyelocytic leukaemia. Br J Haematol. 1997;96:314-21.
20. Olshanskaya YV, Gutorova DS, Shuravina EN, et al. The incidence and prognostic significance of additional chromosome restructuring in acute promyelocytic leukemia. Gematologiya I Transfuziologiya. 2007;52:27-32.
21. Slack JL, Arthur DC, Lawrence D, et al. Secondary cytogenetic changes in acute promyelocytic leukemia—prognostic importance in patients treated with chemotherapy alone and association with the intron 3 breakpoint of the PML gene: a Cancer and Leukemia Group B study. J Clin Oncol. 1997;15:1786-95.
22. Hernandez JM, Martin G, Gutierrez NC, et al. Additional cytogenetic changes do not influence the outcome of patients with newly diagnosed acute promyelocytic leukemia treated with an ATRA plus anthracyclin based protocol. A report of the Spanish group PETHEMA. Haematologica. 2001;86:807-13.
23. Paschka P, Du J, Schlenk RF, et al. Secondary genetic lesions in acute myeloid leukemia with inv(16) or t(16;16): a study of the German-Austrian AML Study Group (AMLSG). Blood. 2013;121:170-7.
24. Jones D, Yao H, Romans A, et al. Modeling interactions between leukemia-specific chromosomal changes, somatic mutations, and gene expression patterns during progression of core-binding factor leukemias. Genes Chromosomes Cancer. 2010;49:182-91.
25. Wakita S, Yamaguchi H, Miyake K, et al. Importance of c-kit mutation detection method sensitivity in prognostic analyses of t(8;21)(q22;q22) acute myeloid leukemia. Leukemia. 2010;25:1423-32.
26. Lin P, Chen L, Luthra R, Konoplev SN, Wang X, Medeiros LJ. Acute myeloid leukemia harboring t(8;21)(q22;q22): a heterogeneous disease with poor outcome in a subset of patients unrelated to secondary cytogenetic aberrations. Mod Pathol. 2008;21:1029-36.
27. Peniket A, Wainscoat J, Side L, et al. Del (9q) AML: clinical and cytological characteristics and prognostic implications. Br J Haematol. 2005;129:210-20.
28. Park SH, Chi HS, Min SK, Park BG, Jang S, Park CJ. Prognostic impact of c-KIT mutations in core binding factor acute myeloid leukemia. Leuk Res. 2011;35:1376-83.
29. Paschka P, Marcucci G, Ruppert AS, et al. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. J Clin Oncol. 2006;24:3904-11.
30. Gustafson SA, Lin P, Chen SS, et al. Therapy-related acute myeloid leukemia with t(8;21) (q22;q22) shares many features with de novo acute myeloid leukemia with t(8;21)(q22;q22) but does not have a favorable outcome. Am J Clin Pathol. 2009;131:647-55.
31. Estey E, Keating MJ, Pierce S, Stass S. Change in karyotype between diagnosis and first relapse in acute myelogenous leukemia. Leukemia. 1995;9:972-6.
32. Garson OM, Hagemeijer A, Sakurai M, et al. Cytogenetic studies of 103 patients with acute myelogenous leukemia in relapse. Cancer Genet Cytogen. 1989;40:187-202.
33. Kern W, Haferlach T, Schnittger S, Ludwig WD, Hiddemann W, Schoch C. Karyotype instability between diagnosis and relapse in 117 patients with acute myeloid leukemia: implications for resistance against therapy. Leukemia. 2002;16:2084-91.
34. Kern W, Haferlach C, Haferlach T, Schnittger S. Monitoring of minimal residual disease in acute myeloid leukemia. Cancer. 2008;112:4-16.
35. Larson RA, Kondo K, Vardiman JW, Butler AE, Golomb HM, Rowley JD. Evidence for a 15;17 translocation in every patient with acute promyelocytic leukemia. Am J Med. 1984;76:827-41.
36. Schnittger S, Alpermann T, Nadarajah N, Grossmann V, Eder C, Kohlmann A, et al. Comparison of Mutation Patterns Between Diagnosis and Relapse in 444 Patients with Acute Myeloid Leukemia Shows High Variability of Stability and Influence On Time to Relapse. American Society of Hematology. Atlanta; 2012.
37. Tsukamoto N, Karasawa M, Tanaka Y, et al. Recurrence of acute myelogenous leukemia with the same AML1/ETO breakpoint as at diagnosis after complete remission lasting 15 years: analysis of stored bone marrow smears. Int J Hematol. 2003;78:362-9.
38. Imagawa J, Harada Y, Shimomura T, et al. Clinical and genetic features of therapy-related myeloid neoplasms after chemotherapy for acute promyelocytic leukemia. Blood.

2010;116:6018-22.
39. Wiemels JL, Xiao Z, Buffler PA, et al. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. Blood. 2002;99:3801-5.
40. McHale CM, Smith MT. Prenatal origin of chromosomal translocations in acute childhood leukemia: implications and future directions. Am J Hematol. 2004;75:254-7.
41. Smith MT, McHale CM, Wiemels JL, et al. Molecular biomarkers for the study of childhood leukemia. Toxicol Appl Pharmacol. 2005;206:237-45.
42. Chang P, Kang M, Xiao A, et al. FLT3 mutation incidence and timing of origin in a population case series of pediatric leukemia. BMC Cancer. 2010;10:513.
43. Burjanivova T, Madzo J, Muzikova K, et al. Prenatal origin of childhood AML occurs less frequently than in childhood ALL. BMC Cancer. 2006;6:100.
44. Bacher U, Haferlach T, Alpermann T, et al. Comparison of cytogenetic clonal evolution patterns following allogeneic hematopoietic transplantation versus conventional treatment in patients at relapse of AML. Biol Blood Marrow Transplant. 2010;16:1649-57.
45. Pedersen-Bjergaard J, Timshel S, Andersen MK, Andersen AS, Philip P. Cytogenetically unrelated clones in therapy-related myelodysplasia and acute myeloid leukemia: experience from the Copenhagen series updated to 180 consecutive cases. Gene Chromosome Cancer. 1998;23:337-49.
46. Hayashi Y, Raimondi SC, Behm FG, et al. Two karyotypically independent leukemic clones with the t(8;21) and 11q23 translocation in acute myeloblastic leukemia at relapse. Blood. 1989;73:1650-5.
47. Waterhouse M, Pfeifer D, Pantic M, Emmerich F, Bertz H, Finke J. Genome-wide profiling in AML patients relapsing after allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2011 Oct;17(10):1450-1459.e1. doi: 10.1016/j.bbmt.2011.07.012. Epub 2011 Jul 23.
48. Kadam PS, Jain HV, Parikh PM, Saikia TK, Agarwal S, Ambulkar I. Emergence of an unrelated highly aberrant clone in an AML patient at relapse four months after peripheral blood stem cell transplantation. Indian J Hum Genet. 2007;13:114-8.
49. Potter NE, Ermini L, Papaemmanuil E, Vijayaraghavan G, Cazzaniga G, Ford AM, et al. Composite Single Cell Genetics and Clonal Phylogeny in Acute Lymphoblastic Leukaemia. American Society of Hematology, Atlanta; 2012.
50. Nakano Y, Kiyoi H, Miyawaki S, et al. Molecular evolution of acute myeloid leukaemia in relapse: unstable N-ras and FLT3 genes compared with p53 gene. Br J Haematol. 1999;104:659-64.
51. Kristensen T, Moller MB, Friis L, Bergmann OJ, Preiss B. NPM1 mutation is a stable marker for minimal residual disease monitoring in acute myeloid leukaemia patients with increased sensitivity compared to WT1 expression. Eur J Haematol. 2011;87:400-8.
52. Boissel N, Renneville A, Biggio V, et al. Prevalence, clinical profile, and prognosis of NPM mutations in AML with normal karyotype. Blood. 2005;106:3618-20.
53. Suzuki T, Kiyoi H, Ozeki K, et al. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood. 2005;106:2854-61.
54. Bachas C, Schuurhuis GJ, Assaraf YG, et al. The role of minor subpopulations within the leukemic blast compartment of AML patients at initial diagnosis in the development of relapse. Leukemia. 2012;26:1313-20.
55. Tiesmeier J, Muller-Tidow C, Westermann A, et al. Evolution of FLT3-ITD and D835 activating point mutations in relapsing acute myeloid leukemia and response to salvage therapy. Leuk Res. 2004;28:1069-74.
56. Wakita S, Yamaguchi H, Omori I, et al. Mutations of the epigenetics-modifying gene (DNMT3a, TET2, IDH1/2) at diagnosis may induce FLT3-ITD at relapse in de novo acute myeloid leukemia. Leukemia. 2012 Nov 8. doi: 10.1038/leu.2012.317. [Epub ahead of print].
57. Hou HA, Kuo YY, Liu CY, et al. DNMT3A mutations in acute myeloid leukemia: stability during disease evolution and clinical implications. Blood. 2012;119:559-68.
58. McCormick SR, McCormick MJ, Grutkoski PS, et al. FLT3 mutations at diagnosis and relapse in acute myeloid leukemia: cytogenetic and pathologic correlations, including cuplike blast morphology. Arch Pathol Lab Med. 2010;134:1143-51.
59. Nazha A, Cortes J, Faderl S, et al. Activating internal tandem duplication mutations of the fms-like tyrosine kinase-3 (FLT3-ITD) at complete response and relapse in patients with acute myeloid leukemia. Haematologica. 2012;97:1242-5.
60. Wang ES, Sait SN, Gold D, et al. Genomic, immunophenotypic, and NPM1/FLT3 mutational studies on 17 patients with normal karyotype acute myeloid leukemia (AML) followed by aberrant karyotype AML at relapse. Cancer Genet Cytogen. 2010;202:101-7.
61. Hou HA, Huang TC, Lin LI, et al. WT1 mutation in 470 adult patients with acute myeloid leukemia: stability during disease evolution and implication of its incorporation into a survival scoring system. Blood. 2010;115:5222-31.
62. Chou WC, Lei WC, Ko BS, et al. The prognostic impact and stability of Isocitrate dehydrogenase 2 mutation in adult patients with acute myeloid leukemia. Leukemia. 2011;25:246-53.
63. Cloos J, Goemans BF, Hess CJ, et al. Stability and prognostic influence of FLT3 mutations in paired initial and relapsed AML samples. Leukemia. 2006;20:1217-20.
64. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukemia revealed by whole-genome sequencing. Nature. 2012;481:506-10.
65. Walter MJ, Shen D, Ding L, et al. Clonal architecture of secondary acute myeloid leukemia. N Engl J Med. 2012;366: 1090-8.
66. Busque L, Mio R, Mattioli J, et al. Nonrandom X-inactivation patterns in normal females: lyonization ratios vary with age. Blood. 1996;88:59-65.
67. Gale RE, Fielding AK, Harrison CN, Linch DC. Acquired skewing of X-chromosome inactivation patterns in myeloid cells of the elderly suggests stochastic clonal loss with age. Br J Haematol. 1997;98:512-9.
68. Laurie CC, Laurie CA, Rice K, et al. Detectable clonal mosaicism from birth to old age and its relationship to cancer. Nat Genet. 2012;44:642-50.
69. Busque L, Patel JP, Figueroa ME, et al. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. Nat Genet. 2012;44:1179-81.
70. Price CM, Marshall CJ, Bashey A. Sequential acquisition of trisomy 8 and N-ras mutation in acute myeloid leukaemia demonstrated by analysis of isolated leukaemic colonies. Br J Haematol. 1994;88:338-42.
71. McCormack E, Bruserud O, Gjertsen BT. Review: genetic models of acute myeloid leukaemia. Oncogene. 2008;27:3765-79.
72. Fortier JM, Graubert TA. Murine models of human acute myeloid leukemia. Cancer Treat Res. 2010;145:183-96.
73. Kogan SC. Mouse models of acute promyelocytic leukemia. Curr Top Microbiol Immunol. 2007;313:3-29.
74. Pratz KW, Sato T, Murphy KM, Stine A, Rajkhowa T, Levis M. FLT3-mutant allelic burden and clinical status are predictive of response to FLT3 inhibitors in AML. Blood. 2010;115:1425-32.
75. Smith CC, Wang Q, Chin CS, et al. Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. Nature. 2012;485:260-3.
76. Moore AS, Faisal A, Gonzalez de Castro D, et al. Selective FLT3 inhibition of FLT3-ITD+ acute myeloid leukaemia resulting in secondary D835Y mutation: a model for emerging clinical resistance patterns. Leukemia. 2012;26:1462-70.
77. Thiede C, Steudel C, Mohr B, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99:4326-35.
78. Chen W, Jones D, Medeiros LJ, Luthra R, Lin P. Acute myeloid leukaemia with FLT3 gene mutations of both internal tandem duplication and point mutation type. Br J Haematol. 2005;130:726-8.
79. Carnicer MJ, Nomdedeu JF, Lasa A, et al. FLT3 mutations are associated with other molecular lesions in AML. Leuk Res. 2004;28:19-23.

Genetics guided therapeutic approaches in acute myeloid leukemia

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A B S T R A C T

Over the past years the development of genomics technologies, such as single nucleotide polymorphism (SNP) microarray analysis and next generation sequencing (NGS), have made a significant contribution to comprehensively deciphering the genetic changes underlying acute myeloid leukemia (AML). An increasing number of genomic aberrations and gene mutations have been identified that cause epigenetic changes and lead to deregulated gene expression. These recent insights further unravel the enormous molecular heterogeneity of AML and show that each patient presents with a distinct and almost individual combination of somatically acquired genetic alterations. While some of these are known to perturb normal mechanisms of self-renewal, proliferation, and differentiation of the hematopoietic progenitor cells, others most likely represent passenger mutations that do not significantly contribute to the disease. Future challenges will be to not only discriminate driver from passenger mutations, but also to evaluate the prognostic and predictive value of a specific mutation in the concert of the various concurrent mutations. Nevertheless, first genetic markers started to translate into the clinic and to impact treatment decisions, especially in case of availability of molecular targeted therapies. To further improve the response to these drugs, that often do not show the expected effects as monotherapy, promising new compounds will need to be put into perspective of the interplay of mutations and ultimately personalized combination treatment approaches might be able to eradicate the disease.

Learning goals

At the completion of this activity, participants should be able to:
- interpret the value of currently available genetic markers with regard to their predictive and prognostic value;
- gain an overview of genetics guided treatment approaches that have already been translated into the clinic.
- better understand future challenges that will have to consider the interplay of genomic aberrations and the network of deregulated cancer relevant pathways in order to design optimal effective novel treatment strategies.

Introduction

Since cytogenetic markers have significantly improved the risk stratification of acute myeloid leukemia (AML),[1] the development of genomics technologies, such as single nucleotide polymorphism (SNP) microarray analysis[2] and next generation sequencing (NGS),[3] has made a significant contribution to deciphering the AML-associated genetic changes. An increasing number of genomic aberrations and gene mutations have been identified that cause epigenetic changes and lead to deregulated gene expression. For example, small genomic losses pointed to a relevant role of the *TET2* gene[4] and NGS helped to identify *IDH1* and *DNMT3A* mutations in AML.[5,6]

These recent insights further highlighted the molecular heterogeneity of AML and showed that individual patients present with a distinct and almost unique combination of somatically acquired genetic aberrations. While some of these are known to perturb a variety of cellular processes of the hematopoietic progenitor cells, including mechanisms of self-renewal, proliferation, differentiation, epigenetic regulation, DNA repair, and RNA splicing, others most likely represent passenger mutations that do not significantly contribute to the disease. In recent years, this growing genetic information has started to translate into the clinic. The current World Health Organization (WHO) classification categorizes more than half of AML cases on the basis of the underlying genetic defects, which in part define distinct entities of clinical importance.[7] First, cytogenetic and molecular genetic changes represent powerful prognostic markers, and second some genetic and epigenetic aberrations can be targeted by novel therapeutic approaches, such as tyrosine kinase inhibitors (TKIs) and demethylating agents.[8]

However, there are still limitations regarding the use of genomic biomarkers in clinical practice as for many novel markers the prog-

nostic impact so far has only been evaluated in retrospective studies. In addition, several new markers still need to be interpreted cautiously as first studies did not take into account interactions with other molecular markers, and often the analysis was based on relatively low patient numbers that might show a selection bias. Furthermore, for improved clinical decision-making there is an unmet need for predictive markers that can be attributed to the clinical benefit of a specific treatment. This might contribute significantly to an improvement in the treatment of AML that has been slow over the past decade with a few subgroups as exceptions, such as younger patients with more favorable genetic disease.[9] Not only novel targeted therapies, but also dose escalation of daunorubicin, the use of alternative nucleoside analogs, antibody-directed chemotherapy as well as allogeneic transplant concepts will benefit from a biomarker guided personalized treatment approach. Finally, a more detailed molecular characterization will provide also the opportunity to more precisely monitor minimal residual disease (MRD) in all AML patients.

In this review we will focus on genetic markers (deregulated gene expression and epigenetic changes will not be discussed) that have already entered clinical practice and affect diagnosis and guidance for therapeutic decisions in adult AML. In addition, we will discuss the prognostic value and potential clinical impact of novel markers that remain investigational.

Established genomic biomarkers

Acute myeloid leukemia with RUNX1-RUNX1T1 and CBFB-MYH11

Acute myeloid leukemia patients who present with a translocation or inversion affecting the core-binding-factor (CBF) complex components RUNX1 or CBFB belong to the genetic favorable-risk category.[6,7] Characterized by either a t(8;21)(q22;q22), leading to a *RUNX1-RUNX1T1* fusion, or an inv(16)(p13.1q22) (or t(16;16)(p13.1;q22)), leading to a *CBFB-MYH11* fusion, CBF-AML shows response rates of approximately 90% to standard '3+7' anthracycline and cytarabine induction chemotherapy in younger adult patients. In this cohort, consolidation therapy with repetitive cycles of high-dose cytarabine (3 g/m^2 every 12 hours on Days 1, 3, and 5) results in 60%-70% long-term survival probabilities in younger adult patients and thus has become a widely accepted standard therapy for CBF-AML. Therefore, older CBF-AML patients have been shown to also benefit from dose intensification of standard chemotherapy (Table 1).[8,11]

Recently, there has also been evidence from the British Medical Research Council (MRC) AML15 trial that CBF-AML patients might also benefit from an antibody-directed chemotherapeutic approach. In this large trial comprising all cytogenetic subgroups, 1113 patients were randomly assigned to receive a single dose of the anti-CD33 immunoconjugate gemtuzumab ozogamicin (GO) at a dose of 3 mg/m^2 in induction course 1 and the first consolidation course.[12] In the overall trial population there was no difference in response and survival, but a pre-defined subgroup analysis showed a significant survival benefit for patients with CBF-AML. Unfortunately, market withdrawal of GO currently prevents the conduction of a confirmatory trial. However, other studies showing a similar beneficial effect warrant reassessment of GO as front-line therapy.[13,14]

Secondary genetic changes in CBF-AML, mutations in the *v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog* (*KIT*) and *FMS-like tyrosine kinase 3* (*FLT3*), have been associated with inferior outcome.[11,15-17] While activating *KIT* mutations are found in approximately one-third of cases, the *KIT* receptor is expressed at significantly higher levels in CBF-AML compared with other subgroups. Recent efforts by the German-Austrian AML Study Group (AMLSG) (www.ClinicalTrials.gov Identifier n. NCT00850382) and Cancer and Leukemia Group B (CALGB) (www.ClinicalTrials.gov Identifier n. NCT01238211) combining conventional induction and consolidation therapy with dasatinib, a potent inhibitor of mutated and wild-type KIT, followed by one year dasatinib maintenance therapy, provided first promising results. In the future, a more refined molecular characterization of CBF-AML based on whole genome approaches might lead to additional molecular targeted approaches.[18,19]

Finally, MRD monitoring in CBF-AML showed that persistence of molecular disease is a highly predictive factor for relapse-free survival (RFS) and overall survival (OS).[16,20-22] Therefore, fusion transcript copy ratios should be monitored in all CBF-AML cases to evaluate whether allogeneic hematopoietic stem cell transplantation (HSCT) or investigational agents can improve outcome as early intervention for molecular relapse.

Acute myeloid leukemia with MLL-fusions

Rearrangements of the *mixed lineage leukemia* (*MLL*) gene are found in approximately 10% of adult AML, especially in secondary acute leukemias that occur following treatment with topoisomerase II inhibitors. Except for the translocation t(9;11)(p22;q23), leading to a *MLLT3-MLL* fusion (also known as *MLL-AF9*), which is a unique WHO classification entity,[7] and the translocation t(11;19)(q23;p13),[23] the presence of an *MLL* rearrangement generally confers a poorer prognosis, although the analysis by MRC did not distinguish between the two different types of t(11;19), i.e. AML with t(11;19)(q23;p13.3) (*MLL-MLLT1*) and AML with t(11;19)(q23;p13.1) (*MLL-ELL*). While there are more than 60 known fusion partners of MLL, *MLL*-rearranged leukemias display remarkable genomic stability, with very few gains or losses of chromosomal regions.[24] Therefore, recent studies suggest that *MLL*-rearranged leukemias are largely driven by epigenetic deregulation as several epigenetic regulators that modify DNA or histones have been implicated in MLL-fusion driven leukemogenesis.[25]

Given that MLL-fusion proteins transform cells via aberrant epigenetic programs including aberrant DNA methylation, modifying the DNA methylation state might have therapeutic efficacy in *MLL*-rearranged leukemia. Indeed, *MLL*-rearranged leukemia cell lines were shown to be sensitive to hypomethylating agents.[25] In addition, epigenetic treatment approaches in *MLL*-rearranged leukemia now also focus on the histone methyltransferase DOT1L that recently has emerged as an important mediator of MLL-fusion mediated leukemic transformation via the modification of histone H3 on lysine 79 (H3K79).[26] There is a strong correlation between elevated H3K79

Table 1. Prognostic value and impact on treatment decision of selected molecular markers in adult AML (adopted from Döhner and Gaidzik).[10]

Biomarker	Prognostic significance	Clinical relevance
AML with *RUNX1-RUNX1T1* and *CBFB-MYH11*	Favorable prognosis in younger and older patients [additional trisomy 22 predicts superior RFS in AML with inv(16)] High relapse probability in patients with molecular disease persistence Secondary *KIT* and possibly also *FLT3* mutations associated with inferior outcome in most but not all studies	'3 + 7' induction followed by repetitive cycles of high-dose cytarabine is the widely accepted standard therapy (older patients with CBF-AML do also benefit from intensive conventional chemotherapy) Allogeneic HSCT may be only considered in individual patients with high-risk factors (e.g. elevated WBC counts, molecular disease persistence) and low transplantation-related mortality KIT inhibitor dasatinib in combination with intensive induction and consolidation therapy in phase II clinical trials Addition of GO significantly improved OS in the MRC15 trial
AML with *MLL* fusions	Unfavorable prognosis, except for AML with t(9;11)	Allogeneic HSCT appears to improve outcome in younger adult patients Experimental therapeutic strategies within clinical trials (e.g. hypomethylating agents, DOT1L inhibitors)
NPM1	Genotype "mutated *NPM1* without *FLT3*-ITD" (in CN-AML) associated with favorable outcome *NPM1* mutations in older patients associated with CR achievement and better outcome, even in patients over 70 years of age Impact of concurrent gene mutations e.g. in *IDH1*, *IDH2*, *DNMT3A*, and *TET2* currently under investigation	Standard induction therapy followed by repetitive cycles of high-dose cytarabine is the reasonable first-line treatment option in patients with the genotype "mutated *NPM1* without *FLT3*-ITD" (CN-AML) Favorable-risk "mutated *NPM1* without *FLT3*-ITD" CN-AML may not benefit from allogeneic HSCT in first CR, except in individual cases (e.g. those with molecular disease persistence) with low transplantation-related risk Older patients with NPM1-mutated AML benefit from intensive conventional chemotherapy Concurrent gene mutations other than *FLT3* (*IDH1*, *IDH2*, *DNMT3A,* etc.) should not yet be used for making treatment decisions
CEBPA	Only *CEBPA*dm cases define this AML entity *CEBPA*dm (CN-AML) associated with favorable outcome Impact in older patients under investigation	Standard induction and consolidation therapy is the reasonable first-line treatment option Patients may not benefit from allogeneic HSCT in first CR
***FLT3*-ITD**	Unfavorable prognosis Particular poor outcome in AML with high burden of mutated *FLT3*-ITD allele (high mutant to wild-type allelic ratio as assessed by DNA fragment analysis) AML with *FLT3*-ITD located outside the JM (non-JM ITD, approximately 30% of cases) appear to do significantly worse than those with AML with JM-ITD	Allogeneic HSCT appears to improve outcome in younger adult patients (no data available for elderly patients) Patients should be entered on clinical trials with FLT3 tyrosine kinase inhibitors whenever possible; 1st-generation (e.g. midostaurin, lestaurtinib, sorafenib) and 2nd-generation TKI (quizartinib) are currently being evaluated in phase II and III clinical trials
TP53	Unfavorable prognosis Mutations/deletions mostly in AML with complex karyotype (56%-78%)	Allogeneic HSCT does not seem to improve outcome; experimental therapeutic approaches within clinical trials warranted
WT1	Prognostic significance somewhat controversial; most studies report a negative prognostic impact Additional studies, preferentially large intra-individual patient meta-analyses, needed to explore the prognostic impact by different post-remission therapies *WT1* SNP rs16754 located in mutational hot spot in exon 7 found to be associated with favorable prognosis in patients with CN-AML	Unknown
RUNX1	Unfavorable prognosis; all studies showed an association of RUNX1 mutations with lower CR rate and adverse outcome	Unknown One study (AMLSG) suggested that allogeneic HSCT may improve outcome; finding needs to be confirmed
TET2	Prognostic significance unclear CALGB study found a negative impact in the subset of molecular favorable-risk (mutated NPM1 without FLT3-ITD) AML; AMLSG study found no impact	Unknown
IDH1	*IDH1* mutations appear to confer higher risk of relapse and inferior OS in CN-AML; however, the effect in the various molecular subsets of CN-AML is controversial *IDH1* SNP rs11554137 (located in the same exon as the R132 mutation) in one study found to be associated with inferior outcome in molecular high-risk CN-AML (either *NPM1* wild-type or *FLT3*-ITD positive)	Unknown IDH inhibitors in pre-clinical development
IDH2	IDH2R172 mutations are only rarely found in concert with other known recurring gene mutations (i.e. *NPM1*, *CEBPA*, *FLT3*-ITD); they are associated with inferior CR rate; impact on outcome unclear Prognostic impact of IDH2R140 mutations controversial, although some studies reported an association with a better prognosis	(see above)
DNMT3A	Associated with intermediate-risk cytogenetics (in particular CN-AML) and with *FLT3*, *NPM1*, and *IDH* mutations Prognostic significance under investigation	Unknown
ASXL1	Unfavorable prognosis; Mutation incidence increases with age	Unknown

methylation and abnormal gene expression in human *MLL*-rearranged leukemia samples on a genome-wide basis,[27] and DOT1L was shown to play an active role in the maintenance of the MLL-fusion mediated gene expression programs important for transformation and leukemogenesis.[26] This supported DOT1L as a potential therapeutic target and a first specific small-molecule inhibitor of DOT1L showed promising anti-proliferative activity that was remarkably selective for cell lines bearing *MLL* rearrangements.[28] Therefore, DOT1L inhibitors are currently further developed as targeted therapeutics for *MLL*-rearranged leukemias, and a first phase I trial testing the DOT1L inhibitor EPZ-5676 was initiated in September 2012 (www.ClinicalTrials.gov Identifier n. NCT0168 4150).

Acute myeloid leukemia with NPM1 mutations

Nucleophosmin 1 (*NPM1*) mutations are the most frequent mutations found in 25%-35% of adult AML, especially in cytogenetically normal (CN)-AML (45%-64%) (Figure 1).[8] While the role of *NPM1* mutations in leukemogenesis has largely remained elusive, in a mouse model mutant *Npm1* knock-in proved to be an AML-initiating lesion leading to *Hox* gene overexpression, increased self-renewal, and expanded myelopoiesis with one-third of mice developing delayed-onset AML.[29] In human AML, *NPM1* mutations were shown to be associated with *FLT3* internal tandem duplications (*FLT3*-ITDs), and more recently also with *IDH* and *DNMT3A* mutations.[6,30-34]

Patients with *NPM1* mutation usually present with higher bone marrow (BM) blast percentages, lactate dehydrogenase serum levels, and white blood cell counts, and blast cells typically show high CD33-antigen, but low or absent CD34-antigen expression.[35] As *NPM1* mutations without concurrent *FLT3*-ITDs have been shown to confer a superior outcome,[1,36] the genotype "mutated *NPM1* without *FLT3*-ITD" (CN-AML only) has been incorporated into the genetic favorable-risk category of the current AML recommendations.[8] However, the prognostic value of the *NPM1*mut/*FLT3*-ITDneg genotype has to be revisited in the context of recently identified concomitant mutations, such as *IDH* and *DNMT3A* mutations.[37]

As younger adult patients with *NPM1*mut/*FLT3*-ITDneg AML have survival probabilities of approximately 60% following conventional induction and consolidation treatment, this patient cohort might not benefit from allogeneic HSCT in first complete remission (CR).[36] However, allogeneic HSCT may be considered in patients with molecular disease persistence,[38] especially those with low transplantation-related risk, or in case new transplantation strategies are investigated within a clinical trial. As the favorable prognostic impact of *NPM1* mutations in the absence of *FLT3*-ITD is also seen in older adults, even in those over 70 years of age,[39,40] *NPM1* mutation screening is clinically relevant in all age groups and helps to select patients who might benefit from intensive conventional chemotherapy.[10]

At present, there is no targeted molecular therapy available for *NPM1* mutated AML, but there are ongoing efforts to target *NPM1* mutation-associated altered transport mechanisms.[35] However, based on high CD33 expression levels, the anti-CD33 antibody GO appears to be an attractive therapeutic strategy. While an MRC AML15 trial subset analysis showed no significant survival benefit for GO in *NPM1* mutant AML,[12] other trials showed a benefit for GO in the low- and intermediate-risk groups.[41] Thus, the GO impact needs to be revisited in the light of concomitant mutations as well as SNPs in CD33 that might impact treatment response.[42] Similarly, based on two controversial reports the potential benefit of all-*trans* retinoic acid (ATRA) in *NPM1*mut/*FLT3*-ITDneg patients remains elusive,[40,43] but a recent AMLSG study in younger AML patients confirms a beneficial effect.[44]

Acute myeloid leukemia with CEBPA mutations

CCAAT/enhancer binding protein alpha (*CEBPA*) mutations are primarily found in CN-AML (10%-18%), and can be divided into two subgroups: single mutation, *CEBPA*^{sm} (one-third of cases), and double mutation cases, *CEBPA*^{dm} (two-thirds of cases).[1] Typically, in *CEBPA*^{dm} AML both alleles are mutated, one showing a N-terminal and one a C-terminal mutation.

Previously, *CEBPA*^{sm} and *CEBPA*^{dm} were considered as one group associated with a favorable outcome.[7] However, recent studies showed that only *CEBPA*^{dm} AML is an independent prognostic factor for favorable outcome.[45] Therefore, the pattern of concurrent gene mutations is different in *CEBPA*^{sm} (significantly higher frequency of *NPM1* mutations and *FLT3*-ITDs than in *CEBPA*^{dm}), and global gene expression studies revealed only a distinct signature for *CEBPA*^{dm} AML cases.[45,46] Consequently, only AML with *CEBPA*^{dm} should be considered as a distinct entity and prognostic category that can be associated with additional genomic alterations such as *GATA2* mutations.[47]

CEBPA^{dm} patients may also not benefit from allogeneic HSCT based on the assumption that, in general, this approach may not improve outcome in favorable-risk AML.[8] While no specific targeted therapies are yet available, molecular insights in *CEBPA*^{dm} pathomechanisms including the deregulation of small non-coding RNAs,

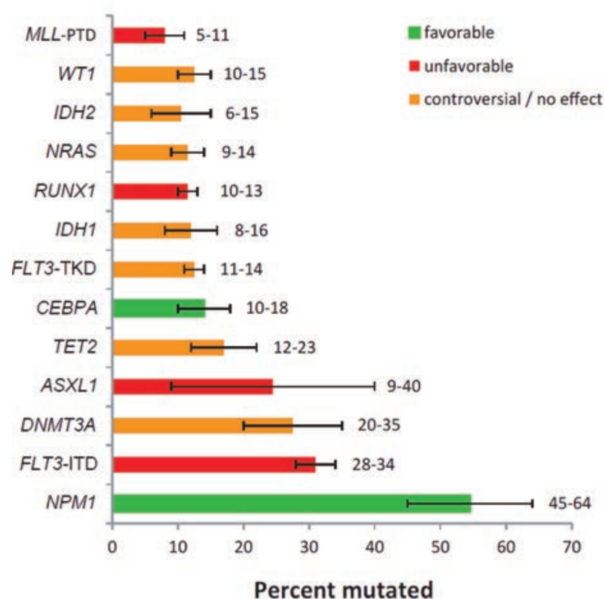

Figure 1. Incidence and prognostic impact of aberrant gene mutations in CN-AML. (adopted from Marcucci *et al.*).[1]

especially miR-181a, point to novel treatment strategies such as lenalidomide.[48]

Acute myeloid leukemia with FLT3 mutations

As a member of the class III receptor tyrosine kinase family, FLT3 plays an important role in proliferation, survival, and differentiation of hematopoietic progenitor cells. *FLT3* mutations are primarily located in the juxtamembrane (JM) domain and the activation loop of the tyrosine kinase domain (TKD); however, other locations are also possible.[49] *FLT3*-ITDs are found in approximately 20% of all AML (CN-AML 28%-34%) and mainly cluster in the JM domain.[1] In CN-AML patients treated with conventional chemotherapy, the presence of *FLT3*-ITDs confers a significantly worse prognosis compared with ITD negative cases.[8] A high mutant-to-wild-type allelic ratio points to an even worse outcome, as it often reflects wild-type allele loss due to uniparental disomy.[1] Occurring in one-third of cases, non-JM ITD insertion is also associated with a particularly poor outcome.[50] In addition, the activation loop of the TKD is affected by point mutations in 11%-14% of CN-AML.[1] However, the prognostic relevance of *FLT3*-TKD mutations remains controversial.

This importance of *FLT3*-ITDs and the fact that the FLT3 pathway is targetable have stimulated huge efforts to develop therapeutic inhibitors of FLT3, e.g. midostaurin (PKC412), lestaurtinib (CEP-701), sunitinib (SU-11248), sorafenib (BAY-43-9006), and the 2nd generation compound quizartinib (AC220).[51] Although these inhibitors have shown promising anti-leukemic activity, efficacy as single agents was limited and thus requires a combination with cytotoxic chemotherapy. A large randomized trial combining chemotherapy with midostaurin for first-line treatment of younger adult patients will hopefully soon provide important answers (www.ClinicalTrials.gov Identifier n. NCT00651261). Notably, the optimized FLT3 inhibitor quizartinib led to high response rates in relapsed and refractory *FLT3*-ITD AML, even if given as a single agent. A phase II trial for relapsed *FLT3*-ITD patients has recently been completed (www.ClinicalTrials.gov identifier n. NCT00989261).[52,53] However, treatment resistance due to acquired mutations is also seen for quizartinib,[54] but ponatinib and crenolanib might be good alternatives in these cases.[55,56]

Besides TKI-based targeted treatment approaches, there is increasing evidence that *FLT3*-ITD positive AML patients might benefit from allogeneic HSCT, especially CN-AML and unfavorable genotypes with *FLT3*-ITD.[36,57,58]

Acute myeloid leukemia with TP53 mutations

Recently, *tumor protein p53* (*TP53*) mutation and/or loss of the *TP53* allele was detected in 69%-78% of AML cases with a complex karyotype (CK-AML), whereas *TP53* mutations were very rare in non-complex karyotype AML (2.1%).[59] Based on an integrative *TP53* mutational screening analysis and array-based genomic profiling in 234 CK-AMLs, *TP53* mutations were found in 60% and *TP53* losses in 40% of CK-AMLs with a total of 70% *TP53* altered cases.[60]

As *TP53*-altered CK-AMLs are characterized by a higher degree of genomic complexity, they more frequently exhibit a monosomal karyotype (MK), which previously was associated with poor AML outcome.[61] *TP53* alterations are also associated with older age, specific DNA copy number alterations, and dismal outcome.[60] In multivariable analysis, *TP53* alteration is the most important prognostic factor in CK-AML, outweighing all other variables, including the MK category.[60] This very unfavorable prognosis of *TP53* mutation was recently confirmed in an independent study showing an overall survival (OS) at three years of 0%.[62]

Therefore, treatment approaches aimed at early allogeneic HSCT in *TP53* altered AML cases, but recent studies showed no improvement in survival for patients with abnl(17p) AML as compared to other adverse cytogenetic risk abnormalities.[63] Thus, *TP53* altered AML cases should be treated within clinical trials evaluating novel therapeutic approaches, e.g. combinations of hypomethylating agents, mTOR (mammalian target of rapamycin) inhibitors, and tosedostat, an orally available aminopeptidase inhibitor.[64] The latter has recently demonstrated significant clinical activity in relapsed or primary refractory AML, including high-risk AML cases.[65,66]

Investigational genomic markers

Over the last years, novel gene mutations have been discovered based on SNP array analysis in combination with sequence analysis of candidate genes in commonly altered regions,[4,67] and NGS studies.[3,68] These biomarkers include mutations in transcription factors like *WT1*, *RUNX1*, and *GATA2,* and in genes influencing transcriptional regulation such as e.g. *NRAS*, *KRAS, CBL*, *KIT,* and *RAD21* to name some of the most recurrent ones. In addition, many mutations were identified in genes impacting epigenetic regulation, such as e.g. *TET2*, *IDH1*, *IDH2*, *DNMT3A*, *ASXL1, MLL*, *TET1, BCOR, NSD1, PHF6, DNMT1, NSD1, EZH2,* and *MLL3.*[1,37,69] While most of these markers remain mainly investigational and are still a controversial subject of debate, many studies are currently evaluating their prognostic and predictive impact on the background of other genomic aberrations. Here, we will focus on mutations for which an important role in AML was supported by recent studies.

Gene mutations affecting transcription factors

WT1 mutations

Wilms' Tumor 1 (*WT1*) gene mutations were among the first of acute leukemias to be reported.[70] Since then, studies of larger cohorts revealed mutations primarily in CN-AML with a frequency of 10%-15%. However, the prognostic impact of the mutation is still inconclusive as studies by the MRC, CALGB, and Acute Leukemia French Association (ALFA) groups showed a negative impact of the mutation on OS, whereas no impact was found in a study of 617 CN-AML by the AMLSG.[70] Interestingly, located in the mutational hot spot of *WT1* in exon 7, a SNP rs16754 was shown to be associated with favorable outcome in patients with CN-AML.[71] While future studies are warranted to validate this finding, it will also be of interest to determine the mechanisms by which this SNP may alter WT1 function and contribute to a more favorable prognosis.

RUNX1 mutations

In AML the *runt-related transcription factor 1* (*RUNX1*) is not only targeted by chromosomal translocation (see above), but also by intragenic mutations that have been associated with inferior outcome in all studies.[72-75] For example, in the largest study by the AMLSG that reported only 6% *RUNX1* mutations in 945 unselected younger adult patients,[72] *RUNX1* mutations clustered in the intermediate-risk cytogenetic group and predicted for resistance to chemotherapy as well as inferior event-free survival (EFS), relapse-free survival (RFS), and OS. In multivariable analysis, *RUNX1* mutation was an independent prognostic marker for shorter EFS, and explorative subgroup analysis suggested that allogeneic HSCT has a favorable impact on RFS in *RUNX1* mutated patients.[72]

Gene mutations impacting epigenetic regulation

TET2 mutations

The identification of microdeletion and copy number neutral loss of heterozygosity affecting the *tet oncogene family member 2* (*TET2*) gene locus on 4q24 pointed to heterogeneous *TET2* mutations,[4,76] that are found in 12%-27% of patients with AML and in other myeloid diseases.[1] Like TET1, the protein TET2 converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). This α-ketoglutarate (α-KG) dependent process plays an important role in DNA demethylation.[77,78] In accordance, *TET2*-mutated AML was shown to display uniformly low levels of 5hmC compared with normal controls. The functional relevance of *TET2* mutations was further supported by mouse models demonstrating impaired hematopoietic differentiation and increased self-renewal *in vivo* following loss of Tet2.[79-81] Concerning the prognostic impact of *TET2* mutations, results are still inconclusive. A first large study reporting *TET2* mutations in 23% of 427 CN-AML cases (18-83 years of age) found an association with inferior EFS and disease-free survival (DFS) in favorable-risk CN-AML cases, i.e. *FLT3*-ITD negative AML with either mutated *CEBPA* or *NPM1*.[82] In contrast, *TET2* mutations had no prognostic impact on survival in an AMLSG study analyzing 783 AML cases, neither in the whole cohort, nor in CN-AML or in AML with mutated *NPM1* without *FLT3*-ITDs.[83] However, a recent Eastern Cooperative Oncology Group (ECOG) trial studying 398 AML cases found that *TET2* mutations were associated with reduced OS among patients with intermediate-risk AML.[84]

IDH1 and *IDH2* mutations

First whole-genome sequencing efforts revealed recurrent *isocitrate dehydrogenase 1* (*IDH1*) mutations in AML,[5] and later candidate gene screens identified *IDH2* to be recurrently mutated.[30,32,85] The combined frequency of *IDH* mutations in unselected cohorts of AML varies between 15% and 22%, and mutations typically affect *IDH1* at codon R132 and *IDH2* at codons R140 or R172.[1] Notably, both mutant IDH1 and IDH2 proteins acquire a neomorphic enzymatic activity that converts α-ketoglutarate (α-KG) to 2-hydroxyglutarate (2-HG). This putative oncogenic metabolite 2-HG has been characterized as a competitive inhibitor of α-KG-dependent dioxygenases, including histone demethylases and TET family 5-methylcytosine hydroxylases.[86] Therefore, there is a mutual exclusivity between *IDH* and *TET2* mutations in AML and consistent with a convergent mechanism of leukemic transformation, expression of IDH mutants causes increased expansion of hematopoietic stem cells and impaired hematopoietic differentiation.[87]

The prognostic impact of *IDH* mutations seems to depend both on the context of concurrent mutations and the *IDH* mutation type.[1] For example, R132 *IDH1* and R140 *IDH2* mutations are often associated with CN-AML with *NPM1* mutations,[30-32] whereas R172 *IDH2* mutations show no significant association with other known recurrent gene mutations.[32,85] Furthermore, similar to *TET2* mutations, the prognostic effect of *IDH* mutations in CN-AML is still controversial. CALGB[85] and AMLSG[32] studies showed *IDH1* mutations to predict for inferior outcome in favorable-risk (*NPM1*mut/*FLT3*-ITDneg) AML, whereas MRC[31] and Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON)[30] studies revealed inferior outcome in *FLT3*-ITDneg and *FLT3*-ITDneg/*NPM1* wild-type AML, respectively. Similarly, AML with R172 *IDH2* mutations have been associated with inferior survival,[85] and R140 *IDH2* mutations predicted for inferior outcome in molecular favorable-risk CN-AML.[32] However, a recent MRC study showed R140 *IDH2* mutations to be an independent favorable prognostic factor for RFS and OS,[88] and in accordance with this, a recent ECOG study found R140 *IDH2* mutations, but not R172 *IDH2* mutations or *IDH1* mutations, associated with improved overall survival.[84] These findings further highlight that cohort size, age, and treatment approach have significant effects on prognostication in AML, and thus individual patient data meta-analyses are needed to resolve these controversies and to identify specific treatment effects.

DNMT3A mutations

Using targeted NGS somatic *DNA (cytosine-5-)-methyltransferase 3 alpha* (*DNMT3A*) mutations were first identified in the highly conserved R882 residue,[89] and genome-wide NGS approaches in AML then identified *DNMT3A* mutations targeting the entire open reading frame.[6,90] *DNMT3A* encodes for a *de novo* methyltransferase that methylates cytosines in CpG dinucleotides, thereby pointing to altered epigenetic patterns in *DNMT3A* mutant AML. However, first analyses did not identify characteristic methylation pattern alterations in *DNMT3A* mutant AML,[6] although more recent studies reported distinct changes suggesting that *DNMT3A* mutation effects might be site and context specific.[90,91] Functional studies in mice proved an important pathogenic role as *Dnmt3a* deficiency resulted in impaired hematopoietic cell differentiation, increased self-renewal, and expansion of the hematopoietic stem cell pool.[92]

A first analysis in 281 AMLs detected *DNMT3A* mutations in 22% of cases and found an association with *FLT3*-ITD, *NPM1*, and *IDH* mutations.[6] A second study confirmed mutations in 21% of cases and reported an association with inferior survival.[90] Recently, a large study in 489 younger adult patients showed a mutation frequency of 18% that was associated with *NPM1*, *IDH1*, and *FLT3*-ITD mutations.[33] While *DNMT3A* mutations in this study predicted for inferior OS, analysis of 1770 younger AMLSG AML patients confirmed *DNMT3A* mutations in 21% of cases with a significant association with CN-AML (Gaidzik *et al.,* Blood in press, 2013). In the entire cohort,

DNMT3A mutations showed no correlation with outcome; however, exploratory subset analyses showed a negative prognostic effect in the European LeukemiaNet (ELN) unfavorable CN-AML group. In contrast to other studies, only R882 mutations had an unfavorable prognosis, whereas non-R882 mutations showed a favorable impact on OS. While selection bias in other studies might in part explain these differences, alternative treatment strategies might also impact the observation. A recent study showed that in *DNMT3A* mutated patients high-dose daunorubicin can improve outcome.[84] In line with this, a recent study suggested that *DNMT3A* mutant patients had improved overall survival when treated with 12 mg/m^2 of idarubicin,[93] which has been shown to be nearly equivalent to high-dose daunorubicin, and which was also used in the AMLSG trial.

ASXL1 mutations

Somatic nonsense or frameshift mutations of the polycomb family gene *additional sex combs like 1* (*ASXL1*), that result in loss of ASXL1 function, are found in a spectrum of myeloid malignancies including AML.[69] *ASXL1* mutations result in impaired polycomb repressive complex 2 (PRC2)-mediated histone H3 lysine 27 tri-methylation (H3K27me3), which regulates the expression of the *HOXA* gene cluster including *HOXA9* that plays a known role in myeloid transformation.[94] Notably, impaired PRC2 function seems to be a common theme in myeloid pathogenesis reflected by additional loss-of-function mutations in other PRC2 complex members *EZH2*, *SUZ12*, *EED* and *JARID2*.[69] While *ASXL1* mutations are relatively uncommon in younger AML patients, their prevalence increases with age and confers a poor impact on OS independent of age, thereby showing that *ASXL1* mutations mark a subset of AML patients with adverse outcome.[84,95-97] Further studies are needed to better understand the mechanisms underlying the different epigenetic regulator mutations, including those less common found in *PHF6, TET1, BCOR, NSD1, DNMT1,* and *MLL3,* in order to develop novel therapeutic strategies to restore epigenetic regulation in AML.

Conclusions

Recent progress in the molecular characterization of AML has greatly improved our understanding of the disease and has started to translate into the clinical setting. However, the growing number of molecular markers identified by genomics and NGS-based approaches provides a considerable challenge to establish the prognostic significance of particular constellations of mutations, thereby demanding analysis of large numbers of homogeneously treated patients in whom the effects of treatment on outcome can be controlled and investigated. International collaborations of study groups are warranted to accelerate this progress, and it will be of prime importance to study these markers in the context of novel therapies, which might impact predictive and prognostic relevance.

In addition, to further 'personalize' treatment approaches, we will not only have to consider the complex genotypes, in which the presence and/or absence of other disease alleles have different effects on outcome, but we will also have to examine the evolution and impact of subclonal mutations. These might contribute to the variations observed in response to therapy and risk of relapse,[98,99] especially subclonal driver mutations such as *TP53* mutations.[100] Thus, it will be important to study how treatment affects clonal evolution, as this information may help to select therapies preventing the expansion of highly fit subclones. Then, we will not only be able to target the most prevalent drivers, but also the evolutionary landscape.

Finally, individual tailoring of AML treatment will also depend on the next wave of clinical trials, which will need to establish the most informative molecular markers to guide therapy and to determine their clinical usefulness for comprehensive MRD monitoring. Furthermore, personalized combination therapies should be the goal, which will require changing the paradigm for drug development and study design. Today, many scientifically interesting and potentially useful agents are discontinued early in development because they fail to demonstrate efficacy as monotherapy. Thus, pre-set efficacy benchmarks will have to be redefined, and faster and better trial designs need to be developed in order to accelerate drug development that ultimately will lead to meaningful improvements in patient survival.

References

1. Marcucci G, Haferlach T, Dohner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. J Clin Oncol. 2011;29:475-86.
2. Bullinger L, Frohling S. Array-based cytogenetic approaches in acute myeloid leukemia: clinical impact and biological insights. Semin Oncol. 2012;39:37-46.
3. Kohlmann A, Grossmann V, Nadarajah N, Haferlach T. Next-generation sequencing - feasibility and practicality in haematology. Br J Haematol. 2013;160:736-53.
4. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009;360:2289-301.
5. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med. 2009;361:1058-66.
6. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010;363:2424-33.
7. Swerdlow SH, Campo E, Harris NL, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2008.
8. Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115:453-74.
9. Burnett AK. Treatment of acute myeloid leukemia: are we making progress? Hematology Am Soc Hematol Educ Program. 2012;2012:1-6.
10. Dohner H, Gaidzik VI. Impact of genetic features on treatment decisions in AML. Hematology Am Soc Hematol Educ Program. 2011;2011:36-42.
11. Paschka P. Core binding factor acute myeloid leukemia. Semin Oncol. 2008;35:410-7.
12. Burnett AK, Hills RK, Milligan D, Kjeldsen L, Kell J, Russell NH, et al. Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial. J Clin Oncol. 2011;29:369-77.
13. Ravandi F, Estey EH, Appelbaum FR, Lo-Coco F, Schiffer CA, Larson RA, et al. Gemtuzumab ozogamicin: time to resurrect? J Clin Oncol. 2012;30:3921-3.
14. Castaigne S, Pautas C, Terre C, Raffoux E, Bordessoule D, Bastie JN, et al. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. Lancet. 2012;379:1508-16.
15. Boissel N, Leroy H, Brethon B, Philippe N, de Botton S, Auvrignon A, et al. Incidence and prognostic impact of c-Kit,

FLT3, and Ras gene mutations in core binding factor acute myeloid leukemia (CBF-AML). Leukemia. 2006;20:965-70.
16. Jourdan E, Boissel N, Chevret S, Delabesse E, Renneville A, Cornillet P, et al. Prospective evaluation of gene mutations and minimal residual disease in patients with core binding factor acute myeloid leukemia. Blood. 2013;121:2213-23.
17. Paschka P, Du J, Schlenk RF, Gaidzik VI, Bullinger L, Corbacioglu A, et al. Secondary genetic lesions in acute myeloid leukemia with inv(16) or t(16;16): a study of the German-Austrian AML Study Group (AMLSG). Blood. 2013;121:170-7.
18. Helton HL, Ries RE, Alonzo TA, Gerbing RB, Trevino LR, Farrar J, et al. Clinically Significant Mutations, Deletions and Translocations Involving ETV6 Identified by Whole Genome and Whole Exome Sequencing; Report From NCI/COG Target AML Initiative. ASH Annual Meeting Abstracts. 2012;120:125.
19. Kuhn MW, Radtke I, Bullinger L, Goorha S, Cheng J, Edelmann J, et al. High-resolution genomic profiling of adult and pediatric core-binding factor acute myeloid leukemia reveals new recurrent genomic alterations. Blood. 2012;119:e67-75.
20. Corbacioglu A, Scholl C, Schlenk RF, Eiwen K, Du J, Bullinger L, et al. Prognostic impact of minimal residual disease in CBFB-MYH11-positive acute myeloid leukemia. J Clin Oncol. 2010;28:3724-9.
21. Krauter J, Gorlich K, Ottmann O, Lubbert M, Dohner H, Heit W, et al. Prognostic value of minimal residual disease quantification by real-time reverse transcriptase polymerase chain reaction in patients with core binding factor leukemias. J Clin Oncol. 2003;21:4413-22.
22. Yin JA, O'Brien MA, Hills RK, Daly SB, Wheatley K, Burnett AK. Minimal residual disease monitoring by quantitative RT-PCR in core binding factor AML allows risk stratification and predicts relapse: results of the United Kingdom MRC AML-15 trial. Blood. 2012;120:2826-35.
23. Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH, et al. Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. Blood. 2010;116:354-65.
24. Kühn M, Bullinger L, Edelmann J, Krönke J, Gröschel S, Rücker F, et al. Genome-wide genotyping of de novo and therapy-related acute myeloid leukemia with t(9;11)(p22;q23) reveals novel recurrent genomic alterations. Haematologica. 2012;97:15(Abstract).
25. Bernt KM, Armstrong SA. Targeting epigenetic programs in MLL-rearranged leukemias. Hematology Am Soc Hematol Educ Program. 2011;2011:354-60.
26. Bernt KM, Zhu N, Sinha AU, Vempati S, Faber J, Krivtsov AV, et al. MLL-rearranged leukemia is dependent on aberrant H3K79 methylation by DOT1L. Cancer Cell. 2011;20:66-78.
27. Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU, et al. H3K79 methylation profiles define murine and human MLL-AF4 leukemias. Cancer Cell. 2008;14:355-68.
28. Daigle SR, Olhava EJ, Therkelsen CA, Majer CR, Sneeringer CJ, Song J, et al. Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. Cancer Cell. 2011;20:53-65.
29. Vassiliou GS, Cooper JL, Rad R, Li J, Rice S, Uren A, et al. Mutant nucleophosmin and cooperating pathways drive leukemia initiation and progression in mice. Nat Genet. 2011;43:470-5.
30. Abbas S, Lugthart S, Kavelaars FG, Schelen A, Koenders JE, Zeilemaker A, et al. Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. Blood. 2010;116:2122-6.
31. Green CL, Evans CM, Hills RK, Burnett AK, Linch DC, Gale RE. The prognostic significance of IDH1 mutations in younger adult patients with acute myeloid leukemia is dependent on FLT3/ITD status. Blood. 2010;116:2779-82.
32. Paschka P, Schlenk RF, Gaidzik VI, Habdank M, Kronke J, Bullinger L, et al. IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. J Clin Oncol. 2010;28:3636-43.
33. Thol F, Damm F, Ludeking A, Winschel C, Wagner K, Morgan M, et al. Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. J Clin Oncol. 2011;29:2889-96.
34. Wagner K, Damm F, Gohring G, Gorlich K, Heuser M, Schafer I, et al. Impact of IDH1 R132 mutations and an IDH1 single nucleotide polymorphism in cytogenetically normal acute myeloid leukemia: SNP rs11554137 is an adverse prognostic factor. J Clin Oncol. 2010;28:2356-64.
35. Falini B, Gionfriddo I, Cecchetti F, Ballanti S, Pettirossi V, Martelli MP. Acute myeloid leukemia with mutated nucleophosmin (NPM1): any hope for a targeted therapy? Blood Rev. 2011;25:247-54.
36. Schlenk RF, Dohner K, Krauter J, Frohling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358:1909-18.
37. Patel JP, Levine RL. How do novel molecular genetic markers influence treatment decisions in acute myeloid leukemia? Hematology Am Soc Hematol Educ Program. 2012;2012:28-34.
38. Kronke J, Schlenk RF, Jensen KO, Tschurtz F, Corbacioglu A, Gaidzik VI, et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. J Clin Oncol. 2011;29:2709-16.
39. Becker H, Marcucci G, Maharry K, Radmacher MD, Mrozek K, Margeson D, et al. Favorable prognostic impact of NPM1 mutations in older patients with cytogenetically normal de novo acute myeloid leukemia and associated gene- and microRNA-expression signatures: a Cancer and Leukemia Group B study. J Clin Oncol. 2010;28:596-604.
40. Schlenk RF, Dohner K, Kneba M, Gotze K, Hartmann F, Del Valle F, et al. Gene mutations and response to treatment with all-trans retinoic acid in elderly patients with acute myeloid leukemia. Results from the AMLSG Trial AML HD98B. Haematologica. 2009;94:54-60.
41. Walter RB, Appelbaum FR, Estey EH, Bernstein ID. Acute myeloid leukemia stem cells and CD33-targeted immunotherapy. Blood. 2012;119:6198-208.
42. Mortland L, Alonzo TA, Walter RB, Gerbing RB, Mitra AK, Pollard JA, et al. Clinical Significance of CD33 Nonsynonymous Single-Nucleotide Polymorphisms in Pediatric Patients with Acute Myeloid Leukemia Treated with Gemtuzumab-Ozogamicin-Containing Chemotherapy. Clin Cancer Res. 2013;19:1620-7.
43. Burnett AK, Hills RK, Green C, Jenkinson S, Koo K, Patel Y, et al. The impact on outcome of the addition of all-trans retinoic acid to intensive chemotherapy in younger patients with nonacute promyelocytic acute myeloid leukemia: overall results and results in genotypic subgroups defined by mutations in NPM1, FLT3, and CEBPA. Blood. 2010;115:948-56.
44. Schlenk RF, Dohner K, Krauter J, Gaidzik VI, Paschka P, Heuser M, et al. All-Trans Retinoic Acid Improves Outcome in Younger Adult Patients with Nucleophosmin-1 Mutated Acute Myeloid Leukemia - Results of the AMLSG 07-04 Randomized Treatment Trial. ASH Annual Meeting Abstracts. 2011;118:80.
45. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009;113:3088-91.
46. Taskesen E, Bullinger L, Corbacioglu A, Sanders MA, Erpelinck CA, Wouters BJ, et al. Prognostic impact, concurrent genetic mutations, and gene expression features of AML with CEBPA mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for CEBPA double mutant AML as a distinctive disease entity. Blood. 2011;117:2469-75.
47. Greif PA, Dufour A, Konstandin NP, Ksienzyk B, Zellmeier E, Tizazu B, et al. GATA2 zinc finger 1 mutations associated with biallelic CEBPA mutations define a unique genetic entity of acute myeloid leukemia. Blood. 2012;120:395-403.
48. Hickey CJ, Schwind S, Radomska HS, Dorrance AM, Santhanam R, Mishra A, et al. Lenalidomide-mediated enhanced translation of C/EBPalpha-p30 protein up-regulates expression of the antileukemic microRNA-181a in acute myeloid leukemia. Blood. 2013;121:159-69.
49. Frohling S, Scholl C, Levine RL, Loriaux M, Boggon TJ, Bernard OA, et al. Identification of driver and passenger mutations of FLT3 by high-throughput DNA sequence analysis and functional assessment of candidate alleles. Cancer Cell. 2007;12:501-13.
50. Kayser S, Schlenk RF, Londono MC, Breitenbuecher F, Wittke K, Du J, et al. Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. Blood.

2009;114:2386-92.
51. Swords R, Freeman C, Giles F. Targeting the FMS-like tyrosine kinase 3 in acute myeloid leukemia. Leukemia. 2012;26:2176-85.
52. Cortes JE, Perl AE, Dombret H, Kayser S, Steffen B, Rousselot P, et al. Final Results of a Phase 2 Open-Label, Monotherapy Efficacy and Safety Study of Quizartinib (AC220) in Patients >= 60 Years of Age with FLT3 ITD Positive or Negative Relapsed/Refractory Acute Myeloid Leukemia. ASH Annual Meeting Abstracts. 2012;120:48.
53. Levis MJ, Perl AE, Dombret H, Dohner H, Steffen B, Rousselot P, et al. Final Results of a Phase 2 Open-Label, Monotherapy Efficacy and Safety Study of Quizartinib (AC220) in Patients with FLT3-ITD Positive or Negative Relapsed/Refractory Acute Myeloid Leukemia After Second-Line Chemotherapy or Hematopoietic Stem Cell Transplantation. ASH Annual Meeting Abstracts. 2012;120: 673.
54. Albers C, Leischner H, Verbeek M, Yu C, Illert AL, Peschel C, et al. The secondary FLT3-ITD F691L mutation induces resistance to AC220 in FLT3-ITD(+) AML but retains in vitro sensitivity to PKC412 and Sunitinib. Leukemia. 2013 Jan 16. [Epub ahead of print]
55. Smith CC, Lasater EA, Zhu X, Lin KC, Stewart WK, Damon LE, et al. Activity of ponatinib against clinically-relevant AC220-resistant kinase domain mutants of FLT3-ITD. Blood. 2013 Feb 21. [Epub ahead of print]
56. Galanis A, Rajkhowa T, Muralidhara C, Ramachandran A, Levis M. Crenolanib: a next generation FLT3 inhibitor. Cancer Res. 2012;72:3660(Abstract).
57. DeZern AE, Sung A, Kim S, Smith BD, Karp JE, Gore SD, et al. Role of allogeneic transplantation for FLT3/ITD acute myeloid leukemia: outcomes from 133 consecutive newly diagnosed patients from a single institution. Biol Blood Marrow Transplant. 2011;17:1404-9.
58. Brunet S, Labopin M, Esteve J, Cornelissen J, Socie G, Iori AP, et al. Impact of FLT3 internal tandem duplication on the outcome of related and unrelated hematopoietic transplantation for adult acute myeloid leukemia in first remission: a retrospective analysis. J Clin Oncol. 2012;30:735-41.
59. Haferlach C, Dicker F, Herholz H, Schnittger S, Kern W, Haferlach T. Mutations of the TP53 gene in acute myeloid leukemia are strongly associated with a complex aberrant karyotype. Leukemia. 2008;22:1539-41.
60. Rucker FG, Schlenk RF, Bullinger L, Kayser S, Teleanu V, Kett H, et al. TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. Blood. 2012;119:2114-21.
61. Breems DA, Van Putten WL, De Greef GE, Van Zelderen-Bhola SL, Gerssen-Schoorl KB, Mellink CH, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. J Clin Oncol. 2008;26:4791-7.
62. Grossmann V, Schnittger S, Kohlmann A, Eder C, Roller A, Dicker F, et al. A novel hierarchical prognostic model of AML solely based on molecular mutations. Blood. 2012;120:2963-72.
63. Mohr B, Schetelig J, Schafer-Eckart K, Schmitz N, Hanel M, Rosler W, et al. Impact of allogeneic haematopoietic stem cell transplantation in patients with abnl(17p) acute myeloid leukaemia. Br J Haematol. 2013;161(2):237-44.
64. Szer J. The prevalent predicament of relapsed acute myeloid leukemia. Hematology Am Soc Hematol Educ Program. 2012;2012:43-8.
65. Cortes J, Feldman E, Yee K, Rizzieri D, Advani AS, Charman A, et al. Two dosing regimens of tosedostat in elderly patients with relapsed or refractory acute myeloid leukaemia (OPAL): a randomised open-label phase 2 study. Lancet Oncol. 2013;14(4):354-62.
66. Lowenberg B, Morgan G, Ossenkoppele GJ, Burnett AK, Zachee P, Duhrsen U, et al. Phase I/II clinical study of Tosedostat, an inhibitor of aminopeptidases, in patients with acute myeloid leukemia and myelodysplasia. J Clin Oncol. 2010;28:4333-8.
67. Dolnik A, Engelmann JC, Scharfenberger-Schmeer M, Mauch J, Kelkenberg-Schade S, Haldemann B, et al. Commonly altered genomic regions in acute myeloid leukemia are enriched for somatic mutations involved in chromatin remodeling and splicing. Blood. 2012;120:e83-92.
68. Ley TJ, Mardis ER, Ding L, Fulton B, McLellan MD, Chen K, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature. 2008;456:66-72.
69. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer. 2012;12:599-612.
70. Owen C, Fitzgibbon J, Paschka P. The clinical relevance of Wilms Tumour 1 (WT1) gene mutations in acute leukaemia. Hematol Oncol. 2010;28:13-9.
71. Damm F, Heuser M, Morgan M, Yun H, Grosshennig A, Gohring G, et al. Single nucleotide polymorphism in the mutational hotspot of WT1 predicts a favorable outcome in patients with cytogenetically normal acute myeloid leukemia. J Clin Oncol. 2010;28:578-85.
72. Gaidzik VI, Bullinger L, Schlenk RF, Zimmermann AS, Rock J, Paschka P, et al. RUNX1 mutations in acute myeloid leukemia: results from a comprehensive genetic and clinical analysis from the AML study group. J Clin Oncol. 2011;29: 1364-72.
73. Mendler JH, Maharry K, Radmacher MD, Mrozek K, Becker H, Metzeler KH, et al. RUNX1 mutations are associated with poor outcome in younger and older patients with cytogenetically normal acute myeloid leukemia and with distinct gene and MicroRNA expression signatures. J Clin Oncol 2012;30:3109-18.
74. Schnittger S, Dicker F, Kern W, Wendland N, Sundermann J, Alpermann T, et al. RUNX1 mutations are frequent in de novo AML with noncomplex karyotype and confer an unfavorable prognosis. Blood. 2011;117:2348-57.
75. Tang JL, Hou HA, Chen CY, Liu CY, Chou WC, Tseng MH, et al. AML1/RUNX1 mutations in 470 adult patients with de novo acute myeloid leukemia: prognostic implication and interaction with other gene alterations. Blood. 2009;114:5352-61.
76. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet. 2009;41:838-42.
77. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature. 2010;468:839-43.
78. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;324:930-5.
79. Li Z, Cai X, Cai CL, Wang J, Zhang W, Petersen BE, et al. Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. Blood. 2011;118:4509-18.
80. Moran-Crusio K, Reavie L, Shih A, Abdel-Wahab O, Ndiaye-Lobry D, Lobry C, et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell. 2011;20:11-24.
81. Quivoron C, Couronne L, Della Valle V, Lopez CK, Plo I, Wagner-Ballon O, et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell. 2011; 20:25-38.
82. Metzeler KH, Maharry K, Radmacher MD, Mrozek K, Margeson D, Becker H, et al. TET2 mutations improve the new European LeukemiaNet risk classification of acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol. 2011;29:1373-81.
83. Gaidzik VI, Paschka P, Spath D, Habdank M, Kohne CH, Germing U, et al. TET2 mutations in acute myeloid leukemia (AML): results from a comprehensive genetic and clinical analysis of the AML study group. J Clin Oncol. 2012;30:1350-7.
84. Patel JP, Gonen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. N Engl J Med. 2012;366:1079-89.
85. Marcucci G, Maharry K, Wu YZ, Radmacher MD, Mrozek K, Margeson D, et al. IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol. 2010;28:2348-55.
86. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell. 2011;19:17-30.
87. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell. 2010;18:553-67.
88. Green CL, Evans CM, Zhao L, Hills RK, Burnett AK, Linch

DC, et al. The prognostic significance of IDH2 mutations in AML depends on the location of the mutation. Blood. 2011;118:409-12.
89. Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, et al. Array-based genomic resequencing of human leukemia. Oncogene. 2010;29:3723-31.
90. Yan XJ, Xu J, Gu ZH, Pan CM, Lu G, Shen Y, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet. 2011;43:309-15.
91. Qu Y, Lennartsson A, Gaidzik VI, Deneberg S, Bengtzen S, Arzenani MK, et al. Genome-Wide DNA Methylation Analysis Shows Enrichment of Differential Methylation in "Open Seas" and Enhancers and Reveals Hypomethylation in DNMT3A Mutated Cytogenetically Normal AML (CN-AML). ASH Annual Meeting Abstracts. 2012;120:653.
92. Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet. 2012;44:23-31.
93. LaRochelle O, Bertoli S, Vergez F, Sarry JE, Mansat-De Mas V, Dobbelstein S, et al. Do AML patients with DNMT3A exon 23 mutations benefit from idarubicin as compared to daunorubicin? A single center experience. Oncotarget. 2011;2:850-61.
94. Abdel-Wahab O, Adli M, LaFave LM, Gao J, Hricik T, Shih AH, et al. ASXL1 mutations promote myeloid transformation through loss of PRC2-mediated gene repression. Cancer Cell. 2012;22:180-93.
95. Chou WC, Huang HH, Hou HA, Chen CY, Tang JL, Yao M, et al. Distinct clinical and biological features of de novo acute myeloid leukemia with additional sex comb-like 1 (ASXL1) mutations. Blood. 2010;116:4086-94.
96. Metzeler KH, Becker H, Maharry K, Radmacher MD, Kohlschmidt J, Mrozek K, et al. ASXL1 mutations identify a high-risk subgroup of older patients with primary cytogenetically normal AML within the ELN Favorable genetic category. Blood. 2011;118:6920-9.
97. Schnittger S, Eder C, Jeromin S, Alpermann T, Fasan A, Grossmann V, et al. ASXL1 exon 12 mutations are frequent in AML with intermediate risk karyotype and are independently associated with an adverse outcome. Leukemia. 2013;27:82-91.
98. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, et al. The origin and evolution of mutations in acute myeloid leukemia. Cell. 2012;150:264-78.
99. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481:506-10.
100. Landau DA, Carter SL, Stojanov P, McKenna A, Stevenson K, Lawrence MS, et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell. 2013;152:714-26.

Acute myeloid leukemia in older patients: conventional and new therapies

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A B S T R A C T

Acute myeloid leukemia (AML) in older patients continues to pose significant treatment challenges. In this age group, the benefit associated with intensive chemotherapy remains marginal and the chance for cure continues to be below 10% overall. While treatment outcome is compromised by a higher prevalence of comorbidities, it is now clear that AML in older patients is a biologically distinct disease that is intrinsically less responsive to chemotherapy. Improving risk-assessment tools is critical to identify those patients who are most likely to benefit from intensive chemotherapy, but optimal induction and post-remission therapies have yet to be determined in this population. New strategies and treatments are emerging and under current assessment. In particular, investigations of monoclonal antibodies, hypomethylating agents, signal transduction inhibitors, and novel cytotoxics hold promise for improving the outcome for older patients with AML, including those for whom traditional chemotherapy is not considered appropriate, either because of anticipated lack of efficacy or unacceptable mortality. Further progress in the care of elderly AML is largely dependent upon building a critical mass of patients and physicians willing to participate in clinical trials.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe current and emerging therapies for older patients with newly diagnosed AML;
- select appropriate up-front therapy based upon patient and disease characteristics;
- discuss treatment options for older patients who may or may not be candidates for intensive chemotherapy;
- understand the importance of encouraging older patients to participate in clinical trials for AML.

Introduction

Acute myeloid leukemia (AML), a disease affecting primarily older adults with a median age at presentation of approximately 68 years, continues to pose significant treatment challenges.[1] Although there have been improvements in treatment outcomes for AML in recent years, these have mostly benefited younger patients under the age of 60 years.[2] Advanced age is considered an adverse prognostic indicator resulting from both a more aggressive underlying disease biology and a decreased capacity of patients to tolerate chemotherapy due to the frequent presence of significant co-morbidities and poor organ reserve. In clinical trials, which typically exclude patients with severe co-morbidities, complete remissions are observed in 40-65% of patients treated intensively, of whom almost 90% relapse within three years.[3,4] Age continuously affects treatment results, as do other independent prognostic factors including performance status, organ dysfunction, white blood cell count, cytogenetics, molecular abnormalities, overexpression of multidrug resistance proteins, and secondary leukemia. Because of this, it is difficult to recommend precise age cut offs for clinical decision-making.[5-7] Much interest is currently being directed at the development of multifactorial risk scores to more accurately predict the outcome for patients who may then be given the choice of intensive or alternative treatment approaches, including less intensive therapy, investigational therapy or palliative care.[8-11] The importance of patient selection is apparent in a review of 2657 elderly patients with AML collected by Medicare and the Surveillance, Epidemiology, and End Results (SEER).[12] Only approximately 30% of patients underwent induction chemotherapy and the median survival across all study population was 2.4 months with a 2-year survival of 6%. However, the analysis also showed that patients who did receive chemotherapy had a survival benefit, even though this was modest. As the general population lives longer, the number of patients in this age group will increase. Therefore, there is an urgent need to find new treatments that are more effective and less toxic for these patients who are traditionally not catered for in most trials. In this review, we provide an outline of the current and developing treatments for older patients with newly diagnosed AML.

Choice of treatment

Despite the reluctance to treat older patients with intensive chemotherapy because of toxicity concerns, induction of a complete remission (CR), even if short-lived, is an appropriate goal for most AML patients over 60 years of age. This concept was established in the late 1980s based on the results of the EORTC AML-7 trial which prospectively compared induction therapy with daunorubicin, vincristine and cytarabine *versus* supportive care with palliative chemotherapy (hydroxyurea or low-dose cytarabine) in patients over 65 years of age.[13] The patients who received induction chemotherapy had a higher CR rate (58% *vs.* 0%), lower incidence of early mortality (3 of 31 *vs.* 18 of 29), longer median survival (21 *vs.* 11 weeks) and greater chance of survival at 2.5 years (13% *vs.* 0%). Importantly, there was no difference in the number of days that patients were hospitalized. Furthermore, registry data from nearly 3000 unselected older patients in Sweden showed reduced rates of early mortality for those who received intensive chemotherapy *versus* palliative care, as well as improved long-term survival in geographical regions where the use of intensive treatment approaches was more common.[14] Thus, achieving CR is a requisite end point for better survival and improved quality of life in elderly AML, and data from large population-based studies have validated the use of intensive chemotherapy over less intensive treatment approaches in patients up to the age of 80 years.

Although it is clear that intensive chemotherapy produces the highest response and survival rates in selected elderly patients with AML, it is ineffective and highly toxic in many others. The challenge is to appropriately identify which patients, based on their disease biology and clinical characteristics, are likely to benefit more from intensive chemotherapy and which require alternative treatment approaches. Several risk scores are available that account for age, performance status, cytogenetics, secondary AML and other covariates to arrive at a prognosis for patients over 60 years of age treated with intensive chemotherapy (Table 1). Despite the differences in variables and end points and methods used, these tools can be used to more accurately individualize the treatment prospects. Patients with the expectation of a low early mortality, high CR rate, and a reasonable long-term survival should be treated with intensive chemotherapy, while those with the expectation of a high risk of early mortality or a poor chance of long-term survival should be offered low-intensity investigational therapy.

Conventional remission induction therapy

For over 30 years, the “3+7” regimen combining daunorubicin (45-50 mg/m^2 for 3 days) and cytarabine (100-200 mg/m^2 by continuous infusion for 7 days) has been the mainstay of induction therapy for older patients with AML.[1] On average, this regimen offers older patients a CR rate of 40-65% with an attendant treatment-related mortality of 15-20%, a median survival of 8-12 months, and a less than 15% probability of sustained remission for three years. Multiple attempts have been made to improve outcome by substituting newer anthracyclines (idarubicin or mitoxantrone) for daunorubicin, escalating the dose of cytarabine, adding other cytotoxic drugs, and priming with growth factors, but none of these strategies has emerged as convincingly superior to “3+7”.[4] However, a recent com-

Table 1. Selected prognostic risk scores in elderly AML.

	Prognostic factors	CR rate (%)	Early death rate (%)	Overall survival (%)
ALFA-9803[8] (n=416)	Poor cytogenetics Age ≥75 PS ≥2 WBC ≥50x10^9/L			(1-year) P-CG or 2/3 factors: 19 Others: 58
MRC AML11/14[9] (n=1071)	Cytogenetics Age WBC PS *De novo vs.* sAML			(1-year) Good: 53 Standard: 43 Poor: 16
MDACC[10] (n=446)	Age ≥80 Complex karyotype PS ≥2 Creatinine >1.3	# 0: 57 # 1: 52 # 2: 29 # ≥ 3: 16	# 0: 16 # 1: 31 # 2: 55 # ≥ 3: 71	(2-year) # 0: 30 # 1: 15 # 2: 7 # ≥ 3: 0
SAL AML-96[11] (n=909)	Cytogenetics Age >65 WBC > 20x10^9/L LDH >700 CD34 >10% NPM1 mutation			(3-years) F-CG: 39.5 I-CG (good): 30 I-CG-(adverse): 10.6 P-CG: 3.3

P-CG: poor-risk cytogenetics; F-CG: favorable-risk cytogenetics; I-CG: intermediate-risk cytogenetics.

bined analysis of two randomized ALFA trials (9801 and 9803), enrolling a total of 727 AML patients age 50 years and over (median 67 years) showed a somewhat superior long-term outcome with idarubicin compared to daunorubicin (cure rate 16.6% *vs.* 9.8%; *P*=0.018). Interestingly, the long-term impact of idarubicin was also evident in the cohort of patients under 65 years of age, although all of the younger patients in the control arm received daunorubicin at higher doses (80 mg/m² × 3).[15]

Options for improvement

Most recently, efforts to improve the complete remission rate and long-term outcome beyond that which is achieved with the traditional "3+7" regimen, have concentrated on anthracycline dose escalation, the addition of novel agents, and alternatives to cytarabine. Lowenberg *et al.* showed a higher CR rate when 813 patients over 60 years of age with newly diagnosed AML were randomized to receive three days of daunorubicin 90 mg/m² *versus* 45 mg/m² in combination with cytarabine 200 mg/m² daily for seven days (64% *vs.* 54%; *P*=0.002).[16] The early death rate was similar between the two groups. Although, overall, there was no difference in survival between patients treated with the standard-dose *versus* the escalated-dose regimen, patients aged 60-65 years gained advantage from daunorubicin intensification with regards to all the major clinical end points. In this subgroup, substantial improvements in CR rate, event-free and overall survival were observed, while patients with core binding factor abnormalities appeared to benefit from high-dose daunorubicin irrespective of age. On the other hand, a randomized trial by the French ALFA group failed to show any clinically relevant superiority of high-dose daunorubicin (80 mg/m² x 3 days) over three or four days of idarubicin (12 mg/m²) when combined with cytarabine for remission induction in 468 patients aged 50-70 years, suggesting therapeutic equivalence between these two drugs at these doses.[17] Whether these studies justify a higher anthracycline dose as the standard of care for older patients with AML is not clear, but they do convincingly demonstrate that there is no increase in toxicity with these regimens.

Other agents with novel mechanisms of action and with non-overlapping toxicity can potentially improve the outcome when added to standard chemotherapy. One option to improve the remission rate and overall outcome could be to incorporate gemtuzumab ozogamicin (GO), an immunoconjugate consisting of a humanized anti-CD33 monoclonal antibody linked to the toxin calicheamicin, into treatment. This strategy has been investigated in three large European studies, two of which show a significant improvement in survival (Table 2). The French ALFA group randomized 280 patients aged 50-70 years (median 62 years) with newly diagnosed AML to standard induction therapy ("3+7") with or without GO given in a fractionated schedule of 3 mg/m² on Days 1, 4 and 7.[18] Although remission rates were much the same in the two groups, patients given GO had lower relapse rates and significantly longer event-free (40.8% *vs.* 17.1%; *P*=0.0003) and overall survival (53.2% *vs.* 41.9%; *P*=0.03) at two years than did controls. This benefit was mainly seen in patients with better-risk disease, but not in those with poor-risk cytogenetics. A more recent study reported by Burnett and colleagues came to the same conclusion as the previous trial.[19] The United Kingdom NCRI AML16 trial randomly assigned 1115 patients (median age 67 years) with newly diagnosed AML to receive induction therapy with daunorubicin and either cytarabine or clofarabine, with or without a single dose of GO 3 mg/m². While there was no difference in the rate of response between the two arms, the cumulative incidence of relapse at three years was significantly reduced with GO (68% *vs.* 76%; *P*=0.007) and overall survival was improved (25% *vs.* 20%; *P*=0.05). Again, the benefit was more evident in those subsets with favorable and intermediate-risk cytogenetics. Importantly, in none of these two trials was the addition of GO associated with excess toxicity. Contrary to the design of these two trials, a sequential rather than concomitant administration of GO and chemotherapy was investigated in a study reported by the EORTC/GIMEMA consortium.[20] This randomized trial compared pre-treatment with GO (6 mg/m² on Days 1 and 15) before initiating induction chemotherapy with the MICE regimen (mitoxantrone, etoposide and cytarabine) in 472 patients aged 61-75 years with previously untreated AML. However, when used in this way, there was no overall benefit, but induction response and survival rates were significantly compromised with GO in patients aged 70 years or older due to excess early mortality. A randomized study from the Ulm group evaluated the effect of all-trans retinoic acid (ATRA) administered in combination with standard induction and consolidation therapy to 242 elderly patients with AML. They showed that addition of ATRA significantly improved CR rate, and event-free and overall survival in these patients.[21] A retrospective analysis of three trials by the French GOELAMS group suggested better response and survival outcomes when lomustine, an alkylating agent, was added to conventional chemotherapy for first-line treatment in older patients with *de novo* AML.[22] A confirmatory randomized study of lomustine in elderly AML is currently ongoing. The already mentioned

Table 2. Randomized trials of gemtuzumab ozogamicin (GO) in combination with conventional chemotherapy in older patients with previously untreated AML.

Trial	N. of patients (age range in years)	GO dose/ schedule	Results
ALFA-0701[18]	278 (50-70)	3 mg/m² Day 1/4/7 with D + A	Similar response rate Longer EFS, RFS, OS No benefit in pts with P-CG
NCRI AML16[19]	1.115 (51-84)	3 mg/m² Day 1 with D + A or Clo + A	Similar response rate Longer RFS, OS Less benefit in pts with P-CG
EORTC/GIMEMA AML-17[20]	472 (61-75)	6 mg/m² Day 1/15 with sequential MICE	Similar response rate Higher induction mortality No benefit in OS, EFS, DFS Too toxic for pts aged > 70 years

D: daunorubicin; A: cytarabine; Clo: clofarabine; MICE: mitoxantrone+cytarabine+etoposide; OS: overall survival; EFS: event-free survival; DFS: disease-free survival; P-CG: poor-risk cytogenetics; pts: patients.

NCRI AML16 trial also compared the purine nucleoside analog clofarabine (20 mg/m^2 x 5 days) against cytarabine, both in combination with standard-dose daunorubicin, in 806 patients age 56-84 years (median 67 years), but there was no evidence of clinical benefit in any risk subgroup.[23]

Postremission therapy

It is generally accepted that the ability to prolong remission and cure patients with AML depends heavily on the administration of some form of postremission therapy.[2] Standard approaches in older patients typically involve cytarabine, either alone or in combination with anthracyclines, for 1-2 cycles. However, there are no randomized trials confirming the benefit of postremission therapy in older patients. Studies of dose-escalated cytarabine in the postremission setting did not produce therapeutic benefits in these patients, and toxicity was prominent.[24,25] Some trials have also failed to show a clinical benefit with increased numbers of consolidation cycles.[26,27] Indeed, there is evidence from the French ALFA-9803 study that multiple less intensive cycles delivered on an outpatient basis may improve survival as compared to a single intensive consolidation course.[28] Randomized trials of postremission maintenance therapy with low-dose cytarabine or attenuated multi-agent chemotherapy have produced improvement of disease-free survival but not overall survival.[29,30] Recent studies of gemtuzumab ozogamicin or interleukin-2 failed to show a benefit in favor of postremission therapy.[31,32]

Allogeneic stem cell transplantation is a curative treatment option for patients with AML, but its application to the elderly population had previously been limited by high rates of transplant-related mortality caused by toxicities from traditional myeloablative conditioning regimens. However, with the use of reduced-intensity conditioning (RIC-SCT) regimens, allogeneic transplantation has become a plausible option to consider for older patients in first complete remission. As suggested by recent reports, these transplants are feasible in selected patients up to 75 years of age and may yield better outcomes than consolidative chemotherapy, but prospective trials are necessary.[33-35]

Alternative treatment approaches

Given the limited success of intensive chemotherapy in providing short- and long-term disease control, and in consideration of the fact that a substantial proportion of patients are deemed unlikely to benefit from traditional regimens based on their disease and clinical characteristics, more contemporary trials have focused on less intensive treatment approaches that may have the potential of preserving efficacy while reducing toxicity in older patients with AML. Low-intensity chemotherapy, investigational new agents, and palliative care represent the spectrum of current alternatives for these patients.

Low-intensity chemotherapy

Subcutaneous administration of low-dose cytarabine (LDAC) is a practical treatment for older patients with AML, and many uncontrolled trials have shown that useful responses, including complete remissions, are achievable with various dose schedules in approximately 15-30% of patients.[36] As part of the United Kingdom NCRI AML14 trial, 217 patients (median age 74 years) who were felt to be unfit for intensive chemotherapy were randomized to either 20 mg cytarabine twice daily subcutaneously for ten days every 4-6 weeks or hydroxyurea.[37] Treatment with LDAC did not increase toxicity and produced a higher CR rate (18% *vs.* 1%; P=0.00006) and better overall survival (P=0.0009). This was accounted for by the achievement of CR (median survival 19 months compared with 2 months in non-responders). However, patients with adverse cytogenetics did not benefit from LDAC. While the overall survival in patients receiving LDAC was still poor (median 5 months), this trial does provide a simple and tolerable low-intensity regimen that could be used as the standard comparator for randomized trials of novel agents in this group of patients. Combining LDAC with either arsenic trioxide, gemtuzumab ozogamicin, or the farnesyl transferase inhibitor tipifarnib produced no survival benefit in older patients unfit for intensive chemotherapy entered into the randomized NCRI AML16 trial ("Pick a Winner" design), although the remission rate was almost doubled with the addition of GO to LDAC (30% *vs.* 17%; P=0.006).[38-40]

Clofarabine, a 2nd generation purine nucleoside analog, has been shown to have activity in elderly AML as a single agent or in combination with cytarabine. A multicenter phase II study of clofarabine monotherapy (30 mg/m^2 daily for 5 days) in 112 previously untreated AML patients aged 60 years and over with at least one adverse prognostic feature (aged 70 years or over, performance status 2, antecedent hematologic disorder, or non-favorable cytogenetics) showed an overall response rate (ORR) of 46%, with a CR rate of 38% and a 30-day all cause mortality of 10%.[41] Interestingly, the ORR was 42% among patients with poor-risk cytogenetics and 38% for patients presenting with multiple risk factors. Median disease-free survival was 37 weeks, and median survival was 41 weeks for all patients. In two consecutive European studies of 106 untreated older patients considered unfit for intensive chemotherapy, patients were given four to six 5-day courses of single agent clofarabine (30 mg/m^2 per day).[42] Median age was 71 years (range 60-84 years), 30% had adverse-risk cytogenetics, 36% had a WHO performance score of 2 or higher, and 46% had Wheatley poor-risk disease. The ORR was 48% (32% CR, 16% CRi) and 18% died within 30 days. The median survival was 19 weeks for all and 45 weeks for those who achieved a CR or a CRi. Importantly, the ORR was consistently high in patients with adverse cytogenetics (44%), patients with secondary AML (31%), and in patients over 70 years of age (49%). While these results suggest encouraging activity in older patients with poor-risk AML, in a recently reported randomized trial of 406 newly diagnosed older patients considered unsuitable for intensive treatment, clofarabine (20 mg/m^2 daily for 5 days) has been shown to significantly improve the response rate compared to LDAC (CR+CRi 38% *vs.* 20%; P<0.0001). However, disappointingly, it did not result in a survival benefit overall, or identify any demographic or risk subgroup.[43] Since clofarabine can potentiate the intracellular metabolism of cytarabine, a study of low-intensity therapy compared treatment with clofarabine (30 mg/m^2 daily for 5 days) with or without LDAC in 70 patients aged over 60 years with untreated AML.[44] The

CR rate was significantly higher in the combination therapy group (63% *vs*. 31%; *P*=0.025), with a non-significant difference in induction mortality (19% *vs*. 31%). However, there was no difference in overall survival. An alternative approach is to take advantage of inhibiting the hypermethylation of tumor suppressor genes thought to play a critical role in the pathobiology of AML. Two hypomethylating agents, azacitidine and decitabine, have been investigated in older patients with AML who are considered not to be candidates for intensive chemotherapy. In a phase III international trial (AZA-001) comparing azacitidine (75 mg/m^2 subcutaneously for 7 days of each 28-day cycle) to conventional care regimens (CCR: doctor's choice of LDAC, intensive chemotherapy or supportive care alone) in patients with intermediate-2 and high-risk myelodysplasia, 113 patients (median age 70 years) had bone marrow blast percentages of 20-29%, which reclassified them as having AML according to the WHO criteria.[45] Although CR rates were similar for azacitidine compared to CCR (18% *vs.* 16%), azacitidine was better tolerated and resulted in a significant survival benefit (median 24.5 *vs.* 16.0 months; *P*=0.005), including higher 2-year survival (38% *vs.* 0%) in patients with adverse cytogenetics. In a phase II study of 55 older patients with untreated AML, intravenous decitabine (20 mg/m^2 daily) was administered for five days monthly until disease progression.[46] An overall response rate of 24% was reported with a 30-day mortality of 7% and a median survival duration of 7.7 months. Notably, responses were seen in all cytogenetic risk groups as well as in patients with prior myelodysplasia. In another study, decitabine was administered at a more myelosuppressive dose schedule (20 mg/m^2 daily for 10 days) to 53 patients (median age 74 years) who were unsuitable for standard chemotherapy.[47] The overall response rate was 64% (CR 47%, CRi 17%), with a 30- and 60-day mortality of 2% and 15%, respectively. Median overall and disease-free survival were 55 and 46 weeks, respectively. Responses occurred in all subgroups, regardless of age, cytogenetics, leukocyte count, and prior myelodysplasia. Recently, decitabine 20 mg/m^2 daily for five days per cycle was compared with conventional care (doctor's choice of supportive care or LDAC) in a large phase III trial of 485 AML patients aged 65 years or older who were unfit for intensive chemotherapy.[48] Treatment with decitabine resulted in a higher response rate (CR+CRp 17.8% *vs.* 7.8%; *P*=0.001) and a non-significant improvement in overall survival (7.7 months *vs.* 5 months) which, however, became significant (*P*=0.03) when more mature survival data were analyzed. Combining decitabine and azacitidine with other epigenetic modulators has been evaluated in several trials.[49-51] Generally, combined epigenetic therapy appears safe and promising, but randomized trials will be required to establish the incremental benefit of this approach on response rates and duration of survival in older patients with AML. Another strategy that is being explored is the integration of epigenetic therapy with low-intensity chemotherapy. Recently, a trial evaluating the combination of clofarabine plus LDAC followed by a prolonged consolidation alternating with decitabine reported an overall response rate of 66% including a CR rate of 58% with few early relapses (median relapse-free survival 14.1 months, median overall survival 12.7 months) in 59 older patients (median age 70 years) with newly diagnosed AML.[52] Based on these promising results, strategies of improving survival with epigenetic therapies without necessarily improving remission rates may be particularly suitable for older patients, but larger studies and long-term follow up are needed to better define the role of this treatment modality in this challenging patient population.

Novel agents

A number of investigational agents that represent alternatives to conventional chemotherapy have shown promise as first-line treatment in older patients with AML. The novel alkylating agent laromustine was reported to have significant single agent activity in 85 previously untreated older patients (median age 72 years) with poor-risk AML, showing an ORR of 32% (CR 23%, CRp 9%) and a 30-day mortality rate of 14%, following a single intravenous infusion at 600 mg/m^2. Response rates were consistent across a spectrum of poor-risk features. The median overall survival was 3.2 months (12.4 months in responders), with a 1-year survival of 21% (52% in responders).[53] CPX-351 is a liposomal formulation of a 5:1 fixed molar ratio of daunorubicin and cytarabine. Among 125 previously untreated patients aged 60-75 years who were randomized between CPX-351 (100 units/m^2 on Days 1, 3 and 5) and standard daurorubicin plus cytarabine induction chemotherapy, the rate of response was increased with CPX-351 (CR+CRp 66.7% *vs*. 51.2%), largely due to a higher CRp rate. The 60-day mortality rate was reduced compared to "3+7" regimen (4.7% *vs*. 14.6%). Interestingly, the trend towards higher response rates was observed particularly for patients with adverse cytogenetics, aged over 70 years, and secondary AML.[54] High-dose lenalidomide (50 mg/day for 28 days for two cycles) for remission induction followed by a lower dose (10 mg/day for 28 days for 12 months) as maintenance was administered to 33 untreated AML patients (median age 71 years) with intermediate- or poor-risk cytogenetics. Responses (CR/CRi) occurred rapidly in 30% of patients, and in 53% of those completing the two induction courses. Importantly, a cytogenetic remission was achieved in 4 of the 5 patients with clonal cytogenetic abnormalities at diagnosis and, similar to the experience with hypomethylating agents, no responses were noted in patients with rapidly progressing, hyperproliferative AML.[55] Sapacitabine, a novel oral cytosine nucleoside analog, has been investigated in a randomized phase II study of three different dose schedules in 105 patients over 70 years of age with AML (86 were previously untreated).[56] The dose schedule with the best efficacy profile was 400 mg twice daily for three days each week for two weeks (cycles repeated every 28 days). Among the 20 patients allocated to receive this schedule, responses were observed in 45% (6 patients had CR or CRi and 3 hematologic improvement), the 30-day mortality was 10%, and the 1-year overall survival was 30%. Randomized trials assessing sapacitabine against LDAC or decitabine are ongoing in elderly AML.

New evidence of the pathobiology and molecular background of the disease has led to the development of a number of targeted agents, and their use, either as single agents or in combination with cytotoxics, may provide us with more effective, less toxic strategies for treating older patients with AML. Tipifarnib, an orally active farnesyl transferase inhibitor, has been assessed in elderly AML, with one phase II study showing good tolerance and an overall response rate of 23% (CR rate 14%) in 158 older

Table 3. Selected targeted agents under investigation in elderly AML.

Class	Agent	Trial status
Farnesyl transferase inhibitors	Tipifarnib	Phase II/III
FLT3 inhibitors	Lestaurtinib, midostaurin, sorafenib, AC220	Phase II/III
Polo-like kinase inhibitors	Volasertib	Phase II/III
Aminopeptidase inhibitors	Tosedostat	Phase I/II
Topoisomerase II inhibitors	Voreloxin	Phase II/III
NF-kB inhibitors	Bortezomib	Phase I/II
PI3K/AKT/mTOR inhibitors	Rapalogs, BKM120	Phase I/II
Hsp90 inhibitors	Ganetespib	Phase I/II
Hedgehog inhibitors	PF-04449913	Phase I/II

patients (median age 74 years) with newly diagnosed, poor-risk AML.[57] However, in a randomized study that compared tipifarnib (600 mg twice a day for the first 21 days, in 28-day cycles) with best supportive care (BSC) for 457 older patients, there was no difference in overall survival (median of 107 days for tipifarnib and 103 days for BSC). In addition, the CR rate for tipifarnib was lower than that previously reported at 8%.[58] Activating mutations of the receptor tyrosine kinase FLT3, in particular internal tandem duplication, are identified in 20-30% of all AML patients and are associated with poor outcome.[59] Despite a strong biological rationale, studies targeting the FLT3 mutations with a number of small-molecule inhibitors (lestaurtinib, midostaurin, sorafenib) have shown modest clinical activity as monotherapy, but trials in combination with chemotherapy are underway.[60,61] Whether more selective and potent 2nd generation inhibitors will have better efficacy in FLT3 mutated AML remains to be seen, but a recently reported phase II trial of quizartinib (AC220) monotherapy in 132 patients aged 60 years or older with first relapse or primary refractory AML showed a high degree of activity in FLT3 mutated patients (n=90: CR+CRp+CRi 53%), suggesting activity also in non-mutated patients (n=42: CR+CRp+CRi 36%).[62] Further studies of quizartinib as monotherapy and in combination with other agents are ongoing or being planned in elderly AML. Volasertib is an inhibitor of Polo-like kinase 1 (Plk1) which is involved in spindle assembly during mitosis. Preliminary results from a phase II study in which 87 newly diagnosed AML patients (median age 75 years) ineligible for intensive chemotherapy were randomized to treatment with LDAC alone or LDAC plus volasertib, showed an improved complete remission rate with the combination regimen compared to controls (CR+CRi 31% *vs.* 11%; *P*=0.02). Responses with LDAC plus volasertib were observed across genetic subgroups, including patients with adverse cytogenetics.[63] A randomized phase III trial is about to start. A number of other agents that target various aspects of the leukemia cell machinery are currently under investigation, including tosedostat (an aminopeptidase inhibitor), vosaroxin (a novel topoisomerase II inhibitor), bortezomib (a NF-kB inhibitor), ganetespib (a Hsp90 inhibitor), PI3K/AKT/mTOR inhibitors, and hedgehog inhibitors (Table 3).

Conclusion

Treatment of AML in the elderly remains a challenge. A higher frequency of unfavorable biological and clinical prognostic factors, rather than age *per se*, is the major determinant for the poor outcome in this patient population. Conventional intensive chemotherapy is frequently inappropriate and unsuccessful for older patients with this disease. Therefore, treatment approaches should be personalized to the individual patient. It is becoming critically important to appropriately define and select patients who will benefit from intensive chemotherapy, and recently developed prognostic risk models can be used by physicians to guide treatment decisions. Intensive chemotherapy with curative intent should be offered to older patients who are otherwise healthy and without adverse prognostic factors, but current induction and postremission strategies need to be optimized. Patients who are unlikely to benefit from intensive chemotherapy should enter investigational trials of lower intensity or targeted therapies. After so many years of therapeutic nihilism, the development of risk-adapted and more targeted treatment approaches has introduced an era of personalized antileukemia therapy that may bring new hope to older patients with AML. In order to ensure progress continues, it is imperative that all patients be offered the opportunity to participate in clinical trials.

References

1. Estey E. Acute myeloid leukemia and myelodysplastic syndromes in older patients. J Clin Oncol. 2007;25:1908-15.
2. Burnett A, Wetzler M, Lowenberg B. Therapeutic advances in acute myeloid leukemia. J Clin Oncol. 2011;29:487-94.
3. Burnett AK. Treatment of acute myeloid leukemia: are we making progress? Hematology Am Soc Hematol Educ Program. 2012;2012:1-6.
4. Luger SM. Treating the elderly patient with acute myelogenous leukemia. Hematology Am Soc Hematol Educ Program. 2010;2010:62-9.
5. Appelbaum FR, Gundacker H, Head DR, et al. Age and acute myeloid leukemia. Blood. 2006;107:3481-5.
6. Buchner T, Berdel WE, Haferlach C, et al. Age-related risk profile and chemotherapy dose response in acute myeloid leukemia: A study by the German Acute Myeloid Leukemia Cooperative Group. J Clin Oncol. 2009;27:61-9.
7. Dohner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel on behalf of the European LeukemiaNet. Blood. 2010;115:453-74.
8. Malfuson JV, Etienne A, Turlure P, et al. Risk factors and decision criteria for intensive chemotherapy in older patients with acute myeloid leukemia. Haematologica. 2008;93:1806-13.
9. Wheatley K, Brookes CL, Howman AJ, et al. Prognostic factor analysis of the survival of elderly patients with AML in the MRC AML11 and LRF AML14 trials. Br J Haematol. 2009;145:598-605.
10. Kantarjian H, Ravandi F, O'Brien S, et al. Intensive chemotherapy does not benefit most older patients (age 70 years or older) with acute myeloid leukemia. Blood. 2010;116:4422-9.
11. Rollig C, Thiede C, Gramatzki M, et al. A novel prognostic model in elderly patients with acute myeloid leukemia: results of 909 patients entered into the prospective AML96 trial.

Blood. 2010;116:971-8.
12. Menzin J, Lang K, Earle CC, Kerney D, Mallick R. The outcomes and costs of acute myeloid leukemia among the elderly. Arch Intern Med. 2002;162:1597-603.
13. Lowenberg B, Zittoun R, Kerkhofs H, et al. On the value of intensive remission-induction chemotherapy in elderly patients of 65+ years with acute myeloid leukemia: a randomized phase III study of the European Organization for Research and Treatment of Cancer Leukemia Group. J Clin Oncol. 1989;7:1268-74.
14. Juliusson G, Antunovic P, Derolf A, et al. Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. Blood 2009;113:4179-87.
15. Gardin C, Chevret S, Pautas C, Raffoux E, Thomas X, et al. Superior long-term outcome with idarubicin compared with high-dose daunorubicin in patients with acute myeloid leukemia age 50 years and older. J Clin Oncol. 2013;31:321-7.
16. Lowenberg B, Ossenkoppele GJ, van Putten W, et al. High-dose daunorubicin in older patients with acute myeloid leukemia. N Engl J Med. 2009;361:1235-48.
17. Pautas C, Merabet F, Thomas X, et al. Randomized study of intensified anthracycline doses for induction and recombinant interleukin-2 for maintenance in patients with acute myeloid leukemia age 50 to 70 years: results of the ALFA-9801 study. J Clin Oncol. 2010;808-14.
18. Castaigne S, Pautas C, Terré C, et al. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. Lancet. 2012;379:1508-16.
19. Burnett AK, Russell NH, Hills RK, et al. Addition of gemtuzumab ozogamicin to induction chemotherapy improves survival in older patients with acute myeloid leukemia. J Clin Oncol. 2012;30:3924-31.
20. Amadori S, Suciu S, Salih H, et al. The sequential combination of gemtuzumab ozogamicin and intensive chemotherapy does not benefit older patients with untreated AML: results of the EORTC-GIMEMA AML-17 randomized trial. Haematologica (EHA Annual Meeting Abstracts). 2012;97:521-2.
21. Schlenk RF, Frohling S, Hartmann F, et al. Phase III study of all-trans retinoic acid in previously untreated patients 61 years or older with acute myeloid leukemia. Leukemia. 2004;18:1798-803.
22. Pigneux A, Harousseau JL, Witz F, et al. Addition of lomustine to idarubicin and cytarabine improves the outcome of elderly patients with de novo acute myeloid leukemia: a report from the GOELAMS. J Clin Oncol. 2010;28:3028-34.
23. Burnett AK, Russell NH, Kell J, et al. A comparison of daunorubicin/Ara-c (DA) versus daunorubicin/clofarabine (DClo) and two versus three courses of total treatment for older patients with AML and high risk MDS: results of the UK NCRI AML16 trial. Blood (ASH Annual Meeting Abstracts). 2012;120:892.
24. Mayer RJ, Davis RB, Schiffer CA, et al. Intensive postremission chemotherapy in adults with acute myeloid leukemia. N Engl J Med. 1994;331:896-903.
25. Stone R, Berg D, George S, et al. Postremission therapy in older patients with de novo acute myeloid leukemia: a randomized trial comparing mitoxantrone and intermediate-dose cytarabine with standard-dose cytarabine. Blood. 2001;98:548-53.
26. Goldstone AH, Burnett AK, Wheatley K, et al. Attempts to improve treatment outcomes in acute myeloid leukemia in older patients: results of the United Kingdom Medical Research Council AML11 trial. Blood. 2001;98:1302-11.
27. Burnett AK, Milligan D, Goldstone A, et al. The impact of dose escalation and resistance modulation in older patients with acute myeloid leukaemia and high risk myelodysplastic syndrome: the results of the LRF AML14 trial. Br J Haematol. 2009;145:318-32.
28. Gardin C, Turture P, Fagot T, et al. Postremission treatment of elderly patients with acute myeloid leukemia in first complete remission after intensive induction chemotherapy: results of the multicenter randomized Acute Leukemia French Association (ALFA) 9803 trial. Blood. 2007;109:5129-35.
29. Lowenberg B, Suciu S, Archimbaud E, et al. Use of recombinant GM-CSF during and after remission induction chemotherapy in patients aged 61 years and older with acute myeloid leukemia: final report of AML-11, a phase III randomized study of the Leukemia Cooperative Group of the European Organisation for the Research and Treatment of Cancer and the Dutch Belgian Hemato-Oncology Cooperative Group. Blood. 1997;90:2952-61.
30. Buchner T, Hiddemann W, Berdel WE, et al. 6-Thioguanine, cytarabine, and daunorubicin (TAD) and high-dose cytarabine and mitoxantrone (HAM) for induction, TAD for consolidation, and either prolonged maintenance by reduced monthly TAD or TAD-HAM-TAD and one course of intensive consolidation by sequential HAM in adult patients at all ages with de novo acute myeloid leukemia (AML): a randomized trial of the German AML Cooperative Group. J Clin Oncol. 2003; 21:4496-504.
31. Lowenberg B, Beck J, Graux C, et al. Gemtuzumab ozogamicin as postremission treatment in AML at 60 years of age or more: results of a multicenter phase 3 study. Blood. 2010;115:2586-91.
32. Baer MR, George SL, Caligiuri MA, et al. Low-dose interleukin-2 immunotherapy does not improve outcome of patients age 60 years and older with acute myeloid leukemia in first complete remission: Cancer and Leukemia Group B study 9720. J Clin Oncol. 2008;26:4934-9.
33. McClune BL, Weisdorf DJ, Pedersen TL, et al. Effect of age on outcome of reduced-intensity hematopoietic cell transplantation for older patients with acute myeloid leukemia in first complete remission or with myelodysplastic syndrome. J Clin Oncol. 2010;28:1878-87.
34. Koreth J, Aldridge J, Kim HT, et al. Reduced-intensity conditioning hematopoietic stem cell transplantation in patients over 60 years of age: hematologic malignancy outcomes are not impaired in advanced age. Biol Blood Marrow Transplant. 2010;16:792-800.
35. Estey E, de Lima M, Tibes R, et al. Prospective feasibility analysis of reduced-intensity conditioning (RIC) regimens for hematopoietic stem cell transplantation (HSCT) in elderly patients with acute myeloid leukemia (AML) and high-risk myelodysplastic syndrome. Blood. 2007;109:1395-400.
36. Cheson BD, Jasperse DM, Simon R, Friedman MA. A critical appraisal of low-dose cytosine arabinoside in patients with acute non-lymphocytic leukemia and myelodysplastic syndromes. J Clin Oncol. 1986;4:1857-64.
37. Burnett AK, Milligan D, Prentice AG, et al. A comparison of low-dose cytarabine and hydroxyurea with or without all-trans retinoic acid for acute myeloid leukemia and high-risk myelodysplastic syndrome in patients not considered fit for intensive treatment. Cancer. 2007;109:1114-24.
38. Burnett AK, Hills RK, Hunter A, et al. The addition of arsenic trioxide to low-dose Ara-C in older patients with AML does not improve outcome. Leukemia. 2011;25:1122-7.
39. Burnett AK, Russell NH, Culligan D, et al. The addition of the farnesyl transferase inhibitor, tipifarnib, to low dose cytarabine does not improve outcome for older patients with AML. Br J Haematol. 2012;158:519-22.
40. Burnett AK, Hills RK, Hunter AE, et al. The addition of gemtuzumab ozogamicin to low-dose Ara-C improves remission rate but does not significantly prolong survival in older patients with acute myeloid leukaemia: results from the LRF AML14 and NCRI AML16 pick-a-winner comparison. Leukemia 2012 Aug 14 [Epub ahead of print].
41. Kantarjian HM, Erba HP, Claxton D, et al. Phase II study of clofarabine monotherapy in previously untreated older adults with acute myeloid leukemia and unfavorable prognostic factors. J Clin Oncol. 2010;28:549-55.
42. Burnett AK, Russell NH, Kell J, et al. European development of clofarabine as treatment for older patients with acute myeloid leukemia considered unsuitable for intensive chemotherapy. J Clin Oncol. 2010;28:2389-95.
43. Burnett AK, Russell NH, McMullin MF, et al. A randomised comparison of clofarabine versus low dose Ara-C as first line treatment for older patients with AML. Blood (ASH Annual Meeting Abstracts). 2012;120:889.
44. Faderl S, Ravandi F, Huang X, et al. A randomized study of clofarabine versus clofarabine plus low-dose cytarabine as front-line therapy for patients aged 60 years and older with acute myeloid leukemia and high-risk myelodysplastic syndrome. Blood. 2008;112:1638-45.
45. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. J Clin Oncol. 2010;28:562-9.
46. Cashen AF, Schiller GJ, O'Donnell MR, et al. Multicenter, phase II study of decitabine for the first-line treatment of older patients with acute myeloid leukemia. J Clin Oncol. 2010;28:556-61.
47. Blum W, Garzon R, Klisovic RB, et al. Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine. Proc Natl Acad Sci

USA. 2010;107:7473-8.
48. Kantarjian HM, Thomas X, Dmoszynska A, et al. Multicenter, randomized, open-label, trial of decitabine versus patient choice, with physician advice, of either supportive care or low-dose cytarabine for the treatment of older patients with newly diagnosed acute myeloid leukemia. J Clin Oncol. 2012;30:2670-7.
49. Soriano AO, Yang H, Faderl S, et al. Safety and clinical activity of the combination of 5-azacytidine, valproic acid, and all-trans retinoic acid in acute myeloid leukemia and myelodysplastic syndrome. Blood. 2007;110:2302-8.
50. Blum W, Klisovic RB, Hackanson B, et al. Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. J Clin Oncol. 2007;25:3884-91.
51. Kirshbaum M, Ivana G, Goldberg SL, et al. Vorinostat in combination with decitabine for the treatment of relapsed or newly diagnosed acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS): a phase I, dose-escalation study. Blood (ASH Annual Meeting Abstracts). 2009;114:2089.
52. Faderl S, Ravandi F, Huang X, et al. Clofarabine plus low-dose cytarabine followed by clofarabine plus low-dose cytarabine alternating with decitabine in acute myeloid leukemia frontline therapy for older patients. Cancer. 2012;118:4471-7.
53. Schiller GJ, O'Brien SM, Pigneux A, et al. Single-agent laromustine, a novel alkylating agent, has significant activity in older patients with previously untreated poor-risk acute myeloid leukemia. J Clin Oncol. 2010;28:815-21.
54. Lancet JE, Cortes JE, Hogge DE, et al. Phase 2B randomized study of CPX-351 vs, cytarabine (CYT) + daunorubicin (DNR) (7+3 regimen) in newly diagnosed AML patients aged 60-75. Blood (ASH Annual Meeting Abstracts). 2010;116:655.
55. Fehninger TA, Uy GL, Trinkaus K, et al. A phase 2 study of high-dose lenalidomide as initial therapy for older patients with acute myeloid leukemia. Blood. 2011;117:1828-33.
56. Kantarjian H, Faderl S, Garcia-Manero G, et al. Oral sapacitabine for the treatment of acute myeloid leukaemia in elderly patients: a randomised phase 2 study. Lancet Oncol. 2012;13:1096-104.
57. Lancet JE, Gojo I, Gotlib J, et al. A phase 2 study of the farnesyl transferase inhibitor tipifarnib in poor-risk and elderly patients with previously untreated acute myelogenous leukemia. Blood. 2007;109:1387-94.
58. Harousseau JL, Martinelli G, Jedrzejczak WW, et al. A randomized phase 3 study of tipifarnib compared with best supportive care, including hydroxyurea, in the treatment of newly diagnosed acute myeloid leukemia in patients 70 years or older. Blood. 2009;114:1166-73
59. Kottaridis PD, Gale RE, Frew ME, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98:1752-9.
60. Knapper S, Burnett AK, Littlewood T, et al. A phase 2 trial of the FLT3 inhibitor lestaurtinib (CEP701) as first-line treatment for older patients with acute myeloid leukemia not considered fit for intensive chemotherapy. Blood. 2006;108:3262-70.
61. Fischer T, Stone RM, Deangelo DJ, et al. Phase IIB trial of oral midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multitargeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. J Clin Oncol. 2010;28:4339-45.
62. Cortes JE, Perl AE, Dombret H, et al. Final results of a phase 2 open-label, monotherapy efficacy and safety study of quizartinib (AC220) in patients ≥60 years of age with FLT3 ITD positive or negative relapsed/refractory acute myeloid leukemia. Blood (ASH Annual Meeting Abstracts). 2012;120:48.
63. Maertens J, Lubbert M, Fiedler W, et al. Phase I/II study of volasertib (BI 6727), an intravenous Polo-like kinase (Plk) inhibitor, in patients with acute myeloid leukemia (AML): results from the randomized phase II part for volasertib in combination with low-dose cytarabine (LDAC) versus LDAC monotherapy in patients with previously untreated AML ineligible for intensive treatment. Blood (ASH Annual Meeting Abstracts). 2012;120:411.

PML/RARA as the master driver of acute promyelocytic leukemia pathogenesis and basis for therapy response

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A B S T R A C T

Acute promyelocytic leukemia (APL) is caused by a chromosomic translocation that always implicates the retinoic acid receptor alpha (RARA) gene. The PML/RARA fusion is by far the most frequent, present in 98% of patients. Over the past 20 years, multiple studies have outlined how PML/RARA interferes with transcriptional regulation and also with assembly of PML nuclear bodies, domains implicated in control of senescence and stem cell self-renewal. However, the respective contribution of each of those defects to APL pathogenesis remains poorly characterized. APL is the model disease for targeted cure of leukemia. Indeed, soon after the demonstration of their clinical activity, retinoic acid (RA) and arsenic trioxide were found to directly target PML/RARA, RA through its RARA moiety, arsenic through the PML one. Analysis of murine APL models has given us an unprecedented level of understanding of the basis for therapy response, highlighting the key role of PML/RARA degradation in the loss of APL self-renewal. Consequently, therapeutic strategies combining RA and arsenic have shown an extraordinary potency in mice and were successfully transposed to patients. While the molecular basis for loss of APL self-renewal remains under study, cure of most patients without any chemotherapy is now clinically achievable.

Learning goals

At the conclusion of this activity, participants should know that:
- PML/RARA is the single APL driver;
- arsenic cures 70% of patients, and its front-line association with retinoic acid cures almost all of them;
- PML/RARA degradation is closely associated with loss of self-renewal and definitive cures.

PML/RARA: the sole APL driver

APL was identified as a separate clinical entity over 50 years ago.[1] One of the key steps in unraveling the disease genetics was the identification of the t(15,17) translocation present in most patients.[2] The latter was characterized at the molecular level in 1990, either through chromosome walking[3] or by direct exploration of the structure of the *RARA* gene,[4] based on the observation of the disease sensitivity to retinoic acid (RA), the ligand of RARA.[5] More than 98% of APLs are associated with the fusion of the promyelocytic gene (*PML*) with *RARA*[6-8] resulting from the t(15,17) translocation (Figure 1). Others are APL patients who harbor alternative translocations involving *RARA*, the most common being t(11;17) that involves the promyelocytic leukemia zinc finger (*PLZF*) gene.[9,10] The constant implication of RARA in these translocations points to a central role of the deregulation of RARA (and nuclear receptor) signaling in APL pathogenesis.

Cancers arise from the accumulation of multiple genetic and epigenetic lesions co-operating to enforce cellular transformation.[11] Leukemias or sarcomas associated with (or defined by) specific translocations may constitute an exception to this model. Indeed, in APL, only rare lesions, often shared with other leukemias or malignancies, have been implicated in progression, such as *MYC* amplification, Fms-like tyrosine kinase 3 activation, or *RAS* mutations,[12,13] findings recently confirmed by pan-genomic approaches in patients or APL mice.[14,15] These do not radically change the presentation of the disease, although activating FLT3 mutations are more often observed in the APLs with hyper-leukocytosis and are associated with a less favorable outcome.[16] The possibility of obtaining transplantable mouse models faithfully recapitulating the human disease, by the mere expression of the PML/RARA transgene in myeloid precursors, provides additional evidence that the fusion protein is the master driver of APL leukemogenesis.[17,18] Human APL has an almost constant incidence with age, suggesting that it arises from a single rate-limiting genetic event.[19] Similarly, studies in APL that develop following chemotherapy have all demonstrated a short (less than a year) time interval between DNA-damaging chemotherapy and disease onset.[20] APL can thus be considered as a quasi-monogenic, X/RARA-driven, disease.[21]

RARA and PML: the constant and major partners of the fusions

Retinoic acid is involved in a variety of physiological regulatory mechanisms, in particular morphogenesis and stem cell self-renewal or myeloid differentiation.[22,23] RARA is a nuclear receptor for RA that exhibits a highly conserved zinc finger-containing, sequence-specific, DNA-binding domain and a complex ligand-binding domain that enable heterodimerization and transcriptional activation.[24] Two other RA receptors have been characterized: RARB and RARG. But surprisingly these have never been implicated in leukemia-associated oncogenic fusions, although RARB was implicated in development of an HBV-driven hepatocellular carcinoma.[25]

RARA is bound to a member of the RXR family of nuclear receptors as an obligatory heterodimer (Figure 1). The RAR and RXR DNA-binding domains each recognize an AGGTCA core motif, in a direct repeat orientation and separated by a spacing of 2 or 5 nucleotides.[26] RARs are versatile transcriptional switches that can either repress or activate transcription. RAR/RXR complexes bind co-repressors in their unliganded state and recruit co-activators in the presence of ligands. Interestingly, RARA appears to be a stronger binder for co-repressors than other RARs.[27]

PML protein initiates the formation of nuclear bodies (NBs), sub-nuclear spherical structures involved in the fine-tuning of several biological processes, such as senescence or stem cell self-renewal, at least in part through the control of P53 signaling.[28] A specific posttranslational modification of PML, sumoylation, controls the recruitment onto NBs of a wide variety of proteins. NBs then modulate the posttranslational modification of these PML partners, resulting in their sequestration or activation[28] (Figure 1). Apart from senescence and stem cell self-renewal, these partner proteins have been implicated in a number of biological and biochemical processes, including DNA repair, apoptosis, or more recently, lipid metabolism (Figure 2).[29,30] Importantly, PML loss is associated with changes in the self-renewal of tissue stem cells, reduced apoptosis and senescence, as well as changes in metabolism.[28,29,31-33]

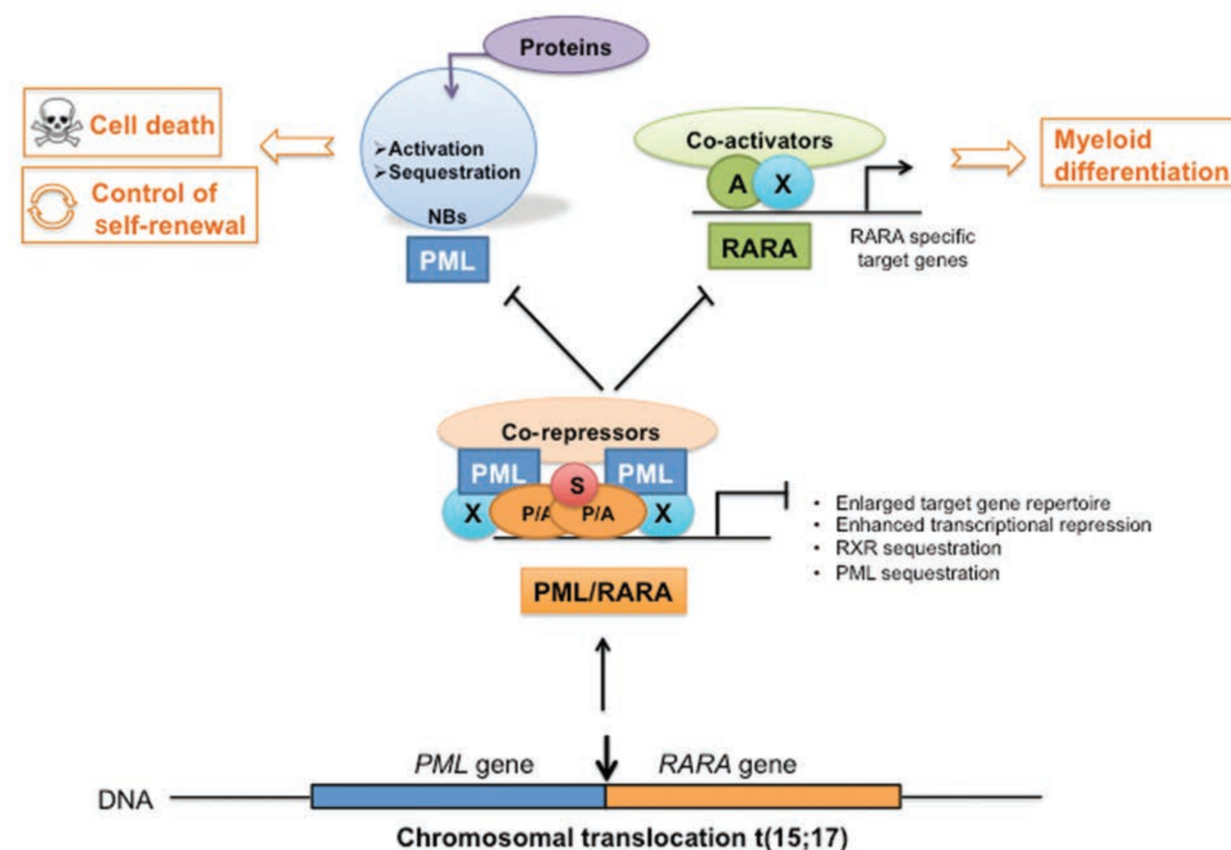

Figure 1. The PML/RARA fusion is a transcriptional repressor that also disrupts PML nuclear bodies. PML/RARA (P/A) binds RXR (X), PML and is sumoylated (S). PML/RARA represses target genes through the recruitment of co-repressors. This blocks RARA (A) targets that are implicated in myeloid differentiation. This also blocks the assembly of PML nuclear bodies, domains that recruit a large number of partner proteins to promote their posttranslational modifications, allowing their activation or sequestration. Defective nuclear bodies were associated to defects in apoptosis control or stem cell self-renewal.

PML/RARA: from a dominant negative to a gain of function oncoprotein

PML/RARA behaves as an altered transcription factor repressing its targets[6] (Figure 1). It was proposed that this results from the ability of PML to impose homo-dimerization to RARA, enhancing its binding to co-repressors and hence the repression of its targets. Interestingly, this capacity of the oncoprotein to self-dimerize is shared by all RARA fusions.[34] In the specific case of PLZF, the most studied RARA fusion partner apart from PML, an additional repression domain was identified in the N-terminus and proposed to explain RA-resistance of this specific subtype of APL.[35] Repression was primarily attributed to recruitment of histone deacetylases, a proposal that was supported by some pharmacological evidence.[36,37] Thus, a simple textbook model emerged whereby PML/RARA behaves as a super-repressor explaining the differentiation block. RA treatment could then release both the transcriptional and differentiation blocks, yielding remissions through induction of differentiation.[38]

Yet, other properties were also demonstrated for PML/RARA, including the ability to sequester PML, RXR, or to regulate transcription from novel DNA-binding sites[39,40] (Figure 1). Further studies shifting from cell lines to *in vivo* models, progressively strengthened the hypothesis that these properties were also important, if not essential, to APL pathogenesis. First, PML/RARA dimerizes with PML, leading to the replacement of the normal speckled nuclear distribution of PML by a micro-speckled one.[41,42] This alteration in nuclear architecture could participate in APL pathogenesis, notably by fostering aberrant self-renewal. Second, in APL cells, PML/RARA is constantly bound to RXRA and RXR-binding is required for *in vivo* transformation.[40,43-45] This PML/RARA//RXRA hetero-tetramer recognizes a wide range of DNA binding sites consisting of 2-3 AGGTCA sites, in any orientation and/or spacing, exemplifying a major gain of function of this oncoprotein.[45,46] Importantly, some of the recognized sequences are targets of other nuclear receptors (VDR, TR, PPAR) controlling myeloid differentiation or stem cell self-renewal. Relaxed binding site specificity through dimerization is a common feature in deregulated oncogenic transcription factors, in particular in myeloid leukemias.[47]

Clarification of the respective contribution of all these features to actual oncogenesis is ongoing. Yet, it should be noted that while in cell lines forced RARA dimerization is sufficient to confer strong repressive ability on RARA signaling and some inhibition of differentiation, attempts to induce APL *in vivo* with RARA dimers were largely unsuccessful.[48] These only succeeded when using the PML dimerization domain,[49] suggesting a key contribution to interference with PML function beyond providing a dimerization interface. Finally, some studies found that the PML moiety itself contributes to transcriptional repression by PML/RARA, through its conjugation by SUMO, a posttranscriptional modification that confers repression ability to transcription factors.[43,50,51] Collectively, while it is evident that deregulation of RARA transcriptional control is a key central feature of APL pathogenesis, the molecular details and respective contributions of the multiple mechanisms proposed remain to be clarified.

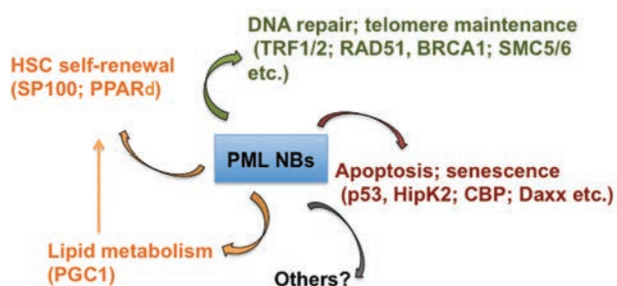

Figure 2. PML nuclear bodies control multiple pathways through modifications of partner proteins. Functions and PML partner proteins associated with them are indicated.

Two drugs for one disease

The introduction of RA for APL treatment in 1985[5] constituted the first example of differentiation therapy.[52] *Ex vivo* and *in vivo*, RA triggers rapid APL cell differentiation into granulocytes, which correlates with patient remissions. With single-agent RA therapy, remissions are usually transient,[53,54] suggesting that differentiation alone cannot abolish cancer cell self-renewal.[21,55] Yet, it should be noted that single agent liposomal RA cured some patients, implying that RA-triggered cure is possible under favorable dosage/pharmacokinetic conditions,[56] in line with mouse models[57] (*see below*).

The other potent anti-APL agent, arsenic, is considerably more efficient than RA as single agent.[58-61] Interestingly, while arsenic is primarily apoptotic *ex vivo*[62] it induces both apoptosis and terminal differentiation *in vivo,* in striking similarity to RA.[21,59] Actually, both agents trigger the so-called differentiation syndrome. As for RA, clinical trials in non-APL cancer patients have been largely disappointing, demonstrating that these compounds exhibit a great specificity for APL cells.[59,63] Such exquisite sensitivity for APL of two completely unrelated agents was puzzling, in particular because arsenic does not control RARA-mediated transcription!

Retinoic acid and arsenic are both PML/RARA-targeted therapies!

Molecular studies performed after demonstration of their clinical efficacy have revealed that both RA and arsenic directly trigger the degradation of the PML/RARA oncoprotein.[21,64-68] In a remarkable and unexpected symmetry, RA targets the RARA part of PML/RARA, while arsenic directly targets its PML part[64] (Figure 3). Thus, these two empirically discovered agents hit PML/RARA through its two constitutive moieties, making them *a posteriori* targeted therapies. This strongly suggested an important, if not essential, contribution of PML/RARA degradation to therapy response.[55,59]

With respect to RA activity, this proposal raised two key issues. What are the molecular mechanisms involved and what are the respective contributions of RA-induced transcriptional activation and degradation to clinical responses. Mechanistically, RA: 1) releases co-repressor binding from PML/RARA; 2) induces AF2-dependent transactivation through the PML/RARA-mediated recruitment of co-activators; 3) induces proteasome-enforced PML/RARA degradation (Figures 3 and 4). In contrast to transcriptional activation, which is already very significant at 10^{-8}M, full degradation requires high RA concentration, presumably because it constitutes a normal feedback mechanism on activation.[67] Accordingly, the therapeutic concentrations of RA required for APL regression are several orders of magnitudes higher than its physiological concentrations, an important observation that was long overlooked. With respect to arsenic, PML/RARA targeting is enforced both by direct binding and by arsenic-induced reactive oxygen species that elicit PML oxidation through the formation of disulfide bridges.[21,70,71] Arsenic targets both PML and PML/RARA. Since these are tightly bound to one another,[39] this dual targeting could enhance response.[70] Therefore, the mechanistic analysis of arsenic activity on APL was intimately linked to the analysis of nuclear body biogenesis. Reformation of NBs and PML degradation occur sequentially.[67,72] As extensively reviewed elsewhere, arsenic-binding and arsenic-triggered oxidation initiate formation of a PML mesh, its hyper-sumoylation, then allowing recruitment of the SUMO-dependent ubiquitin ligase RNF4, which subsequently triggers PML or PML/RARA degradation[72-75] (Figure 3). The role of PML/RARA degradation in arsenic-based therapy is supported by significant genetic evidence. Mutation of the arsenic-binding or arsenic-sensitive sumoylation site in PML/RARA impairs degradation and *ex vivo* response to treatment.[50,70,72] Mutations immediately adjacent to the arsenic-binding site of PML/RARA were observed in arsenic-resistant patients.[76] Finally, vitamin E derivatives with mitochondrial toxicity which, like arsenic, generate oxidative stress, also induce prolonged remissions in murine models of AP.[77] Importantly, arsenic does not induce PLZF/RARA degradation and is accordingly inefficient in PLZF/RARA APL models.[70,78]

Analysis of therapy resistant patients strongly supported these findings. Primary RA-resistance often reflects insufficient levels of RA in the blood, as the result of RA-induced activation of the cytochrome that catabolizes the hormone.[79,80] Then patient cells remain susceptible to RA-induced differentiation *ex vivo*. Some cases of secondary resistance were also linked to mutations in the RA-binding domain in the RARA moiety of PML/RARA.[81,82] They exhibit resistance to RA *ex vivo*. These PML/RARA mutations impede transcriptional activation and degradation, precluding clarification of their respective contributions to therapy response (Figures 2 and 4). Upregulation of cellular export or RA-trapping mechanisms, were proposed to further contribute to decreased RA intra-cellular concentrations. That only pharmacological levels of RA elicit therapy response and full PML/RARA degradation supports an important role for the latter in long-term disease response.[65] With respect to arsenic, mutations adjacent to the arsenic-binding site in the PML moiety of PML/RARA were observed in 2 therapy-resistant patients,[76] although other mechanisms, notably pharmacogenomics, have not been fully explored.[83] Deciphering the respective roles of PML/RARA degradation and transcriptional activation, in an attempt to unify the modes of action of arsenic and RA, was only possible through *in vivo* modeling in mice.

Differentiation and/or self-renewal?

At the cellular level, the concept of differentiation-based therapy in APL primarily relies on the correlation between clinical remissions and morphological maturation of leukemia blasts.[52] However, this cannot explain why only few patients are cured by RA alone, nor why arsenic cures 70% of APL patients, although it does not induce differentiation *ex vivo*. Accordingly, there have been recent controversies as to the exact contribution of cell differentiation to APL cure.[55,84] Studies have addressed this issue by exhaustively examining the effect of therapy, not only on tumor clearance and leukemia cell differentiation, but also on the loss of self-renewal,[55,57] which can only be assessed in transplantation experiments. While it was considered that the first two cellular responses were tightly coupled, recent evidence has dissociated these two end points, and only loss of self-renewal predicts disease eradication *in vivo*.[55,57,84,85] Indeed, in PML/RARA-driven APL, terminal differentiation of the leukemia is achieved even at low RA doses, but complete APL clearance only appears with treatments at the highest concentrations.[57] Similarly, complete loss of clonogenic activity *in vivo* was observed in APL mice treated with the RA/arsenic combination, although the combination actually delays morphological differentiation.[57,78,86,87] Careful examination of PLZF/RARA-driven APLs revealed that they fully differentiate upon RA treatment, while the latter has only modest effects on self-renewal, explaining their clinical RA-resistance and providing the most striking dissociation

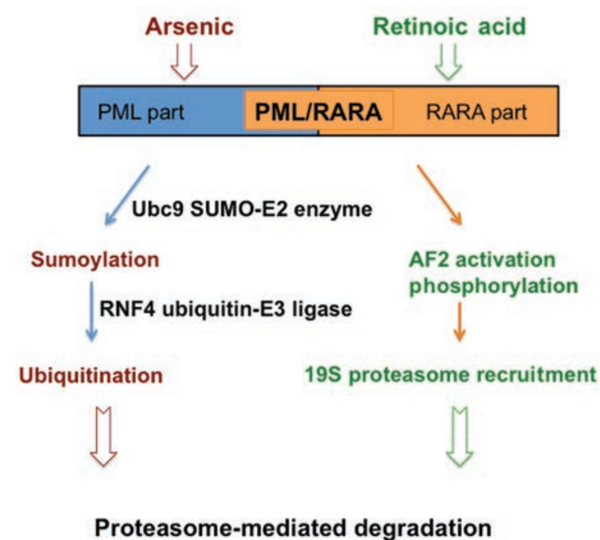

Figure 3. Schematic representation of retinoic acid- and arsenic-triggered PML/RARA catabolism. Note that retinoic acid degrades RARA and arsenic degrades PML.

between APL differentiation and eradication.[57] The fortuitous identification of retinoids that activate RARA-dependent transcription but fail to degrade RARA has provided evidence that only PML/RARA degradation entails loss of self-renewal *ex vivo* or *in vivo,* whereas transcriptional regulation correlates with induction of differentiation[85] (Figure 4). In primary resistance, insufficient RA levels allow differentiation, but not loss of clonogenic activity, resulting in continued APL development. While these observations unify the molecular bases for the antileukemic activity of RA and arsenic (and also explain the potency of their combination, see below), they raise the issue of how arsenic, which does not affect transcriptional regulation, actually induces *in vivo* differentiation. Unpublished evidence from our laboratory has demonstrated that excision of RXRA in APL cells elicits *ex vivo* or *in vivo* differentiation, in the absence of any positive inducer of retinoid signaling. This unexpected result suggests that transcriptional derepression is actually sufficient to trigger differentiation (J Halftermeyer, unpublished observations, 2012). It in turn explains the differentiating effect of arsenic, which clears PML/RARA from promoters, allowing RARA to perform its physiological action.[88] Similarly, the artificial downregulation of PML/RARA (J Ablain, unpublished observations, 2012) or the reversal of histone deacetylation may restore cell maturation processes through mere transcriptional derepression.[89]

What is the basis for loss of clonogenic activity?

PML/RARA degradation entails loss of self-renewal.[85] In principle, full PML/RARA loss should revert all of the proposed effects of the fusion on survival or self-renewal pathways. One of these deserves a particular mention: interference with PML nuclear bodies. Indeed, in normal progenitors or in the context of other leukemic fusion proteins, PML controls self-renewal,[31,32] consistent with the proposal that NBs tune several critical pathways involved in 'stemness' and self-renewal (Figures 2 and 4), such as P53, AKT/PTEN, HIF1A.[90,91]

The triumph of combined approaches

Initial studies performed *ex vivo* demonstrated that RA and arsenic failed to synergize, and even actually antagonize, for APL cell differentiation.[62,86] Yet, as argued above, differentiation is not the most relevant end point to predict clinical efficacy.[55,84,85] Studies performed *in vivo* using genetically defined mouse models or human xenograft, all demonstrated dramatic synergy between these two drugs for survival, through the immediate (3-4 days) loss of self-renewal and clonogenic activity.[57,78,87,92] In retrospect, this can now be

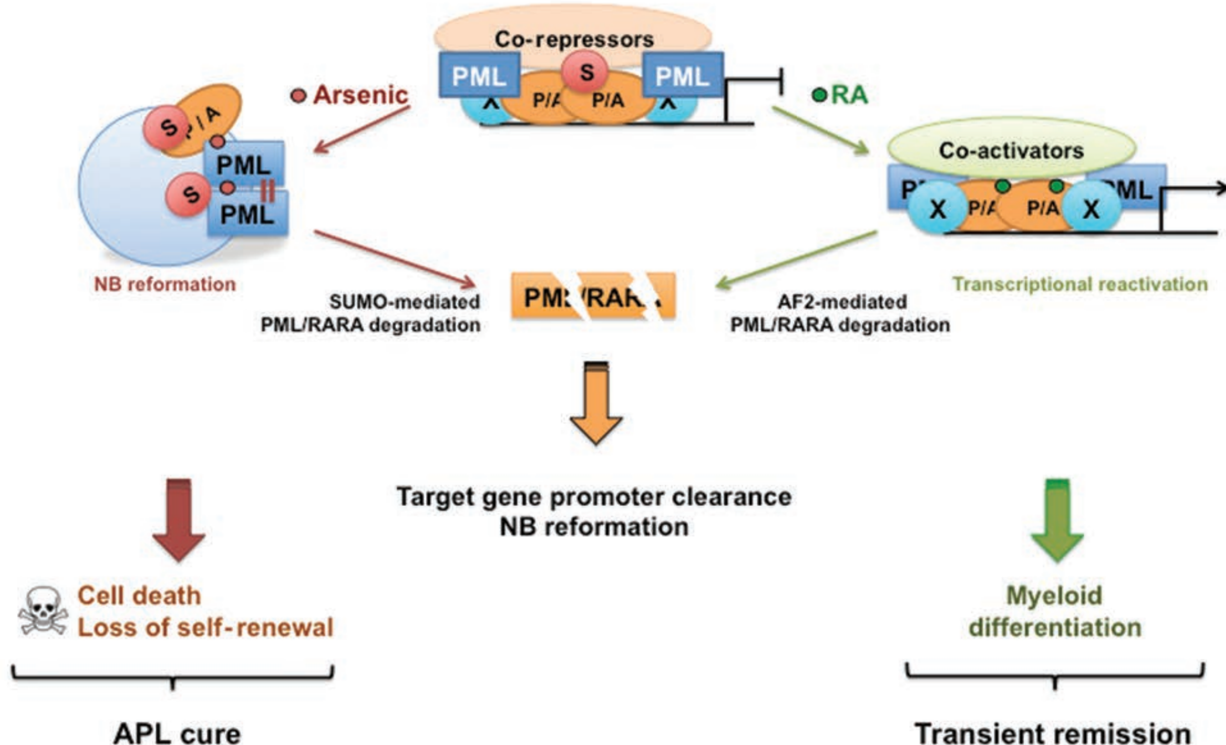

Figure 4. Uncoupling differentiation and cure. (Right) RA activates PML/RARA repressed genes, initiating myeloid differentiation. AF2-mediated degradation also indirectly yields NB reformation. (Left) Arsenic enforces NB reformation, through direct binding and oxidation. NB reformation is tightly linked to loss of self-renewal and apoptosis, correlating with APL eradication. PML/RARA degradation by arsenic also clears promoter and could thus indirectly explain differentiation through promoter clearance. Collectively, through their shared ability to degrade PML/RARA via different mechanisms (Figure 3), both drugs clear target promoters and restore PML nuclear bodies, promoting *in vivo* differentiation and varying degrees of APL clearance.

attributed to the fact that RA and arsenic induce PML/RARA degradation by different mechanisms, predicting accelerated degradation and absence of cross-resistance *in vivo*. In addition, assuming that NB-reformation plays a role in loss of 'stemness', the direct targeting of the normal PML allele by arsenic[70,71] to enforce reformation of NBs may be found to be critical in the eradication process. Front-line combined regimens were successfully transposed to patients, with over 95% of them definitively cured by the association of RA and arsenic[21,54,93-95] (F Lo-Coco, personal communication, 2012), providing a spectacular illustration of the power of mouse models to optimize treatments in patients.[18]

What are the specificities of APL that ensured the success of targeted therapies?

As a paradigm for targeted therapies, APL underscores the superiority of proteolysis over enzymatic inhibition. Indeed, complete degradation abolishes all of the functions of oncoproteins, including those linked to protein/protein interactions, which may be very important in controlling self-renewal.

In APL, the extraordinary clinical potency of RA and arsenic reflects the fact that RARA and PML are both dispensable (in mice), while APL cells are addicted to the continuous expression of PML/RARA. Thus, agents that fully degrade RARA, PML and PML/RARA, exert maximal efficacy on APL cells without any toxicity on normal cells, explaining the high therapeutic index of these agents or their association.[18,21,96] Another reason for the curative activity of these drugs is the great stability of the APL genome, as assessed by next generation sequencing studies.[14,15] Indeed, the APL genome does not seem to be globally instable, contrasting with chronic myeloid leukemias, where resistance to kinase inhibitors gradually occurs as time progresses.[97] Because RA and arsenic degrade PML/RARA by non-overlapping mechanisms, combining RA and arsenic front line reduces the risk of cross-resistance in APL patients. Collectively, the stability of the APL genome, together with rapid tumor debulking by differentiation and the immediate abrogation of all properties of PML/RARA, particularly self-renewal, all contributed to the success of the only example of cancer cure without DNA-damaging therapies.

Diagnosis and monitoring

With the efficiency of the current treatment, the biggest remaining challenge is to reverse the coagulation disorders as early as possible to avoid sudden death through hemorrhage before or in the course of induction. Apart from molecular typing (*see below*), diagnosis may also be achieved through observation of the disruption of PML NBs.[41,98] This highly efficient and straightforward procedure is now used in many centers, as treatment with RA and arsenic can then be started immediately. As in other leukemias driven by fusion genes, PCR on the gene junction has allowed rapid molecular diagnosis, but also the follow up of minimal residual disease. Pioneering studies demonstrated that molecular relapses preceded clinical ones, offering the possibility to re-treat the disease while the leukemic clone remained small. Today, for PML/RARA-driven APLs, the rates of complete remission achieved with current treatments actually question the clinical utility of monitoring the fusion during treatment. This remains an option in the variant APLs for which tools have been recently obtained that have clarified the issue of RA-induced APL clearance in these conditions.[99]

The differentiation syndrome also remains an issue, both with respect to its actual physiopathology and treatment.[100-102] In particular, it is not currently known whether the front-line association of RA and arsenic will decrease its incidence or severity. Intriguingly, how RA reverses the disorders of hemostasis remains to be understood.[103] Finally, the specific issue of hyperleukocytosis at presentation, which still indicates an unfavorable prognosis, should be further evaluated.[104]

References

1. Hillestad LK. Acute promyelocytic leukemia. Acta Med Scand. 1957;159(3):189-94.
2. Rowley JD, Golomb HM, Dougherty C. 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia. Lancet. 1977;1(8010):549-50.
3. Borrow J, Goddart A, Sheer D, Solomon E. Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. Science. 1990;249:1577-80.
4. de Thé H, Chomienne C, Lanotte M, Degos L, Dejean A. The t(15;17) translocation of acute promyelocytic leukemia fuses the retinoic acid receptor a gene to a novel transcribed locus. Nature. 1990;347:558-61.
5. Huang M, Ye Y, Chen R, Chai J, Lu J, Zhoa L, et al. Use of all trans retinoic acid in the treatment of acute promyelocytic leukaemia. Blood. 1988;72:567-72.
6. de Thé H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A. The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell. 1991;66(4):675-84.
7. Kakizuka A, Miller W Jr, Umesono K, Warrell R Jr, Frankel SR, Murty VV, et al. Chromosomal translocation t(15; 17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. Cell. 1991;66(4):663-74.
8. Goddard AD, Borrow J, Freemont PS, Solomon E. Characterization of a zinc finger gene disrupted by the t(15; 17) in acute promyelocytic leukemia. Science. 1991;254 (5036):1371-4.
9. Chen Z, Brand N, Chen A, Chen S, Tong J, Wang Z, et al. Fusion between a novel Kruppel-like zinc finger gene and the retinoic acid receptor a locus due to a variant t(11,17) translocation in acute promyelocytic leukemia. EMBO J. 1993;12:1161-7.
10. Piazza F, Gurrieri C, Pandolfi PP. The theory of APL. Oncogene. 2001;20(49):7216-22.
11. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
12. Akagi T, Shih LY, Kato M, Kawamata N, Yamamoto G, Sanada M, et al. Hidden abnormalities and novel classification of t(15;17) acute promyelocytic leukemia (APL) based on genomic alterations. Blood. 2009;113(8):1741-8.
13. Jones L, Wei G, Sevcikova S, Phan V, Jain S, Shieh A, et al. Gain of MYC underlies recurrent trisomy of the MYC chromosome in acute promyelocytic leukemia. J Exp Med. 2010;207 (12):2581-94.
14. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, et al. The origin and evolution of mutations in acute myeloid leukemia. Cell. 2012;150(2):264-78.
15. Wartman LD, Larson DE, Xiang Z, Ding L, Chen K, Lin L, et al. Sequencing a mouse acute promyelocytic leukemia genome reveals genetic events relevant for disease progression. J Clin Invest. 2011;121(4):1445-55.
16. Barragan E, Montesinos P, Camos M, Gonzalez M, Calasanz MJ, Roman-Gomez J, et al. Prognostic value of FLT3 mutations in patients with acute promyelocytic leukemia treated with all-trans retinoic acid and anthracycline monochemotherapy. Haematologica. 2011;96(10):1470-7.

17. Bernardi R, Grisendi S, Pandolfi PP. Modelling haematopoietic malignancies in the mouse and therapeutical implications. Oncogene. 2002;21(21):3445-58.
18. Lallemand-Breitenbach V, Zhu J, Kogan S, Chen Z, de The H. Opinion: how patients have benefited from mouse models of acute promyelocytic leukaemia. Nat Rev Cancer. 2005;5(10):821-7.
19. Vickers M, Jackson G, Taylor P. The incidence of acute promyelocytic leukemia appears constant over most of a human lifespan, implying only one rate limiting mutation. Leukemia. 2000;14(4):722-6.
20. Mistry AR, Felix CA, Whitmarsh RJ, Mason A, Reiter A, Cassinat B, et al. DNA topoisomerase II in therapy-related acute promyelocytic leukemia. N Engl J Med. 2005;352(15): 1529-38.
21. de The H, Chen Z. Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. Nat Rev Cancer. 2010;10(11):775-83.
22. Kastner P, Lawrence HJ, Waltzinger C, Ghyselinck NB, Chambon P, Chan S. Positive and negative regulation of granulopoiesis by endogenous RARalpha. Blood. 2001;97(5):1314-20.
23. Strickland S, Mahdavi V. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell. 1978;15(2): 393-403.
24. Kastner P, Mark M, Chambon P. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell. 1995;83(6):859-69.
25. Dejean A, Bougueleret L, Grzeschick K, Tiollais P. Hepatitis virus DNA integration in a sequence homologous to v-erbA and steroid receptors genes in a hepatocellular carcinoma. Nature. 1986;322:70-2.
26. de Thé H, Vivanco-Ruiz MdM, Tiollais P, Stunnenberg H, Dejean A. Identification of a retinoic acid responsive element in the retinoic acid receptor beta gene. Nature. 1990;343:177-80.
27. Farboud B, Hauksdottir H, Wu Y, Privalsky ML. Isotype-restricted corepressor recruitment: a constitutively closed helix 12 conformation in retinoic acid receptors beta and gamma interferes with corepressor recruitment and prevents transcriptional repression. Mol Cell Biol. 2003;23(8):2844-58.
28. Lallemand-Breitenbach V, de The H. PML nuclear bodies. Cold Spring Harb Perspect Biol. 2010;2:a000661.
29. Carracedo A, Weiss D, Leliaert AK, Bhasin M, de Boer VC, Laurent G, et al. A metabolic prosurvival role for PML in breast cancer. J Clin Invest. 2012;122(9):3088-100.
30. Bernardi R, Pandolfi PP. Role of PML and the PML-nuclear body in the control of programmed cell death. Oncogene. 2003;22(56):9048-57.
31. Ito K, Bernardi R, Morotti A, Matsuoka S, Saglio G, Ikeda Y, et al. PML targeting eradicates quiescent leukaemia-initiating cells. Nature. 2008;453(7198):1072-8.
32. Regad T, Bellodi C, Nicotera P, Salomoni P. The tumor suppressor Pml regulates cell fate in the developing neocortex. Nat Neurosci. 2009;12(2):132-40.
33. Ito K, Carracedo A, Weiss D, Arai F, Ala U, Avigan DE, et al. A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. Nat Med. 2012 Aug 19. [Epub ahead of print.]
34. Licht JD. Reconstructing a disease: What essential features of the retinoic acid receptor fusion oncoproteins generate acute promyelocytic leukemia? Cancer Cell. 2006;9(2):73-4.
35. Lin RJ, Nagy L, Inoue S, Shao WL, Miller WH, Evans RM. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature. 1998;391:811-4.
36. He LZ, Tolentino T, Grayson P, Zhong S, Warrell RP Jr, Rifkind RA, et al. Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. J Clin Invest. 2001;108(9):1321-30.
37. Warrell RP, He L-Z, Richon V, Calleja E, Pandolfi PP. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J Natl Cancer Inst. 1998;90:1621-5.
38. Melnick A, Licht JD. Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. Blood. 1999;93:3167-215.
39. Kastner P, Perez A, Lutz Y, Rochette-Egly C, Gaub M-P, Durand B, et al. Structure, localization and transcriptional properties of two classes of retinoic acid receptor alpha fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncoproteins. EMBO J. 1992;11(2):629-42.
40. Perez A, Kastner P, Sethi S, Lutz Y, Reibel C, Chambon P. PML/RAR homodimers: distinct binding properties and heteromeric interactions with RXR. EMBO J. 1993;12(8):3171-82.
41. Daniel M-T, Koken M, Romagné O, Barbey S, Bazarbachi A, Stadler M, et al. PML protein expression in hematopoietic and acute promyelocytic leukemia cells. Blood. 1993;82:1858-67.
42. Koken MHM, Puvion-Dutilleul F, Guillemin MC, Viron A, Linares-Cruz G, Stuurman N, et al. The t(15;17) translocation alters a nuclear body in a RA-reversible fashion. EMBO J. 1994;13:1073-83.
43. Zhu J, Nasr R, Peres L, Riaucoux-Lormiere F, Honore N, Berthier C, et al. RXR is an essential component of the oncogenic PML/RARA complex in vivo. Cancer Cell. 2007;12(1): 23-35.
44. Zeisig BB, Kwok C, Zelent A, Shankaranarayanan P, Gronemeyer H, Dong S, et al. Recruitment of RXR by homotetrameric RARalpha fusion proteins is essential for transformation. Cancer Cell. 2007;12(1):36-51.
45. Martens JH, Brinkman AB, Simmer F, Francoijs KJ, Nebbioso A, Ferrara F, et al. PML-RARalpha/RXR Alters the Epigenetic Landscape in Acute Promyelocytic Leukemia. Cancer Cell. 2010;17(2):173-85.
46. Kamashev DE, Vitoux D, De Thé H. PML/RARA-RXR oligomers mediate retinoid- and rexinoid- /cAMP in APL cell differentiation. J Exp Med. 2004;199:1-13.
47. So CW, Cleary ML. Dimerization: a versatile switch for oncogenesis. Blood. 2004;104(4):919-22.
48. Sternsdorf T, Phan VT, Maunakea ML, Ocampo C, Sohal J, Siletto A, et al. Forced retinoic acid receptor a homodimer prime mice for APL-like leukemia. Cancer Cell. 2006;9:81-94.
49. Occhionorelli M, Santoro F, Pallavicini I, Gruszka A, Moretti S, Bossi D, et al. The self-association coiled-coil domain of PML is sufficient for the oncogenic conversion of the retinoic acid receptor (RAR) alpha. Leukemia. 2011;25(5):814-20.
50. Zhu J, Zhou J, Peres L, Riaucoux F, Honore N, Kogan S, et al. A sumoylation site in PML/RARA is essential for leukemic transformation. Cancer Cell. 2005;7(2):143-53.
51. Verger A, Perdomo J, Crossley M. Modification with SUMO. A role in transcriptional regulation. EMBO Rep. 2003;4(2):137-42.
52. Degos L, Dombret H, Chomienne C, Daniel MT, Miclea JM, Chastang C, et al. All-trans retinoic acid as a differentiating agent in the treatment of acute promyelocytic leukemia. Blood. 1995;85(10):2643-53.
53. Warrell R, de Thé H, Wang Z, Degos L. Acute promyelocytic leukemia. New Engl J Med. 1993;329:177-89.
54. Tallman MS, Altman JK. How I treat acute promyelocytic leukemia. Blood. 2009;114(25):5126-35.
55. Kogan SC. Curing APL: differentiation or destruction? Cancer Cell. 2009;15(1):7-8.
56. Tsimberidou AM, Tirado-Gomez M, Andreeff M, O'Brien S, Kantarjian H, Keating M, et al. Single-agent liposomal all-trans retinoic acid can cure some patients with untreated acute promyelocytic leukemia: an update of The University of Texas M. D. Anderson Cancer Center Series. Leuk Lymphoma. 2006;47(6):1062-8.
57. Nasr R, Guillemin MC, Ferhi O, Soilihi H, Peres L, Berthier C, et al. Eradication of acute promyelocytic leukemia-initiating cells through PML-RARA degradation. Nat Med. 2008;14(12): 1333-42.
58. Chen SJ, Zhou GB, Zhang XW, Mao JH, de The H, Chen Z. From an old remedy to a magic bullet: molecular mechanisms underlying the therapeutic effects of arsenic in fighting leukemia. Blood. 2011;117(24):6425-37.
59. Zhu J, Chen Z, Lallemand-Breitenbach V, de Thé H. How acute promyelocytic leukemia revived arsenic. Nat. Rev. Cancer 2002;2:705-13.
60. Mathews V, George B, Chendamarai E, Lakshmi KM, Desire S, Balasubramanian P, et al. Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: long-term follow-up data. J Clin Oncol. 2010;28(24):3866-71.
61. Ghavamzadeh A, Alimoghaddam K, Rostami S, Ghaffari SH, Jahani M, Iravani M, et al. Phase II Study of Single-Agent Arsenic Trioxide for the Front-Line Therapy of Acute Promyelocytic Leukemia. J Clin Oncol. 2011;29(20):2753-7.
62. Chen G-Q, Zhu J, Shi X-G, Ni J-H, Zhong H-J, Si G-Y, et al. In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia. As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. Blood. 1996;88:1052-61.
63. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood. 2008;111(5):2505-15.
64. Quignon F, Chen Z, de Thé H. Retinoic acid and arsenic: towards oncogene targeted treatments of acute promyelocytic

leukaemia. Biochim Biophys Acta. 1997;1333:M53-M61.
65. Zhu J, Lallemand-Breitenbach V, de The H. Pathways of retinoic acid- or arsenic trioxide-induced PML/RARalpha catabolism, role of oncogene degradation in disease remission. Oncogene. 2001;20(49):7257-65.
66. Zhu J, Gianni M, Kopf E, Honore N, Chelbi-Alix M, Koken M, et al. Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor alpha (RAR alpha) and oncogenic RAR alpha fusion proteins. Proc Natl Acad Sci USA. 1999;96:14807-12.
67. Zhu J, Koken MHM, Quignon F, Chelbi-Alix MK, Degos L, Wang ZY, et al. Arsenic-induced PML targeting onto nuclear bodies: implications for the treatment of acute promyelocytic leukemia. Proc Natl Acad Sci USA. 1997;94:3978-83.
68. Chen GQ, Shi XG, Tang W, Xiong SM, Zhu J, Cai X, et al. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): I. As2O3 exerts dose-dependent dual effects on APL cells. Blood. 1997;89(9):3345-53.
69. Ablain J, Nasr R, Bazarbachi A, de The H. Oncoprotein proteolysis, an unexpected Achille's Heel of cancer cells? Cancer Discovery. 2011;1:117-27.
70. Jeanne M, Lallemand-Breitenbach V, Ferhi O, Koken M, Le Bras M, Duffort S, et al. PML/RARA oxidation and arsenic binding initiate the antileukemia response of As2O3. Cancer Cell. 2010;18(1):88-98.
71. Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY, Sun HB, et al. Arsenic trioxide controls the fate of the PML-RARalpha oncoprotein by directly binding PML. Science. 2010;328 (5975):240-3.
72. Lallemand-Breitenbach V, Zhu J, Puvion F, Koken M, Honore N, Doubeikovsky A, et al. Role of Promyelocytic Leukemia (PML) Sumolation in Nuclear Body Formation, 11S Proteasome Recruitment, and As(2)O(3)-induced PML or PML/Retinoic Acid Receptor alpha Degradation. J Exp Med. 2001;193(12):1361-72.
73. Lallemand-Breitenbach V, Jeanne M, Benhenda S, Nasr R, Lei M, Peres L, et al. Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. Nat Cell Biol. 2008;10(5):547-55.
74. Tatham MH, Geoffroy MC, Shen L, Plechanovova A, Hattersley N, Jaffray EG, et al. RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. Nat Cell Biol. 2008;10(5):538-46.
75. Lallemand-Breitenbach V, Zhu J, Chen Z, de The H. Mechanisms of APL cure through PML/RARA degradation by As2O3. Trends in Molecular Medicine. 2012;18:36-42.
76. Goto E, Tomita A, Hayakawa F, Atsumi A, Kiyoi H, Naoe T. Missense mutations in PML-RARA critical for the lack of responsiveness to arsenic trioxide treatment. Blood. 2011 May 25. [Epub ahead of print.]
77. Dos Santos GA, Abreu ELRS, Pestana CR, Lima AS, Scheucher PS, Thome CH, et al. (+)alpha-Tocopheryl succinate inhibits the mitochondrial respiratory chain complex I and is as effective as arsenic trioxide or ATRA against acute promyelocytic leukemia in vivo. Leukemia. 2011 Aug 26. [Epub ahead of print.]
78. Rego EM, He LZ, Warrell RP Jr, Wang ZG, Pandolfi PP. Retinoic acid (RA) and As2O3 treatment in transgenic models of acute promyelocytic leukemia (APL) unravel the distinct nature of the leukemogenic process induced by the PML-RARalpha and PLZF-RARalpha oncoproteins. Proc Natl Acad Sci USA. 2000;97:10173-8.
79. Muindi J, Frankel SR, Miller WH Jr, Jakubowski A, Scheinberg DA, Young CW, et al. Continuous treatment with all-trans retinoic acid causes a progressive reduction in plasma drug concentrations: implications for relapse and retinoid "resistance" in patients with acute promyelocytic leukemia. Blood. 1992;79(2):299-303.
80. Delva L, Cornic M, Balitrand N, Guidez F, Miclea JM, Delmer A, et al. Resistance to all-trans retinoic acid (ATRA) therapy in relapsing acute promyelocytic leukemia: study of in vitro ATRA sensitivity and cellular retinoic acid binding protein levels in leukemic cells. Blood. 1993;82(7):2175-81.
81. Gallagher RE, Schachter-Tokarz EL, Zhou DC, Ding W, Kim SH, Sankoorikal BJ, et al. Relapse of acute promyelocytic leukemia with PML-RARalpha mutant subclones independent of proximate all-trans retinoic acid selection pressure. Leukemia. 2006;20(4):556-62.
82. Gallagher RE. Retinoic acid resistance in acute promyelocytic leukemia. Leukemia. 2002;16(10):1940-58.
83. Kroemer G, de The H. Arsenic trioxide, a novel mitochondriotoxic anti-cancer agent? J Natl Cancer Inst. 1999;91:743-5.
84. Ablain J, de The H. Revisiting the differentiation paradigm in acute promyelocytic leukemia. Blood. 2011;117(22):5795-802.
85. Ablain J, Leiva M, Peres L, Fonsart J, Anthony E, de The H. Uncoupling RARA transcriptional activation and degradation clarifies the bases for APL response to therapies. J Exp Med. 2013. In press.
86. Shao W, Fanelli M, Ferrara FF, Riccioni R, Rosenauer A, Davison K, et al. Arsenic trioxide as an inducer of apoptosis and loss of PML/RARalpha protein in acute promyelocytic leukemia cells. J Natl Cancer Inst. 1998;90:124-33.
87. Lallemand-Breitenbach V, Guillemin M-C, Janin A, Daniel M-T, Degos L, Kogan SC, et al. Retinoic acid and arsenic synergize to eradicate leukemic cells in a mouse model of acute promyelocytic leukemia. J Exp Med. 1999;189:1043-52.
88. Cassinat B, Zassadowski F, Ferry C, Llopis L, Bruck N, Lainey E, et al. New role for granulocyte colony-stimulating factor-induced extracellular signal-regulated kinase 1/2 in histone modification and retinoic acid receptor alpha recruitment to gene promoters: relevance to acute promyelocytic leukemia cell differentiation. Mol Cell Biol. 2011;31 (7):1409-18.
89. Leiva M, Moretti S, Soilihi H, Pallavicini I, Peres L, Mercurio C, et al. Valproic acid induces differentiation and transient tumor regression, but spares leukemia-initiating activity in mouse models of APL. Leukemia. 2012 Feb 15. [Epub ahead of print.]
90. Ito K, Bernardi R, Pandolfi PP. A novel signaling network as a critical rheostat for the biology and maintenance of the normal stem cell and the cancer-initiating cell. Curr Opin Genet Dev. 2009;19(1):51-9.
91. Song MS, Salmena L, Carracedo A, Egia A, Lo-Coco F, Teruya-Feldstein J, et al. The deubiquitinylation and localization of PTEN are regulated by a HAUSP-PML network. Nature. 2008;455(7214):813-7.
92. Jing Y, Wang L, Xia L, Chen G, Chen Z, Miller WH, et al. Combined effect of all-trans retinoic acid and arsenic trioxide in acute promyelocytic leukemia cells in vitro and in vivo. Blood. 2001;97(1):264-9.
93. Shen ZX, Shi ZZ, Fang J, Gu BW, Li JM, Zhu YM, et al. All-trans retinoic acid/As2O3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci USA. 2004;101:5328-35.
94. Hu J, Liu YF, Wu CF, Xu F, Shen ZX, Zhu YM, et al. Long-term efficacy and safety of all-trans retinoic acid/arsenic trioxide-based therapy in newly diagnosed acute promyelocytic leukemia. 2009;106(9):3342-7.
95. Estey E, Garcia-Manero G, Ferrajoli A, Faderl S, Verstovsek S, Jones D, et al. Use of all-trans retinoic acid plus arsenic trioxide as an alternative to chemotherapy in untreated acute promyelocytic leukemia. Blood. 2006;107(9):3469-73.
96. Nardella C, Lunardi A, Patnaik A, Cantley LC. The APL paradigm and the co-clinical trial project. Cancer Discovery. 2011;1:108-16.
97. Michor F, Hughes TP, Iwasa Y, Branford S, Shah NP, Sawyers CL, et al. Dynamics of chronic myeloid leukaemia. Nature. 2005;435(7046):1267-70.
98. Dyck JA, Warrell RP, Evans RM, Miller WH. Rapid diagnosis of acute promyelocytic leukemia by immunohistochemical localization of PML/RAR alpha protein. Blood. 1995;86:862-7.
99. Jovanovic JV, Rennie K, Culligan D, Peniket A, Lennard A, Harrison J, et al. Development of real-time quantitative polymerase chain reaction assays to track treatment response in retinoid resistant acute promyelocytic leukemia. Front Oncol. 2011;1:35.
100. Camacho LH, Soignet SL, Chanel S, Ho R, Heller G, Scheinberg DA, et al. Leukocytosis and the retinoic acid syndrome in patients with acute promyelocytic leukemia treated with arsenic trioxide. J Clin Oncol. 2000;18(13):2620-5.
101. Vhadat L, Maslak P, Miller W, Eardley A, Heller G, Scheinberg D, et al. Early mortality and the retinoic acid syndrome in APL: impact of leukocytosis, low dose chemotherapy, PML/RARa isoform and CD13 expression in patients teated with ATRA. Blood. 1994;84:3843-9.
102. Zuckerman T, Ganzel C, Tallman MS, Rowe JM. How I treat hematologic emergencies in adults with acute leukemia. Blood. 2012;120(10):1993-2002.
103. Stein E, McMahon B, Kwaan H, Altman JK, Frankfurt O, Tallman MS. The coagulopathy of acute promyelocytic leukaemia revisited. Best Pract Res Clin Haematol. 2009;22(1):153-63.
104. Sanz MA, Grimwade D, Tallman MS, Lowenberg B, Fenaux P, Estey EH, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2009;113(9):1875-91.

Management of special situations in acute promyelocytic leukemia

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A B S T R A C T

The introduction of all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO) into the therapy of acute promyelocytic leukemia (APL) has turned this hematologic malignancy into one of the first to receive a targeted treatment. Using treatment strategies that include these agents in combination with cytotoxic agents have provided excellent therapeutic results in most patients. Nevertheless, there are some special situations that require the implementation of changes from the conventional therapeutic approach. We will review the management of APL in children, elderly patients, and pregnant women among other singular situations.

Learning goals

At the conclusion of this activity, participants should be able to:
- know which are the special situations that require an alternative therapeutic approach;
- discuss the treatment approach in each of these special situations and, more specifically, why and how it varies from conventional therapy;
- discuss the evidence-based management for elderly patients, children, pregnant women, therapy-related APL, genetic variants, and extramedullary relapse;
- identify the specific issues that need to be addressed in future studies.

Introduction

The introduction of all-*trans* retinoic acid (ATRA) and, more recently, arsenic trioxide (ATO) into the therapy of acute promyelocytic leukemia (APL) has revolutionized the management and outcome of this disease. Several treatment strategies using these agents, usually in combination with chemotherapy, have provided excellent therapeutic results. These strategies are generally designed for patients whose clinical situation does not generate special difficulty for the administration of conventional therapy. However, when a patient with APL presents a special situation, and by this we mean clinical circumstances that prevent partially or completely the administration of ATRA, ATO or chemotherapy, the management of these patients is a bigger challenge and has to be analyzed separately.

The present article aims to review some of these clinical situations that differ from the standard patient with APL. We will discuss the management of APL in children, elderly patients and pregnant women.

Management of special situations

Older patients

APL has a median age of around forty years and is relatively uncommon in older patients, in contrast to most cases of acute myeloid leukemia (AML). The outcome in these patients has been proven to be more favorable if we compare it with other subtypes of AML. Older patients with APL seem at least as responsive to therapy as do younger patients, maybe due to the fact that they are more likely to present with low-risk features when compared to younger patients.[1] This fact may, to some extent, explain the relatively low relapse rate observed in patients over 70 years of age receiving ATRA and moderately reduced anthracycline-based chemotherapy.[1,2] A potential selection bias due to a higher proportion of non-eligible patients among those of an older age has been ruled out in a large series of patients registered in the PETHEMA database (M. Sanz *et al.,* unpublished data, 2013).

On the other hand, it should be taken into account that older patients are more vulnerable to therapy-related toxicity, with higher rates of neutropenic sepsis and increased treatment-related mortality. Zhang *et al.*[3] found a significantly higher mortality in comparison with younger patients during consolidation therapy, mainly due to infections following chemotherapy-induced myelosuppression. The reported mortality rate in complete remission (CR) ranges from less than 1% in patients under 60 years of age to 19% in patients over 70 years.[1] Therefore, it seems reasonable to design less intensive therapeutic strategies aiming to reduce morbidity and mortality in this last group. It should be taken into account that this

population has a high rate of non-eligibility for treatment;[4] this means that even though conventional treatment is highly effective in this age group, only a small number of patients will satisfy the rigorous selection criteria.

For those frail patients who are considered unfit for chemotherapy, less toxic treatment approaches are needed to allow a broader applicability. Today, ATO with or without ATRA appears to be one of the best alternatives to the standard ATRA plus chemotherapy approach, although current supporting scientific data are still limited.[5] The results reported by Zhang *et al*.[3] indicate that a single-agent ATO regimen is safe and effective with long-term durable remission. Based on these results, ATO may be considered to be an option for first-line treatment in elderly patients with APL. Nevertheless, more data are needed on this subject to turn it into the standard of care for patients unfit for conventional therapy.

Patients with severe comorbidities

Similar to the outlined approach for older and frail patients, in younger patients who are not candidates for first-line intensive chemotherapy due to certain comorbidities (severe cardiac impairment or other organ dysfunction), there are several alternative treatment approaches to minimize the use of cytotoxic agents. These would be based on the use of ATRA, ATO, and gemtuzumab ozogamicin, with minimal or no chemotherapy.[5]

The outcome in this particular setting is not sufficiently documented. It should be noted that any therapeutic strategy used in these patients should aim to achieve molecular remission, and guide the need for additional therapy with monitoring of minimal residual disease (MRD).

Children

Compared with adults, children with APL have a higher incidence of hyperleukocytosis (roughly 40% *vs*. 20-25%) and M3v morphology.[1] It has been suggested that the difference in WBC count is mainly observed in children under the age of 12 years.[6]

Apart from two relatively small pediatric series from the German-Austrian-Swiss group[7] and the European APL group,[8] as well as two larger series from the GIMEMA[9] and PETHEMA[10] groups, a recent analysis from the European APL group[6] reports the outcome in different age groups of children and adolescents. In this analysis, children under 4 years of age presented the highest relapse rate (52% *vs*. 18% in children aged 5-12 years old); this is a new finding given the lack of studies of children in different age groups. This observation was not attributed to a higher WBC count or other high-risk features.

Given the long life span in children cured from this disease, there is a wide concern about the potential cardiac toxicity that high-dose anthracyclines can produce in the long term. Therefore, there have been some attempts to simply reduce the exposure to these agents without any additional treatment modification. This therapeutic strategy reported worse results in a clinical trial carried out by the US Intergroup.[11] Just as with older patients, other therapeutic strategies are being sought after to reduce the dose of cytotoxic agents; ATO is one of the new possibilities. ATO appears to be effective in pediatric APL,[12,13] just like in adults, but as yet very limited data are available.

To reduce the frequency of some side effects associated with induction therapy that appear more frequently in children, particularly severe headache and pseudotumor cerebri (PTC), most trials use a reduced dose of ATRA (*e.g.* 25 mg/m^2 instead of 45 mg/m^2) in the pediatric age group.[7-10] The study by Castaigne *et al*.[14] showed no difference in terms of pharmacokinetics, therapeutic efficacy, triggering of hyperleukocytosis, or retinoic acid syndrome with ATRA at 25 mg/m^2/d as compared to the standard dose of 45 mg/m^2/d. The apparently lower incidence of PTC and headache, together with the excellent therapeutic results obtained with this reduced dose has led to a recommended 25 mg/m^2 per day as the standard dose in children and adolescents. Headache is a common complication during ATRA therapy and it is, therefore, important to rule out PTC, CNS leukemia or bleeding. The diagnosis of PTC is based on increased intracranial pressure with normal cerebrospinal fluid (CSF) composition and negative cerebral imaging studies. It is usually accompanied by papilledema, but this is not a requirement for the diagnosis of PTC.[15] In this situation, sustained elevations in CSF pressure should be documented through successive lumbar punctures or by intracranial pressure monitoring, if necessary.[16] Sometimes, the symptoms of PTC resolve with the initial 'diagnostic' lumbar puncture. If this occurs, no further medical treatment is required. If symptoms persist, temporary discontinuation or dose reduction of ATRA, analgesics, and administration of steroids and acetazolamide are the mainstays of the medical treatment of this neurological complication. Acetazolamide is administered in an initial dose of 25 mg/kg/day and progressively increased until clinical response is achieved (maximum dose 100 mg/kg/day). Electrolytes must be monitored to allow early detection of hypokalemia and acidosis (common side effects during acetazolamide treatment). If this diuretic treatment is ineffective, then prednisone can be given at a dose of 2 mg/kg/day for two weeks followed by a 2-week taper.[17]

Pregnant women

The diagnosis of APL during pregnancy is not frequent and most reports are based on individual cases or very small series. This is a challenging situation in which decision-making must be carried out with a multidisciplinary perspective, involving the patient, hematologist, obstetrician and neonatologist. With this approach, there is a higher chance of a successful outcome for both mother and baby, as was highlighted in the guidelines on the management of AML in the United Kingdom.[18]

As with any other patient with APL, the start of treatment should not be delayed or the chance of a successful remission could be compromised; it must be considered a medical emergency. The key problem in this special situation is the teratogenic potential of chemotherapy, ATRA and arsenic trioxide on the fetus; the most important factors at the moment of taking a decision are the gestational age and the attitude of the patient towards the risk to both mother and fetus.

Management during the first trimester deserves a different approach to that adopted for the second and third trimesters of pregnancy. Both periods will, therefore, be addressed separately.

Management of APL during the first trimester of pregnancy

The possibility of a conventional therapeutic approach is not possible during the first trimester of pregnancy, due

to the highly teratogenic side effects of ATRA.[19] During this period, the only option is anthracycline-based chemotherapy. The use of daunorubicin is usually preferred over idarubicin. This is probably due to a wider experience with the former drug, and because idarubicin is more lipophilic and can favor an increased placental transfer.[20]

It has to be taken into account that even with only chemotherapy there is an increased risk of fetal malformations, abortion, and low birth weight.[21] This is why the first decision that should take place when APL is diagnosed in the first trimester is whether or not to continue with the pregnancy. If the pregnancy is not interrupted, the mother would receive anthracycline chemotherapy alone. In the case of terminating the pregnancy, she would be able to receive conventional treatment with ATRA and cytotoxic agents. Using chemotherapy alone involves an increased risk of hemorrhage due to release of procoagulants and plasminogen activators from malignant promyelocytes. If remission is achieved with chemotherapy and the pregnancy is progressing normally, treatment with ATRA could be administered during the second and third trimesters.

Although ATO is an alternative treatment in other groups of patients, it is not an option during pregnancy. This agent has a high potential embryotoxicity and cannot be recommended at any stage of pregnancy. Human data are very limited and restricted to people exposed to arsenic from drinking water, or working in or living near metal smelters. Low birthweight, spontaneous abortion, and stillbirth were reported in this population.[22]

Taking all this into account, female patients with APL treated conventionally should be routinely advised against conceiving while exposed to ATRA or ATO for consolidation and maintenance therapy.

Management of APL during the second and third trimesters of pregnancy

During the second and third trimester of pregnancy, conventional treatment with ATRA and chemotherapy is a reasonable option, although scientific literature on this subject is limited. The maternal outcome seems to be the same as in non-pregnant women when conventional therapy is used. If we analyze the therapeutic components separately, ATRA does not seem to cause embryotoxicity past the first trimester.[21] This agent can be safely administered, although it is advisable to monitor cardiac function, given that there have been some reports of reversible fetal arrhythmias and other cardiac complications at birth. On the other hand, although chemotherapy does not seem to cause congenital malformation, in some cases it increases the risk of abortion, premature delivery, low birthweight, neonatal neutropenia, and sepsis. This has recently been reviewed by Culligan *et al.*[21] and leads to two different possible approaches.

1) Sequential use of ATRA and chemotherapy. This implies the administration of ATRA alone until CR, delaying the administration of chemotherapy until elective delivery is possible. A gestational age of at least 32 weeks is considered relatively safe when appropriate neonatal care is provided.[23] For deliveries before 36 weeks of gestation, antenatal corticosteroids are recommended to reduce the risk of respiratory distress syndrome.[24]

Regarding maternal outcome, the expected response rate with ATRA alone is not significantly different to ATRA plus chemotherapy in terms of CR rate, but it can have an unfavorable impact on the risk of relapse.[25] This theoretical disadvantage could be counteracted later with a reinforcement of post-remission therapy. If this strategy is followed, the administration of chemotherapy should not be delayed excessively to avoid resistance and disease recurrence. It has been suggested that molecular assessment of response and subsequent RQ-PCR monitoring can be used to indicate the need for chemotherapy.[21] It should also be noted that when using ATRA alone there is an increased (approximately 25%) risk of APL differentiation syndrome.[26]

2) Simultaneous administration of ATRA and chemotherapy. This approach provides the best chances of cure for the mother and is a clear option for high-risk patients with hyperleukocytosis and for those in whom appropriate RQ-PCR monitoring is not possible. In this case, daunorubicin is also preferred to idarubicin; this is not so clear for patients with advanced gestational age pregnancy.

Vaginal delivery is preferred due to its association with a reduced risk of bleeding. Caesarean section should only be performed if it is required for other reasons.[21] After delivery, breast-feeding is contraindicated if chemotherapy or ATO is needed. Other aspects of management do not differ from non-pregnant women with APL.

Therapy-related APL

There is little information available on the true incidence of therapy-related APL (tAPL) since these patients are less likely to be entered into clinical trials. In addition, available estimates are subject to important methodological limitations, being based on retrospective series[27,28] or the experience of single referral centers.[29,30] In these studies, tAPL cases ranged from less than 5% to 22% of all APL cases. A growing incidence of tAPL has been reported over the last few years paralleling the increased use of topoisomerase II-targeted drugs in both malignant and non-malignant diseases. Breast carcinoma is the most frequent previous cancer, followed by lymphoma, with a large predominance of non-Hodgkin's lymphoma, whereas other tumor types were found with lower incidence.[28] The drugs most commonly implicated in tAPL are epirubicin and mitoxantrone, but a number of cases have been reported to follow exposure to radiotherapy alone.[31-34] It has also been reported that some cases of secondary APL (sAPL) have arisen in patients whose primary tumor was treated by surgery, without chemotherapy or radiotherapy exposure.[27,28] Typically, the latency period between chemotherapy exposure and onset of tAPL is relatively short (<3 years) and occurs without a preceding myelodysplastic phase. Hematologic findings do not seem to differ from those observed in *de novo* APL, as previously reported for other tAML with specific karyotype.[35,36]

Regarding tAPL arising in patients treated for non-malignant diseases, it should be noted that cases of t-APL were increasingly reported during the period of time in which mitoxantrone was approved for treating aggressive forms of multiple sclerosis.[37] The risk of developing this complication was estimated to be approximately 1 in 400 patients with multiple sclerosis treated with

mitoxantrone.[38]

Although current data would suggest that patients with tAPL have a relatively favorable prognosis, a higher incidence of early death during treatment has been reported.[28] However, a more precise knowledge of the outcome of patients with tAPL treated with state-of-the-art therapy should be prospectively established; at present, there is no clear reason to manage these patients in a different manner to those with *de novo* APL. However, in a significant number of patients with tAPL, the use of anthracycline-based regimens is limited by previous exposure to topoisomerase II inhibitors. In such situations, ATO in combination with ATRA provides an option for consolidation following standard induction therapy or as first-line treatment using schedules such as those published by the MD Anderson group.[5,39]

Genetic variants of APL

There are no specific guidelines for rare genetic variants of APL because the available evidence is mostly based on single case reports. Nevertheless, it is a general rule that patients with ATRA-sensitive variants (*NuMA-RARA, NPM1-RARA* and *FIP1L1-RARA*) should be treated with standard protocols involving ATRA combined with anthracycline-based chemotherapy, while those with ATRA-resistant variants (*STAT5b-RARA*) should be managed with AML-like approaches. For those relatively ATRA resistant (*PLZF-RARA*), or with unknown sensitivity to ATRA (*PRKAR1A-RARA*), it is reasonable to combine ATRA with AML-like chemotherapy. Sensitivity to ATO has not been documented outside *PML-RARA* positive APL, except for *PLZF-RARA* positive APL that has been shown to be resistant.[40]

Central nervous system and other extramedullary relapses

Central nervous system (CNS) and other extramedullary relapses are uncommon in APL. CNS involvement can occur as an isolated event or associated with bone marrow involvement as a first relapse, but also after one or more hematologic relapses. The majority of CNS relapses occur in patients with hyperleukocytosis at presentation, and the optimal management of such patients is still controversial.

Even though the contemporary literature on the subject is scarce, it seems pragmatic to pursue an approach derived from experience of the management of extramedullary relapse in acute lymphoblastic leukemia and other subtypes of AML. In this regard, induction treatment of CNS relapse would consist of weekly triple intrathecal therapy (ITT) with methotrexate, hydrocortisone, and cytarabine until complete clearance of blasts in the CSF, followed by 6-10 more spaced out ITT treatments as consolidation. Since CNS disease is almost invariably accompanied by hematologic or molecular relapse in the marrow, systemic treatment should also be given. The timing of this may be dictated by clinical circumstances. One approach could be to give ATO and ATRA as a non-myeloablative treatment approach whilst ITT is being delivered. Chemotherapy regimens with high CNS penetrance (*e.g.* high-dose cytarabine) have been used in this situation, and in patients responding to treatment, allogeneic or autologous transplant should be the consolidation approach of choice with appropriate craniospinal irradiation. In case of granulocytic sarcoma, wherever it is localized, radiation and intensive systemic therapy may be considered.

References

1. Sanz MA, Vellenga E, Rayon C, et al. All-trans retinoic acid and anthracycline monochemotherapy for the treatment of elderly patients with acute promyelocytic leukemia. Blood. 2004;104(12):3490-3.
2. Mandelli F, Latagliata R, Avvisati G, et al. Treatment of elderly patients (> or =60 years) with newly diagnosed acute promyelocytic leukemia. Results of the Italian multicenter group GIMEMA with ATRA and idarubicin (AIDA) protocols. Leukemia. 2003;17(6):1085-90.
3. Zhang Y, Zhang Z, Li J, et al. Long-term efficacy and safety of arsenic trioxide for first-line treatment of elderly patients with newly diagnosed acute promyelocytic leukemia. Cancer. 2013;119(1):115-25.
4. Lengfelder E, Hanfstein B, Haferlach C, et al. Outcome of elderly patients with acute promyelocytic leukemia: results of the German Acute Myeloid Leukemia Cooperative Group. Ann Hematol. 2013;92(1):41-52.
5. Estey E, Garcia-Manero G, Ferrajoli A, et al. Use of all-trans retinoic acid plus arsenic trioxide as an alternative to chemotherapy in untreated acute promyelocytic leukemia. Blood. 2006;107(9):3469-73.
6. Bally C, Fadlallah J, Leverger G, et al. Outcome of acute promyelocytic leukemia (APL) in children and adolescents: an analysis in two consecutive trials of the European APL Group. J Clin Oncol. 2012;30(14): 1641-6.
7. Mann G, Reinhardt D, Ritter J, et al. Treatment with all-trans retinoic acid in acute promyelocytic leukemia reduces early deaths in children. Ann Hematol. 2001;80(7):417-22.
8. de Botton S, Coiteux V, Chevret S, et al. Outcome of childhood acute promyelocytic leukemia with all-trans-retinoic acid and chemotherapy. J Clin Oncol. 2004;22(8):1404-12.
9. Testi AM, Biondi A, Lo Coco F, et al. GIMEMA-AIEOPAIDA protocol for the treatment of newly diagnosed acute promyelocytic leukemia (APL) in children. Blood. 2005;106(2):447-53.
10. Ortega JJ, Madero L, Martin G, et al. Treatment with all-trans retinoic acid and anthracycline monochemotherapy for children with acute promyelocytic leukemia: a multicenter study by the PETHEMA Group. J Clin Oncol. 2005;23(30):7632-40.
11. Powell BL MB, Stock B, et al. Arsenic trioxide improves survival in first line APL consolidation treatment: the NCI/CALGB study results. ASCO meeting, Chicago, USA, 2007.
12. Fox E, Razzouk BI, Widemann BC, et al. Phase 1 trial and pharmacokinetic study of arsenic trioxide in children and adolescents with refractory or relapsed acute leukemia, including acute promyelocytic leukemia or lymphoma. Blood. 2008;111(2):566-73.
13. George B, Mathews V, Poonkuzhali B, Shaji RV, Srivastava A, Chandy M. Treatment of children with newly diagnosed acute promyelocytic leukemia with arsenic trioxide: a single center experience. Leukemia. 2004;18(10):1587-90.
14. Castaigne S, Lefebvre P, Chomienne C, et al. Effectiveness and pharmacokinetics of low-dose all-trans retinoic acid (25 mg/m2) in acute promyelocytic leukemia. Blood. 1993;82(12):3560-3.
15. Wang SJ, Silberstein SD, Patterson S, Young WB. Idiopathic intracranial hypertension without papilledema: a case-control study in a headache center. Neurology. 1998;51(1):245-9.
16. Spence JD, Amacher AL, Willis NR. Benign intracranial hypertension without papilledema: role of 24-hour cerebrospinal fluid pressure monitoring in diagnosis and management. Neurosurgery. 1980;7(4):326-36.
17. Robertson WC Jr, Wilson M-CB, Baker MJ. Pseudotumor Cerebri: Pediatric Perspective. In: Sheth RD, Talavera F, Mack KJ, Benbadis SR, Lorenzo NY, (eds.) eMedicine. Available from: http://www.emedicine.com/neuro/topic537.htm. Accessed February 14 2008.
18. Milligan DW, Grimwade D, Cullis JO, et al. Guidelines on the management of acute myeloid leukaemia in adults. Br J

Haematol. 2006;135(4):450-74.
19. Lammer EJ, Chen DT, Hoar RM, et al. Retinoic acid embryopathy. N Engl J Med. 1985;313(14):837-41.
20. Cardonick E, Iacobucci A. Use of chemotherapy during human pregnancy. Lancet Oncol. 2004;5(5):283-91.
21. Culligan DJ ML, Kell J, et al. The management of acute promyelocytic leucemia presenting during pregnancy. Clinical Leukemia. 2007;1:183-91.
22. US Environmental Protection Agency. Arsenic compounds. Available from: http://www.epa.gov. Accessed Jan 8 2008.
23. Slattery MM, Morrison JJ. Preterm delivery. Lancet. 2002;360 (9344):1489-97.
24. Royal College of Obstetricians and Gynecologists. Antenatal corticosteroids to reduce respiratory distress syndrome. Guideline No. 7. Available from: http://www.rcog.org.uk/resources/Public/pdf/Antenatal_corticosteroids_No7.pdf. Accessed April 1 2007.
25. Fenaux P, Chastang C, Chevret S, et al. A randomized comparison of all transretinoic acid (ATRA) followed by chemotherapy and ATRA plus chemotherapy and the role of maintenance therapy in newly diagnosed acute promyelocytic leukemia. The European APL Group. Blood. 1999;94(4):1192-200.
26. Tallman MS, Andersen JW, Schiffer CA, et al. All-trans-retinoic acid in acute promyelocytic leukemia. N Engl J Med. 1997;337(15):1021-8.
27. Pulsoni A, Pagano L, Lo Coco F, et al. Clinicobiological features and outcome of acute promyelocytic leukemia occurring as a second tumor: the GIMEMA experience. Blood. 2002;100(6):1972-6.
28. Beaumont M, Sanz M, Carli PM, et al. Therapy-related acute promyelocytic leukemia. J Clin Oncol. 2003;21(11):2123-37.
29. Mattson JC. Acute promyelocytic leukemia. From morphology to molecular lesions. Clin Lab Med. 2000;20(1):83-103.
30. Pollicardo N, O'Brien S, Estey EH, et al. Secondary acute promyelocytic leukemia. Characteristics and prognosis of 14 patients from a single institution. Leukemia. 1996;10(1):27-31.
31. Mistry AR, Felix CA, Whitmarsh RJ, et al. DNA topoisomerase II in therapy-related acute promyelocytic leukemia. N Engl J Med. 2005;352(15):1529-38.
32. Andersen MK, Pedersen-Bjergaard J. Therapy-related MDS and AML in acute promyelocytic leukemia. Blood. 2002;100(5):1928-9.
33. Pedersen-Bjergaard J. Insights into leukemogenesis from therapy-related leukemia. N Engl J Med. 2005;352(15):1591-4.
34. Smith SM, Le Beau MM, Huo D, et al. Clinical-cytogenetic associations in 306 patients with therapy-related myelodysplasia and myeloid leukemia: the University of Chicago series. Blood. 2003;102(1):43-52.
35. Pedersen-Bjergaard J, Philip P, Larsen SO, Jensen G, Byrsting K. Chromosome aberrations and prognostic factors in therapy-related myelodysplasia and acute nonlymphocytic leukemia. Blood. 1990;76(6):1083-91.
36. Quesnel B, Kantarjian H, Bjergaard JP, et al. Therapy-related acute myeloid leukemia with t(8;21), inv(16), and t(8;16): a report on 25 cases and review of the literature. J Clin Oncol. 1993;11(12):2370-9.
37. Ledda A, Caocci G, Spinicci G, Cocco E, Mamusa E, La Nasa G. Two new cases of acute promyelocytic leukemia following mitoxantrone treatment in patients with multiple sclerosis. Leukemia. 2006;20(12):2217-8.
38. Ghalie RG, Mauch E, Edan G, et al. A study of therapy-related acute leukaemia after mitoxantrone therapy for multiple sclerosis. Mult Scler. 2002;8(5):441-5.
39. Lo-Coco F, Avvisati G, Orlando SM, et al. ATRA and Arsenic Trioxide (ATO) Versus ATRA and Idarubicin (AIDA) for Newly Diagnosed, Non High-Risk Acute Promyelocytic Leukemia (APL): Results of the Phase III, Prospective, Randomized, Intergroup APL0406 Study by the Italian-German Cooperative Groups Gimema-SAL-AMLSG. ASH meeting, Atlanta, USA,2012.
40. Koken MH, Daniel MT, Gianni M, et al. Retinoic acid, but not arsenic trioxide, degrades the PLZF/RARalpha fusion protein, without inducing terminal differentiation or apoptosis, in a RA-therapy resistant t(11;17)(q23;q21) APL patient. Oncogene. 1999;18(4):1113-8.

The evolving role of stem cell transplantation in acute promyelocytic leukemia

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A B S T R A C T

The availability of several highly effective agents in acute promyelocytic leukemia (APL) including all-trans retinoic acid (ATRA), arsenic trioxide (ATO) and anthracyclines, has transformed this once highly fatal disease into the most frequently curable acute leukemia. While it is firmly established that neither autologous nor allogeneic stem cell transplantation (SCT) are indicated in first remission of the disease, and that patients relapsing after ATRA-containing regimens should be treated with ATO, controversy remains on the selection of the most appropriate consolidation therapy, and in particular on indications for transplantation after second remission. Owing to the lack of randomized comparative studies and the very limited number of relapses, consolidation strategies should be based on several clinical and biological criteria and rely on both available reported experience and published recommendations. These criteria include age and performance status, first remission duration, donor availability, and minimal residual disease status. In this article, we review current recommendations and controversial issues related to use of SCT in APL.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe the available treatment options for patients with acute promyelocytic leukemia in first relapse;
- define the clinical and biological criteria for selecting autologous or allogeneic stem cell transplantation in APL in second remission or beyond;
- describe the available therapeutic consolidation options for patients in second remission or beyond who are ineligible for transplantation.

APL, from highly fatal to highly curable

Over the past two decades, modern treatment with simultaneous all-trans-retinoic acid (ATRA) and anthracycline-based chemotherapy (CHT) has transformed acute promyelocytic leukemia (APL) from a rapidly fatal into a highly curable disease. In fact, more than 80% of patients receiving this combination have been reported to become long-term survivors in large multicenter studies.[1] In addition, excellent outcome results have been reported in APL using arsenic trioxide (ATO) combined or not with ATRA and CHT. Initially shown to be very active in patients relapsing after ATRA-containing regimens, ATO has been tested in several pilot studies in the front-line management of the disease with promising results.[2-5] Moreover, very recent results of a prospective randomized study indicated that combined ATO and ATRA is at least as effective as ATRA and CHT for patients with non-high risk disease (commonly defined as those with WBC at diagnosis $<10x10^9$/L, and accounting for approximately 75% of cases).[6] While the latter trial results will likely change the standard of care in front-line therapy (*i.e.* favoring the use of ATO+ATRA instead of ATRA+CHT), they also help reinforce the concept that APL is a highly curable disease in which targeted drugs and/or limited use of conventional CHT are likely to eradicate the disease.

No role for stem cell transplantation in APL patients in first remission

Based on the availability of the aforementioned highly effective agents in the front-line management, there is a general expert consensus on recommending the use of stem cell transplantation (SCT) in APL only after second or subsequent remission.[1] In this respect, it is worth emphasizing that no particular single (or even combined) features associated to slightly inferior prognosis in patients treated with standard ATRA and CHT should justify the use of SCT in first remission. In fact, outcomes in patients showing these reportedly unfavorable features, including elevated WBC counts at diagnosis.[7] CD56 expression,[8] or FLT3-ITD mutation,[9] still remain considerably good. In addition, the chances of achieving second remission with ATO in relapsed APL are extremely high (approximately 85-90%) and repeated ATO given for re-induction and consolidation is able to induce molecular

remission in almost 80% of patients treated at relapse.[10,11] Based on these considerations, it should be firmly restated that neither allogeneic SCT (ASCT) nor autologous SCT (AuSCT) have any role in APL in first remission. In the following paragraphs, we will review the current recommendations for transplantation as a consolidation strategy for APL patients in second remission or beyond.

Consolidation options for APL patients in second complete remission

Although most patients relapsing after front-line therapy reported to date had received the standard ATRA and CHT, it is likely that this scenario will change in the near future. In fact, an increasing number of relapses are expected to be reported in patients treated with CHT-free approaches such as ATO +/- ATRA. This is due to a growing interest in the use of the latter approach. In principle, relapsing patients who have never been exposed to CHT should receive the standard ATRA plus CHT for re-induction and consolidation, in parallel with investigating their transplantation options. However, data on the outcome of patients relapsing after ATO who are rescued with ATRA-CHT followed by SCT are not currently available. For patients who relapse after the standard front-line treatment of ATRA plus chemotherapy, re-induction with ATO is recommended followed by one consolidation cycle of the same agent combined with ATRA.[1,11,12] There is no current consensus on the best option to further consolidate remission after ATO. The very low number of relapsing patients treated with the current standard treatment has made randomized studies comparing different strategies including ASCT, AuSCT, prolonged ATO with or without ATRA or chemotherapy unfeasible. In addition, patients in most reported studies were not systematically monitored for molecular status pre- and post-SCT. Consequently, it is difficult to establish recommendations based on the impact of SCT and other consolidation options in patients included in these studies.

Selection of the successive consolidation strategy after ATO and ATRA will depend on a number of variables including patient's age and performance status, duration of first remission, donor availability and minimal residual disease (MRD) status after salvage therapy.[1,11,12] It is widely recognized that AuSCT is considered for patients achieving second molecular remission, *i.e.* those who test PCR-negative for the disease-specific *PML/RARA* fusion gene in their marrow after consolidation, with such tests being performed in highly specialized reference laboratories. Autologous SCT is notoriously associated with lower morbidity and mortality as compared to ASCT and can represent a convenient and effective option for patients with late relapse who achieve second molecular remission. As to the definition of early *versus* late relapse, and consequently of short *versus* prolonged first remission duration, here again there is no definitive consensus. Because most standard ATRA plus chemotherapy regimens include prolonged maintenance for two years, we propose that early relapse is considered as that occurring within two years of achieving remission, although this definition may be somewhat arbitrary.

Allogeneic transplant is still an effective therapy and a valid treatment option, especially in fit patients at higher risk of subsequent relapse who have a suitable donor. These include patients with short first remission duration (<2 years) and patients who do not achieve a second molecular remission after 2 cycles of ATO+/-ATRA. Prolonged ATO is a viable alternative for patients unfit for a transplantation procedure, or as a bridge during donor identification.[1,13] It remains uncertain as to whether prolonged ATO +/- ATRA can produce long-term remission in APL patients with late relapse, although a single experience of a limited series suggested that a high proportion of patients receiving this treatment strategy may achieve another long-term remission.[13] An algorithm with recommended consolidation options after second CR and criteria for selecting them is illustrated in Figure 1.

The following review is organized in sections based on reports comparing results of consolidation with and without stem cell transplantation, consolidation with autologous *versus* allogeneic transplantation, and reports of consolidation with ATO alone or in combination regimens in patients not eligible for transplant.

Reports comparing results of consolidation with and without stem cell transplantation

An early report from China on 47 relapsed APL patients treated with ATO suggested that disease-free survival (DFS) was significantly better with more intensified consolidation therapy of combined ATO and chemotherapy as compared to ATO or chemotherapy alone after second CR.[14] There is also considerable evidence that consolidation with SCT may improve the outcome of patients in second remission after ATO treatment and is better than consolidation with ATO or ATRA alone or with chemotherapy.[15-17] Both the updated results of a pivotal US multicenter trial and the European APL group study showed higher overall survival (OS) and DFS rates in patients who received SCT as consolidation compared to those who did not receive transplantation.[15,16] In the latter study, 7-year event-free survival (EFS) and OS were 30.4%, and 39.5% in patients who received no SCT compared to 61% and 60%, and 52.2% and 51.8%, in patients treated with autologous and allogeneic transplants, respectively.[16] Similar results were observed in a more recent series of 37 relapsed patients. In this series, 5-year OS was 100% for patients who underwent autologous transplantation compared to 39% in patients treated with ATO plus ATRA.[17]

Reports of consolidation with autologous and allogeneic stem cell transplantation

Although no prospective randomized trials have been reported, a number of studies compared the role of autologous or allogeneic SCT in APL patients in second remission. Some of these studies documented a similar or even better outcome with autologous than with ASCT. A recurrent observation reported in these retrospective analyses is that transplant-related mortality (TRM) of ASCT hampered a possible OS benefit related to the graft-*versus*-leukemia effect in reducing subsequent relapses.

Among these studies, the European Bone and Marrow Transplant (EBMT) group evaluated the role of either

AuSCT or ASCT in a large number of patients treated in the ATRA era. In this registry study, patients in CR2 achieved a better leukemia-free survival after ASCT compared to those receiving AuSCT (59% *vs.* 51%). However this benefit was at the expense of increased TRM (24% *vs.* 16%).[18] This study lacked information about MRD prior to and following transplant. We reported a single center study on the outcome after ASCT in 17 patients treated after second or subsequent CR for whom pre-transplant MRD assessment was available. We documented a significant anti-leukemic effect of ASCT even in patients with advanced disease including those with pre-transplant evidence of MRD.[19] All patients with MRD positive disease prior to transplant achieved molecular remission after transplant, although the response was less prolonged in more advanced cases. The 10-year actuarial probabilities of OS and DFS were 53% and 46%, respectively; however, TRM was high (32%).[19]

Studies that evaluated prognostic factors for subsequent relapse after SCT showed that the duration of first remission and the achievement of second molecular remission prior to transplant are associated with post-transplant outcome.[16,19-22] The relevance of pre-SCT MRD status is well established in the autologous transplant setting. Meloni *et al.* prospectively monitored MRD status of 15 patients who received autologous transplantation in second remission. Six of 8 patients who received *PML/RARA*-negative marrow achieved prolonged clinical and molecular remissions (median 28 months; range 15-60 months). In contrast, all the 7 patients transplanted with positive MRD relapsed at a median time of five months (range 2-9 months) from transplant.[21]

Similarly, the European APL group retrospectively evaluated the outcome of patients who underwent autologous or allogeneic SCT after second complete remission.[16] EFS and OS were significantly better in the autologous setting. Moreover, when the analysis was limited to patients in molecular remission, the 7-year EFS and OS improved to 77% and 75%, respectively, compared to 52.2% and 51.8% in the allogeneic group. Transplant-related mortality was 7% compared to 39% in the autologous and allogeneic settings, respectively.[16] The results from using AuSCT were comparable to those reported by a CALGB study on AML that included 12 APL patients in second complete remission. The 5-year DFS and OS were both 67%.[22] Two other small studies support the use of autolo-

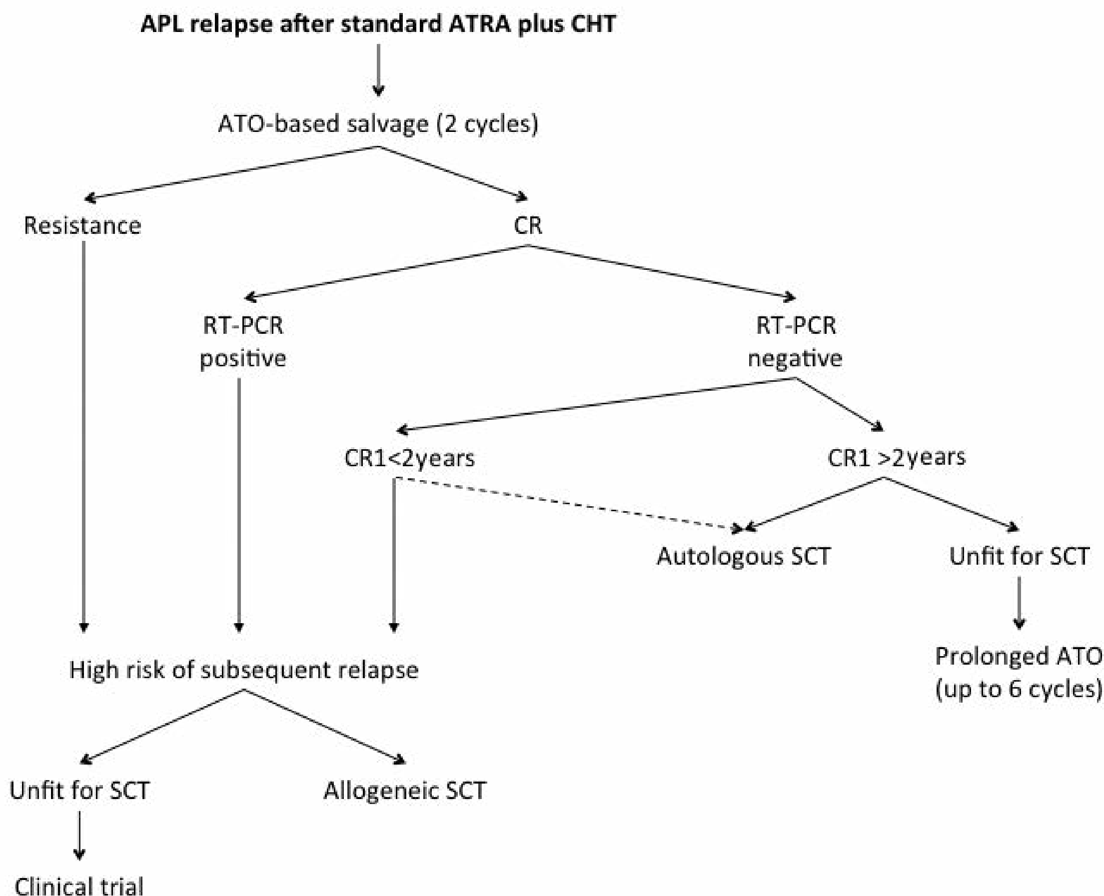

Figure 1. Therapeutic options for APL patients in second CR. Molecular status for PML/RARA after consolidation and CR1 duration are important factors for the choice of successive options. These may include autologous or allogeneic SCT and prolonged ATO+/-ATRA cycles for patients unfit for an SCT procedure. For patients with molecular CR and CR1 duration under two years, the choice between autologous or allogeneic SCT may also vary based on the type of available donor and clinical parameters (*e.g.* age, PS) with impact on TRM.

gous transplant in patients who achieve a second molecular complete remission.[20,23] The earlier one reported that long-term remission after either allograft or autograft is associated with eradication of *PML-RARA* positive cells, and that continued positivity predicts subsequent relapse.[20] The more recent one showed that 11 of 13 patients who received autologous transplants while in second molecular remission were alive.[23] Ten patients in this latter study were in sustained molecular remission after a median follow up of 25 months with no TRM.[23] Together these studies suggest that, for APL patients who had a long first remission duration and are in second molecular remission, autologous transplantation is an effective approach for a second lengthy remission.[16-18,20,21,23]

We recently evaluated the role of allogeneic transplant in patients with advanced disease (CR2 or beyond) treated in the era of ATO. This study included 31 patients (15 CR2, 16≥ CR3) transplanted in 4 Italian institutions. At time of transplant, 16 patients were MRD positive and 15 were negative. The 4-year overall survival was higher for patients transplanted in CR2 and for MRD negative patients (62% and 64%, respectively) compared to patients transplanted in CR3 or over and positive for MRD (31% and 27%, respectively). MRD status prior to transplant was associated with significantly better DFS and the rate of relapse was higher in patients transplanted with RT-PCR-positive disease.[24] The 4-year cumulative incidence of TRM was 19.6% in this series including advanced disease cases and 7 haploidentical transplants.[23] This improvement may reflect recent advances in transplant supportive measures, wider use of peripheral blood stem cells as well as better haploidentical transplant modalities.[25] In conjunction with other reported series, this study confirms that allogeneic transplant continues to be an effective therapeutic option in relapsed APL patients who are eligible for this treatment policy.

Consolidation reports of ATO alone or in combination regimens in patients not eligible for transplant

Given the exquisite efficacy of ATO in APL and the possibility of accurately monitoring response to therapy and re-emerging MRD through PCR analysis, prolonged therapy with ATO-based regimens with or without ATRA may be considered in patients unfit for transplant. Durable molecular remissions were reported in 8 of 9 patients (median CR duration, 25 months) treated with prolonged post-remission therapy consisting of four courses of ATO and seven shorter courses of ATRA. All patients in this recent report had late relapses prior to SCT rescue (at a median time of 1.9 years; range 1-7 years), all except one were treated for molecular relapse, and all were closely monitored for MRD.[13] However, this experience was limited to only a few patients, and the identification of patients with low risk of subsequent relapse after first disease recurrence remains challenging.

Conclusion

Recommendations and indications for remission re-induction and consolidation in APL patients with APL relapse are evolving because of the changing scenario in front-line therapy. ATRA-ATO or ATRA-CHT are the standard approach for patients relapsing after chemotherapy-based or ATO-based treatment, respectively. First consolidation after re-induction with further ATO or CHT is recommended with the aim of achieving second molecular remission. All patients must be tested after consolidation for MRD status in experienced laboratories of reference. The choice for further consolidation will be taken in consideration of first remission duration, the quality of remission (molecular *vs.* hematologic only), patient age and performance status, and donor availability. Autologous SCT can be recommended for patients with prolonged (>2 years) first remission who test negative for MRD after 2 cycles of ATO-based therapy, while patients ineligible for SCT can continue ATO for consolidation and maintenance with close monitoring of MRD. Patients who fail to achieve first complete molecular remission, those who had short first remission, or those who test positive for MRD after ATO induction and consolidation, should be considered for allogeneic SCT if a suitable donor is available. Patients who are candidates for allogeneic SCT should be sent to transplant without delay once they achieve molecular remission.

References

1. Sanz MA, Grimwade D, Tallman MS, Lowenberg B, Fenaux P, Estey EH, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2009;113:1875-91.
2. Ghavamzadeh A, Alimoghaddam K, Ghaffari SH, et al. Treatment of acute promyelocytic leukemia with arsenic trioxide without ATRA and/or chemotherapy. Ann Oncol. 2006; 17:131-4.
3. Hu J, Liu YF, Wu CF, et al. Long-term efficacy and safety of all-trans retinoic acid/arsenic trioxide-based therapy in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci USA. 2009;106:3342-7.
4. Mathews V, George B, Lakshmi KM, et al. Single agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: durable remissions with minimal toxicity. Blood. 2006;107:2627-32.
5. Ravandi F, Estey E, Jones D, et al. Effective treatment of acute promyelocytic leukemia with all-trans-retinoic acid, arsenic trioxide, and gemtuzumab ozogamicin. J Clin Oncol. 2009;27:504-10.
6. Lo-Coco F, Avvisati G, Orlando SM, et al. ATRA and Arsenic Trioxide (ATO) Versus ATRA and Idarubicin (AIDA) for Newly Diagnosed, Non High-Risk Acute Promyelocytic Leukemia (APL): Results of the Phase III, Prospective, Randomized, Intergroup APL0406 Study by the Italian-German Cooperative Groups Gimema-SAL-AMLSG Blood 2012;120:6(Abstract).
7. Sanz MA, Lo Coco F, Martín G. Definition of relapse risk and role of nonanthracycline drugs for consolidation in patients with acute promyelocytic leukemia: a joint study of the PETHEMA and GIMEMA cooperative groups. Blood. 2000;96:1247-53.
8. Montesinos P, Rayón C, Vellenga E. Clinical significance of CD56 expression in patients with acute promyelocytic leukemia treated with all-trans retinoic acid and anthracycline-based regimens Blood. 2011;117:1799-805.
9. Gale RE, Hills R, Pizzey AR. Relationship between FLT3 mutation status, biologic characteristics, and response to targeted therapy in acute promyelocytic leukemia. Blood. 2005;106:3768-76.
10. Douer D, Tallman MS. Arsenic trioxide: new clinical experience with an old medication in hematologic malignancies. J Clin Oncol. 2005;23:2396-410.
11. Tallman MS. Treatment of relapsed or refractory acute promyelocytic leukemia. Best Pract Res Clin Haematol. 2007;20:57-65.

12. Sanz MA, Lo-Coco F. Modern approaches to treating acute promyelocytic leukemia. J Clin Oncol. 2011;29:495-503.
13. Breccia M, Cicconi L, Minotti C, Latagliata R, Gianni L, Lo-Coco F. Efficacy of prolonged therapy with combined arsenic trioxide and ATRA for relapse of acute promyelocytic leukemia. Haematologica. 2011;96(9):1390-1.
14. Niu C, Yan H, Yu T, Sun HP, Liu JX, Li XS, et al. Studies on treatment of acute promyelocytic leukemia with arsenic trioxide: remission induction, follow-up, and molecular monitoring in 11 newly diagnosed and 47 relapsed acute promyelocytic leukemia patients. Blood. 1999;94:3315-24.
15. Douer D, Hu W, Giralt S, Lill M, DiPersio J. Arsenic trioxide (trisenox) therapy for acute promyelocytic leukemia in the setting of hematopoietic stem cell transplantation. Oncologist. 2003;8:132-40.
16. de Botton S, Fawaz A, Chevret S, et al. Autologous and allogeneic stem-cell transplantation as salvage treatment of acute promyelocytic leukemia initially treated with all-trans-retinoic acid: a retrospective analysis of the European acute promyelocytic leukemia group. J Clin Oncol. 2005;23:120-6.
17. Thirugnanam R, George B, Chendamarai E, Lakshmi KM, Balasubramanian P, Viswabandya A, et al. Comparison of clinical outcomes of patients with relapsed acute promyelocytic leukemia induced with arsenic trioxide and consolidated with either an autologous stem cell transplant or an arsenic trioxide-based regimen. Biol Blood Marrow Transplant. 2009;11:1479-84.
18. Sanz MA, Labopin M, Gorin NC, de la Rubia J, Arcese W, Meloni G, et al. Hematopoietic stem cell transplantation for adults with acute promyelocytic leukemia in the ATRA era: a survey of the European Cooperative Group for Blood and Marrow Transplantation. Bone Marrow Transplant. 2007;39: 461-9.
19. Lo-Coco F, Romano A, Mengarelli A, et al. Allogeneic stem cell transplantation for advanced acute promyelocytic leukemia: results in patients treated in second molecular remission or with molecularly persistent disease. Leukemia. 2003;17:1930.
20. Roman J, Martin C, Torres A. Absence of detectable PML-RARa fusion transcripts in long-term remission patients after BMT for acute promyelocytic leukemia. Bone Marrow Transplant. 1997;19:679-83.
21. Meloni G, Diverio D, Vignetti M, et al. Autologous bone marrow transplantation for acute promyelocytic leukemia in second remission: prognostic relevance of pretransplant minimal residual disease assessment by reverse-transcriptase polymerase chain reaction of the PML/RARa fusion gene. Blood. 1997;90:1321-5.
22. Linker CA, Owzar K, Powell B, et al. Auto-SCT for AML in second remission: CALGB study 9620. Bone Marrow Transplant. 2009;44:353.
23. Ferrara F, Finizio O, Izzo T, Riccardi C, Criscuolo C, Carbone A, et al. Autologous stem cell transplantation for patients with acute promyelocytic leukemia in second molecular remission. Anticancer Res. 2010; 30:3845-9.
24. Ramadan SM, Di Veroli A, Camboni A, Breccia M, Iori AP, Aversa F, et al. Allogeneic stem cell transplantation for advanced acute promyelocytic leukemia in the ATRA and ATO era. Haematologica. 2012;97:1731-5.
25. Aversa F. Setting the standard in T-cell-depleted haploidentical transplantation and beyond. Best Pract Res Clin Haematol. 2011;24:325-9.

Genetics of inherited disorders of platelets

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A B S T R A C T

Genetic defects of platelets constitute a group of rare diseases that give rise to bleeding syndromes of autosomal dominant or recessive inheritance. They affect platelet production, giving rise to a low circulating platelet count and changes in platelet morphology, platelet function, or a combination of both an altered megakaryopoiesis and a defective platelet response. As a result, blood platelets fail to fulfill their hemostatic function. The most studied are deficiencies of glycoprotein mediators of adhesion and aggregation while defects of primary receptors for stimuli include that of the P2Y12 ADP receptor. Inherited defects of secretion from storage organelles (dense granules, α-granules) and of the generation of procoagulant activity have led to the identification of many genes involved in megakaryocyte biology. Signaling pathway defects leading to agonist-specific modifications of platelet aggregation are the current target of exome-sequencing strategies. In familial thrombocytopenia, changes in megakaryocyte maturation within the bone marrow mostly lead to a deficient proplatelet formation and an altered timing of platelet release; sometimes defects extend to other cells and in some cases interfere with development. We now review recent advances in the field and highlight genes responsible for inherited diseases of platelets.

Learning goals

At the conclusion of this activity, participants should be able to assess:
- how a wide variety of molecular defects of surface and intracellular constituents of platelets lead to defective platelet function and bleeding;
- how pathologies within each of a series of major gene families put inherited disorders of platelets to the forefront of research into rare diseases;
- how genetic defects of transcription factors and of cytoskeletal proteins can affect megakaryocyte biology leading to an altered platelet morphology and number;
- how the use of modern gene screening procedures including whole exome or genome sequencing will transform practice in a routine hematology laboratory.

The Human Genome Variation Society (HGVS) numbering and nomenclature as used in this review (available from://www.Hgvs.org/mutnomen) is recommended to describe mutations. Since amino acid numbering within each protein is now recommended to start with the initiating methionine, while it was variably used before, current numbering is often different from the one used in the original publications. In cases in which the HGVS numbering is different from the original publications, the original nomenclature is provided in parentheses.

Introduction

This review of inherited disorders of platelet function and platelet production will emphasize recent advances and the identification of genes whose defects are at the origin of a bleeding syndrome.[1-4] Spontaneous bleeding is mostly mucocutaneous in nature; excessive trauma-related bleeding is another feature of milder forms. Treatment has been reviewed elsewhere.[5] Figure 1 illustrates disorders affecting platelet surface constituents, while Figure 2 shows those affecting intracellular components. Tables 1 and 2 summarize gene defects giving familial thrombocytopenia (FT) with or without defects of platelet function and grouped according to platelet morphology. Gene variants identified by candidate gene association and genome-wide association studies should also be born in mind for they can cumulatively lead to hypo-reactive platelets and affect such parameters as platelet size or count. They include novel gene variants such as *PEAR1* (platelet endothelial aggregation receptor 1) that modulate platelet reactivity and bleeding tendency.[45-48]

Defects of platelet function

Defects of platelet adhesion

Abnormalities of GPIb-IX function. Bernard-Soulier syndrome (BSS) associates a moderate to severe macrothrombocytopenia with a decreased von Willebrand factor (VWF)-dependent platelet adhesion under

flow caused by quantitative or qualitative defects of GPIb-IX-V (Table 1). GPIbα contains VWF and thrombin-binding sites within the N-terminal domain. The additional absence of extracellular binding sites on GPIbα for P-selectin, TSP1, coagulation factors (F) XI and XII, αMβ2 and high molecular weight kininogen may extend the phenotype.[6] The products of four genes (*GPIBA*, *GPIBB*, *GP9* and *GP5*) assemble in a 2:4:2:1 ratio within maturing megakaryocytes (MK) in the bone marrow to form GPIb-IX-V as present in the platelet membrane.[6] Mutations within *GPIBA*, *GPIBB* and *GP9* in BSS prevent the composition and/or trafficking of the complex through the endoplasmic reticulum (ER) and Golgi apparatus by changing the quaternary organization of GPIb-IX.[7] The absence of the interaction between GPIbα and filamin A in the membrane cytoskeleton may account for the giant platelets. In rare variant BSS, platelets express non-functional GPIbα.[1,2,4] A common heterozygous p.Ala172Val (formerly Ala156Val) mutation is the cause of inherited thrombocytopenia alone in Southern Italy.[8] Heterozygous mutations in *GPIBB* cause BSS when associated with the DiGeorge/velocardiofacial syndrome, a developmental disorder given by a heterozygous microdeletion at 22q11, the site of localization of *GPIBB*. Correction of murine BSS by lentivirus-mediated gene therapy suggests a promising strategy for gene therapy.[49]

Upregulated VWF-binding to GPIbα. Platelet-type von Willebrand disease (platelet-type VWD) is characterized by thrombocytopenia and increased platelet agglutination by low-dose ristocetin in the presence of normal plasma.

Table 1. Inherited thrombocytopenia with large-sized platelets.

Group of abnormalities	Syndrome	Platelet count and morphology	Platelet function	Associated phenotype	Associated biological abnormalities	Gene defect and transmission	Ref
Platelet adhesion	BSS	Moderate to severe decrease Giant platelets	Loss of platelet adhesion to VWF	Occasionally Di-George syndrome	Impaired platelet production	*GPIBA* (17p13) *GPIBB* (22q11) GP9 (3q21) AR	6-8
	Platelet-type VWD	Decreased Some large platelets	Enhanced VWF/GPIb. Abnormal vessel-wall interaction	-	Blocked GPIb Loss of large VWF multimers from plasma	*GPIBA* (17p13) AD	2,9,10
	VWD2B	Variable, +/- enlarged, sometimes agglutinated platelets	Enhanced VWF/GPIb Abnormal vessel-wall interaction	-	Abnomal VWF Loss of large VWF multimers from plasma	Exon 28 of *VWF* (12p13.3) AD	2,11
Transcription factors	GATA-1	Decreased Enlarged platelets	Aggregation impaired,	Dyserythropoietic anemia, β-thalassemia	Decreased protein (e.g. GPIb-IX) and α-granule expression	*GATA-1* (Xp11) X-linked	2,12,13
	Paris-Trousseau syndrome	Decreased with giant fused granules	-	Psychomotor retardation, facial, cardiac defects	Immature MKs predominate in marrow	*FLI1 (11q23.3)* microdeletion AD	2,12,14,15
α-granule defects	Gray platelet syndrome	Decreased with enlarged platelets lacking α-granules	Variable aggregation response	Myelofibrosis Enlarged spleen	Occasional loss of GPVI Increased Vitamin B12	*NBEAL2* (3p21) mostly AR	16-20
	Quebec syndrome	Sometimes decreased Platelet aniscocytosis	Abnormal response to epinephrine	Excessive fibrinolysis	Proteolytic degradation of α-granule proteins	*PLAU* *(10q24)* tandem duplication	21,22
Cytoskeleton defects	MYH9-RD	Decreased Presence of giant platelets	Abnormal NMMHC-IIA distribution and function	Deafness, cataract, renal dysfunction	Presence of Döhle bodies in leukocytes	*MYH9* (22q12-13) AD	2,12, 23,24
	Filaminopathia	Decreased Variable presence of large platelets	Abnormal thrombus formation	Neurological, gastro-intestinal, cardiologic	Abnormal distribution of FLNA in platelets	*FLNA* *(Xq28)* X-linked	2,25,26
	Tubulin 1	Decreased Presence of large, round, platelets	Normal aggregation	-	Platelets with decreased microtubules	*TBB1* (6p21.3) AD	12,27
Defects of αIIbβ3	Isolated thrombocytopenia	Decreased Presence of large platelets	Variable aggregation response	-	Altered platelet production	ITGA2B ITGB3 (17q21.32) AD or AR	28-31
Lipid metabolic disorder	Hyperabsorption and defect of metabolism of sterols	Normal at birth, rapidly macrothrombocytopenic	Reduced	Premature coronary artery disease and atherosclerotic disease	Increased plant sterols, hypercholesterolemia	ABCG5 or ABCG8 (2p21) AR	12,32

p.Gly249Val (or Ser), p.Asp251Tyr, p.Met255Val (previously Gly233Val/Ser, Asp235Tyr, Met239Val) substitutions provoke changes in the conformation of the GPIbα N-terminal domain that binds soluble VWF directly as does a p.Pro449_Ser457 deletion in the macroglycopeptide-coding region of *GPIBA*.[4,9,10] This clinical condition resembles type 2B VWD and diagnosis requires care.[9] Giant platelets, thrombocytopenia and even circulating platelet aggregates can occur in type 2B VWD given by mutations in exon 28 of the *VWF* gene. Culture of $CD34^+$ cells from the peripheral blood of patients with type 2B VWD showed early association of the up-regulated VWF with GPIb, an altered megakaryopoiesis and modified proplatelet production.[11] Mouse models have confirmed a marked reduction in thrombus formation *in vivo* with modulation of disease severity by ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I motif, 13); platelet-bound large VWF multimers are particularly susceptible to cleavage by this enzyme.[39,50]

Deficient collagen receptor function. Platelet-collagen interaction in flowing blood is a multistep process involving both integrin α2β1 and GPVI that signals through the FcRγ-chain, a process negatively regulated by PECAM-1 (platelet-endothelial adhesion molecule-1). α2β1 is shared with a variety of cell types whereas GPVI is megakaryocyte (MK)-specific. Specific haplotypes in *GP6* and *ITGA2* can account for variations in a wide range of density of both receptors and affect the collagen response.[45] Patients with FT linked to heterozygous mutations in the *ANKRD26* (ankyrin repeat domain 26) gene variably express α2β1, otherwise there is no clear pathology of this integrin.[51] In contrast, individuals in two families with a life-long but mild bleeding syndrome and severely deficient collagen-induced platelet aggregation are compound heterozygotes for mutations of *GP6*.[52,53] Note that acquired antibodies, extracellular proteases and even shear can induce sheddase activity by members of the ADAMTS family and loss of GPVI, a factor to take into account in diagnosis.[54] Sheddase is a name given to cell-bound proteases that cleave membrane receptors close to the transmembrane domain with release of the extracellular domain.

Inherited variants of receptors for soluble agonists and of signaling pathways

Pathology of ADP and T_xA_2 (thromboxane A_2) receptors

Inherited defects of platelet aggregation to specific agonists are a frequent source of bleeding with many patients having as yet undefined abnormalities affecting Gi-receptor signaling, the TXA_2 pathway or dense granule secretion.[55] Platelets possess 2 classes of purinergic receptor for ADP: $P2Y_1$ that mediates Ca^{2+}-mobilization and shape

Table 2. Inherited thrombocytopenia with normal sized or small platelets.

Group of abnormalities	Syndrome	Platelet count and morphology	Platelet function	Associated phenotype	Associated biological abnormalities	Gene defect and transmission	Ref
Associated with orthopedic abnormalities	TAR	Decreased Normalization possible	Abnormal	Orthopedic abnormality. Malformations of the heart and kidneys. Normal hands	Decreased Y14 protein and defective mRNA processing	*RBM8* 1q21.1 AR Deletion of one allele, SNP in the other	2,33-35
	RUS	Decreased	-	Orthopedic abnormality. Hand abnormality	Amegakaryocytosis	*HOXA11* (7p15-14) AD	2,12
Transcription factor	FPD/AML1 RUNX1	Decreased. Variable platelet morphology	Impaired Aspirin-like	Hematological malignant syndrome	Abnormal PCK-θ, platelet type 12-LOX among others	*RUNX1* (21q22.3) AD	2,12,36-38
Role of the corresponding protein unknown	ANKRD26	Decreased	Impaired	Leukemia?	Decreased α-granule number. Increased TPO levels	*ANKRD26* 10p.12 AD	12,39,40
Congenital amegakaryopoiesis	CAMT	Severely decreased	Normal	Possible development of aplasia	Increased TPO levels. Decreased number of MK	*MPL* (1p34) AD	2,12,41
Cytoskeleton/ signaling defect	WAS Small discoid platelets	Decreased	Impaired aggregation, reduced secretion	Cellular and humoral immunodeficiency, increased risk of autoimmune disease and hematologic malignancy	Decreased WASP. Low granule number. Altered platelet production	WAS (Xp11.23) X-linked	2,42-44
X-linked Thrombocytopenia	XLT	Mild thrombocytopenia. Small discoid platelets	Impaired aggregation, defect in secretion	-	Decreased WASP. Low granule number. Altered platelet production	*WAS* (Xp11.23) X-linked	2,42-44

change; and $P2Y_{12}$, responsible for macroscopic platelet aggregation.[56] Only patients with quantitative or qualitative abnormalities of $P2Y_{12}$ have been characterized so far. Their phenotype includes a much decreased and reversible platelet aggregation to high-dose ADP and an inability of ADP to inhibit adenylate cyclase. A specific receptor defect was confirmed when analysis of PCR products from genomic DNA of a French patient revealed a mutant allele at the *P2RY12* locus.[57] Mutations in other patients include nucleotide deletions in the open-reading frame, frameshifts resulting in premature protein truncation, and missense mutations affecting ADP binding and even receptor trafficking.[55-58]

A defective platelet aggregation to TXA_2 in Japanese families linked to a p.Arg60Leu substitution in the TXA_2 receptor α-subunit (*TPXA2R*, TPα) results in impaired signal transmission and a loss of aggregation induced by arachidonic acid and U46619, a TXA_2 receptor agonist.[59] Mutations that disrupt both TPα function and receptor cycling have been reported.[55] An absent platelet response to adrenaline is frequent in routine screening but its contribution as a cause of bleeding remains uncertain.

Defects in intracellular signaling pathways

Pathologies of signal transduction pathways also concern patients with defects of platelet aggregation that affect some stimuli more than others.[1,4] Early studies highlighted patients with abnormalities of: a) phospholipase C; b) protein kinase C; and c) Gαi and Gαq, although gene mutations were not reported. Likewise, patients with congenital deficiencies of cyclooxygenase-1, prostaglandin H synthase-1, thromboxane synthase, phospholipase A_2, lipoxygenase, glycogen synthase and ATP metabolism were all the object of reports largely based on platelet function testing.[1,4] Such defects may directly interfere with αIIbβ3 activation and Fg binding, or intervene secondarily by preventing secretion of ADP or TXA_2. A special category of patients with defects in the G-protein cascade involve second messengers or RGS proteins that affect cAMP levels.[60] Gsα is regulated by the complex imprinted gene cluster *GNAS1*; direct genetic and epigenetic defects of *GNAS1* have been reported and include both Gsα hypofunction and a thrombotic phenotype associated with more generalized hormonal, skeletal defects and sometimes mental retardation.[60-62] Isolated reports of signaling mole-

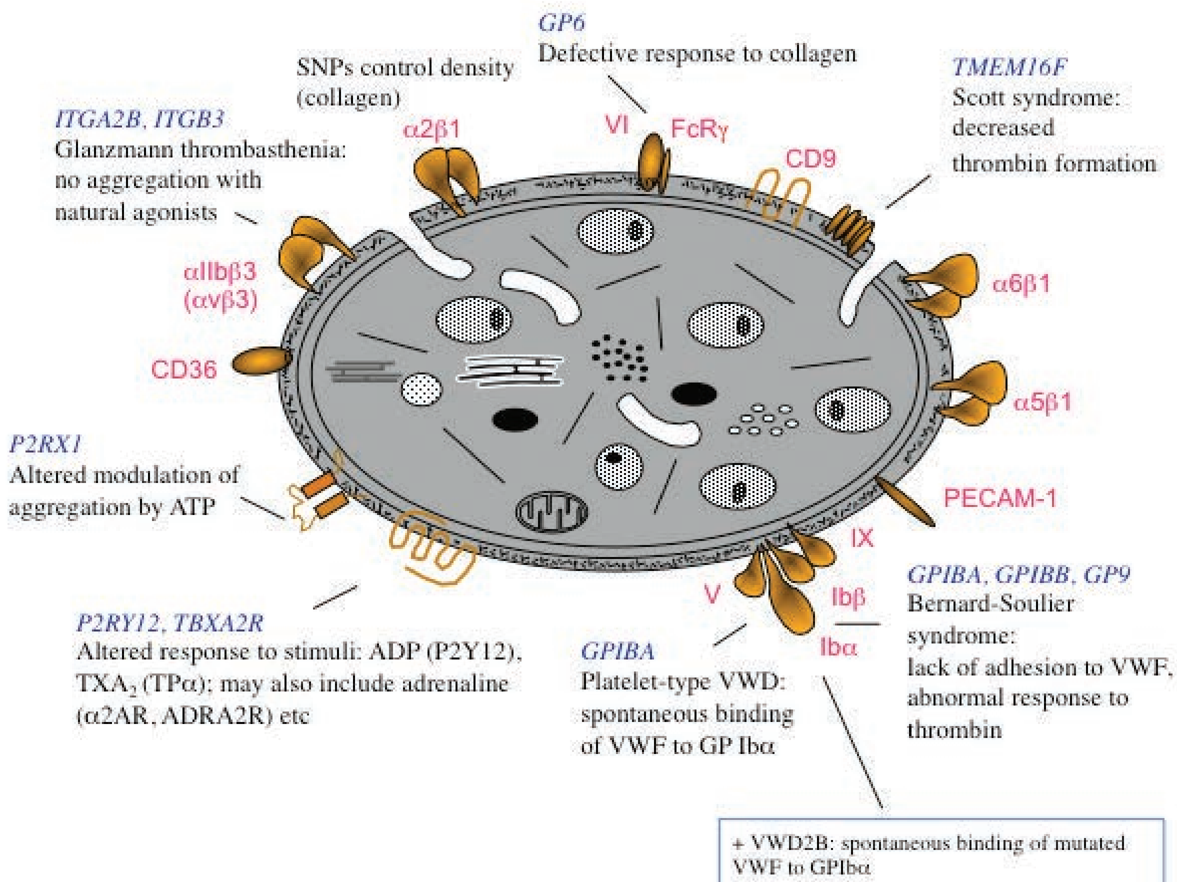

Figure 1. Disorders that principally affect surface components of platelets.

cules involved in platelet dysfunction include human gp91phox (phagocyte oxidase) deficiency with impaired isoprostane formation and decreased thrombus formation, while mutations in the *OCRL* (Lowe oculocerebrorenal syndrome protein) gene encoding an inositol polyphosphate 5-phosphatase characterize the Lowe syndrome.[63,64]

A major effort is underway to uncover the mutations responsible for signaling defects either by a phenotypic approach or by whole exome sequencing.[16,46,55] Signaling defects can be specific for MKs and platelets, or extend to other cell types and be secondary to genetic defects of transcription factors, as we will show in later sections.

Defects of secretion (storage pool disease)

Defects of α-granules

These are the storage sites for many proteins either synthesized in MK or endocytosed from plasma. Most are biologically active and after secretion account for nonhemostatic roles of platelets as well as participating in hemostasis.[65,66] The organelle membranes contain a variety of glycoproteins (e.g. P-selectin, CD40L and CD63) that are translocated to the plasma membrane during secretion. Specific deficiencies of α-granule-stored proteins also occur in inherited deficiencies of the corresponding plasma proteins (e.g. factor V deficiency, fibrinogen (Fg) in afibrinogenemia, VWF in type 3 VWD).[4]

Gray platelet syndrome (GPS) is a mild bleeding disorder characterized by a severe deficiency of α-granules and their contents.[1,4] The molecular defect involves packaging of proteins and α-granule biogenesis in MK. Clinical features include modest macrothrombocytopenia, an early onset of myelofibrosis and enlarged spleens. Secretion-dependent platelet aggregation is abnormal, as is platelet spreading and thrombus formation under flow.[4,67] A low platelet expression of GPVI due to increased sheddase activity by members of the ADAMTS family has been reported in isolated cases.[4] Electron microscopy shows only vestigial α-granules in platelets and MKs, vacuoles are abundant in MKs and α-granule proteins seen in the surface-connected canalicular system. Emperipolesis (passage of other blood cells through MKs) is a feature. Small vesicles containing tissue inhibitors of metalloproteases (TIMPs) are present in GPS platelets and may represent the T-granules described by others.[17,68] In 2011, three groups using new generation sequencing technologies showed mutations in *NBEAL2* (neurobeachin-like 2) in GPS.[16,18,19] *NBEAL2* belongs to a gene family that includes *LYST* (see below). The protein encoded by this gene con-

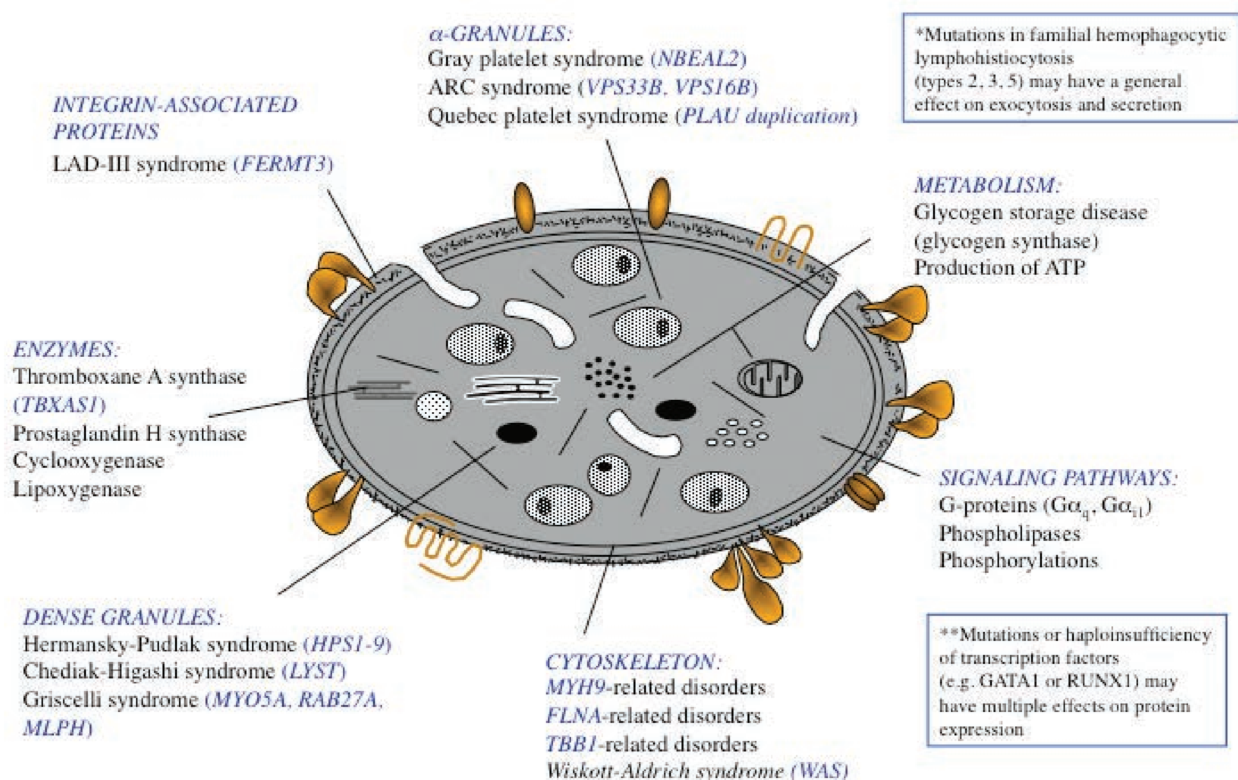

Figure 2. Disorders that affect intracellular organelles or cytosolic proteins of platelets.

tains a beige and Chediak-Higashi (BEACH) domain and multiple WD40 domains (domains terminating in tryptophan (W) and aspartic acid (D)) and appears directly implicated in α-granule biogenesis in MKs. It has been speculated that genetically GPS is a heterogeneous trait whose severity depends on the basis of the α-granule deficiency.[20]

Mutations of *VPS33B*, encoding a regulator of soluble N-ethylmaleimide-sensitive factor activating receptor (SNARE)-dependent membrane fusion and of *VIPAS39* encoding VPS33B-interacting protein, cause the arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome.[69] Mostly lethal for young children, ARC associates platelet dysfunction and low granule content with a multisystem disorder featuring renal tubular and other dysfunctions. The platelet defect extends to stored and membrane components of α-granules.[70] Other variant disorders affecting α-granules include the Medich giant platelet disorder where platelets feature scroll-like membranous inclusions.[1,4]

The autosomal dominant Quebec platelet syndrome (QPS) is unique to French-Canadian families.[21] Here, platelets show protease-related degradation of many α-granule proteins (including P-selectin) despite a normal α-granule ultrastructure. Thrombocytopenia is sometimes observed and there is a characteristic platelet aggregation deficiency with epinephrine. Fibrinolytic inhibitors not platelet transfusions reduce bleeding due to the fact that platelets in QPS possess unusually large amounts of urokinase-type plasminogen activator (u-PA). This promotes intra-granular plasminogen generation and excessive fibrinolysis upon platelet secretion. The genetic basis of QPS is a tandem duplication of the u-PA gene, *PLAU*.[22]

Defects of dense (δ) granules

These are storage sites for serotonin, ADP and ATP. Storage pool disease (SPD) affecting dense granules is a common cause of defects of secretion-dependent platelet aggregation.[55] Secretion deficiency may be severe or partial, in some patients it also extends to α-granules. It may concern granule biogenesis and storage of constituents or the signaling pathways responsible for exocytosis. When dense granules deficiencies are associated with generalized abnormalities of lysosome-related organelles, they lead to clearly defined phenotypes. This is so for the Hermansky-Pudlak (HP), Chediak-Higashi (CH) and Griscelli syndromes where heterogeneous disorders of vesicle biogenesis and melanosomal defects also cause a lack of pigmentation of the skin and hair.[71]

In HPS, oculocutaneous albinism is an additional feature as is ceroid-lipofuchsin storage in the reticuloendothelial system; granulomatous colitis, interstitial lung disease and fatal pulmonary fibrosis occur in some subtypes. Defects in nine genes (*HPS1* through *HPS9*) cause distinct HPS subtypes in man.[71,72] A pathological 16-base duplication in exon 15 of the *HPS1* gene predominates in Puerto Rican patients. HPS proteins interact with each other in complexes called BLOCS (biogenesis of lysosome related organelles complexes). Genetic defects disrupt processing of these and SLC35D3 (a member of the solute carrier family) during dense granule biogenesis.[73] HPS2 is associated with innate immunity defects.[71] The beta3A subunit of the adaptor protein-3 (AP-3) complex encoded by *AP3B1* is abnormal in HPS2, although homozygosity mapping and whole-exome sequencing have revealed candidate mutations in *SLC45A2* and *G6PC3* (glucose-6-phosphatase 3) as potential causes of HPS2 in a patient who associated oculocutaneous albinism with neutropenia.[74] Next generation sequencing has also allowed the rapid identification of a c.597-2 A>T transversion in the intron 7 splice acceptor site of *HPS4* leading to abnormal splicing and a premature stop codon in exon 10.[75]

In CHS, bleeding is associated with severe immunological defects with life-threatening infections and progressive neurological dysfunction if the patient survives to adulthood.[71] The immunodeficiency leads to the development of a lymphoproliferative syndrome and an accelerated phase in approximately 85% of patients. The hallmark of CHS is the presence of giant inclusion bodies in a variety of granule-containing cells. The CHS gene (*LYST,* lysosomal trafficking regulator) has been cloned and a series of frameshift and nonsense mutations described that result in truncated CHS protein and a severe phenotype. Rare missense mutations can be associated with a milder form of the disease. LYST is a large protein with distinct structural domains including 'BEACH' and 'ARM/HEAT' suggestive as for NEABL2 of a function in membrane contact interactions and organelle protein trafficking.

Patients in Griscelli syndrome have partial albinism and silver hair; different subtypes associate neurological defects and/or severe immunodeficiency with a defective cytotoxic lymphocyte activity but no obvious bleeding tendency. Mutations in the genes encoding myosin Va, Rab27a (a small GTPase), or melanophilin cause 3 subtypes of Griscelli syndrome. Differential diagnosis with HPS type II can be difficult, as shown for a child with both heterozygous Rab27a and homozygous *AP3B1* mutations, bleeding and an impaired secretion-dependent platelet aggregation.[76] Defective platelet secretion (dense and α-granules) despite normal granule cargo in familial hemophagocytic lymphohistiocytosis (FHL) types 3, 4 and 5 are caused by defects in Munc (mammalian uncoordinated) 13-4, syntaxin-11 and the Munc18b coding genes; Munc18b may be a key regulator of syntaxin-11 in platelet exocytosis.[77-79] This review highlights how platelets use similar secretory machinery as cytotoxic T lymphocytes and NK (natural killer) cells.

Glanzmann thrombasthenia

Glanzmann thrombasthenia (GT) is the classic inherited platelet disorder; platelets fail to aggregate to all physiological agonists due to quantitative or qualitative defects of the integrin, αIIbβ3. In normal hemostasis, αIIbβ3 on activated platelets binds Fg and other adhesive proteins that link platelets together during aggregation. Other manifestations of GT include a defective platelet spreading on collagen, while clot retraction and αIIbβ3-dependent Fg storage in α-granules are variably defective depending on the nature of the mutation. GT has been comprehensively dealt with in two recent reviews and only essential details will be repeated here.[28,29]

Direct sequencing of the *ITGA2B* and *ITGB3* genes allows the detection of most mutations in GT which has autosomal recessive inheritance, so compound heterozygosity is common except for certain ethnic groups, such as

the French Manouche gypsies in whom consanguinity is widespread.[80] Genetic defects occur across both genes that are closely located at 17q21-23. Nonsense mutations, splice site mutations resulting in frameshifts and missense mutations are all common. They mostly prevent subunit biosynthesis in MKs or inhibit transport of pro-αIIbβ3 complexes from the ER to the Golgi apparatus and/or their export to the cell surface. Particularly abundant are mutations within the β-propeller region of αIIb and within the epithelial growth factor (EGF)-domains of β3 (for specific examples see Mansour *et al*. and Mor-Cohen *et al*.[81,82]). Analysis of GT is now advanced and population studies have started.[83,84] β3 also forms part of the vitronectin receptor (αvβ3) expressed in many tissues. It has but a minor presence in platelets. In GT, αvβ3 is absent if the genetic lesion affects β3 production. Yet patients with β3 gene defects do not have a distinctive phenotype because, unlike β3-/- mouse models, clear evidence for abnormal vessel development, bone thickening, increased rates of abortion or of tumor development has not been forthcoming.[28] An unusually high number of reports of deep vein thrombosis leaves the question open as to whether it may be an unexpected risk factor in GT.[85] In variant GT, platelets express non-functional integrin. Mostly the mutations affect *ITGB3* and amino acid substitutions (e.g. p.Asp145Tyr or p.Arg240Gln or Trp – formerly Asp119Tyr and Arg214Gln/Trp) affecting MIDAS (metal ion dependent adhesion site), ADMIDAS (adjacent to MIDAS) or SyMBS (synergistic metal ion binding site) domains; these helped identify ligand binding sites in the activated integrin.[28,29] Likewise, a p.Ser778Pro (previously Ser752Pro) substitution in the cytoplasmic domain of β3, or stop codons leading to β3 truncated within the cytoplasmic tail, have helped identify domains involved in ‘inside-out’ signaling and activation of αIIbβ3 through the binding of kindlin-3 and talin.[28,29] A p.Cys586Arg (formerly Cys560Arg) in β3 unexpectedly led to platelets expressing residual surface αIIbβ3 able to spontaneously bind Fg; a situation recalling platelet-type VWD where normal VWF multimers spontaneously bind to mutated GPIbαβ and block its function.[4,9] In fact, mutations within many of the disulfides in the EGF domains of β3 both severely interfere with αIIbβ3 expression and lead to partially activated integrin.[82] Correction of dog GT by lentivirus-mediated gene therapy suggests a promising strategy for the future treatment of patients with classic GT.[86]

An interesting new variant-type in GT was identified by mutations within *ITGA2B* and *ITGB3,* which, while affecting αIIbβ3 function also lead to moderate thrombocytopenia and platelet anisocytosis.[28,30] These mostly affect either cytoplasmic domains of both αIIb and β3 and especially the salt-bridge linking αIIb p.Arg1026 (previously Arg995) and β3 p.Asp749 (formerly Asp723) or membrane proximal residues in extracellular domains and also favor αIIbβ3 activation.[30,31] One recent study has shown how such mutations modify MK interaction with matrix proteins and interfere with proplatelet production.[31]

Also to be mentioned is leukocyte adhesion deficiency-III (LAD-III) syndrome in which life-threatening bleeding is associated with a high susceptibility for infections and poor wound healing in early life. The complex clinical features result from mutations in the kindlin-3 coding gene (*FERMT3*) that abolish ‘inside-out’ integrin activation in platelets, white blood and endothelial cells.[87-89]

Scott syndrome

The Scott syndrome is a rare inherited disorder caused by defective scrambling of phospholipids on blood cells.[90] It manifests by a decreased fibrin formation during shear-dependent adhesion of platelets to subendothelium. Scott platelets when activated are unable to translocate phosphatidylserine (PS) to the outer phospholipid leaflet of the membrane bilayer; factors Va and Xa fail to bind leading to a decreased capacity of platelets to convert prothrombin into thrombin. This lack of thrombin generation is sufficient to induce a bleeding syndrome. Physiological stimuli that induce PS translocation include a thrombin and collagen mixture and complement C5b-9. Microvesiculation, a process that can be quantified by flow cytometry using FITC-annexin V, accompanies PS expression and is also defective in Scott syndrome. The disease is given by mutations in *TMEM16F* (also known as *ANO6*, anoctamin 6) that encodes transmembrane protein 16F, a Ca^{2+}-activated channel essential for Ca^{2+}-dependent PS exposure.[90-92]

Familial thrombocytopenias

Inherited defects of platelet production constitute a heterogeneous group of diseases often autosomal dominant in inheritance.[1-4,12] In Tables 1 and 2 we group the disorders according to the presence or not of platelet size changes, in the text we discuss them in terms of their genotype. Some disorders associate low circulating platelet counts with well characterized platelet functional and morphological abnormalities and have been dealt with in preceding sections. In others, while platelet dysfunction is also often present, it has historically been considered as secondary to the low platelet count or little studied.

Defects in transcription factors

An altered megakaryopoiesis resulting from transcription factor defects is a common cause of familial thrombocytopenias (FT). Abnormalities can extend to other marrow cells and interfere with development, e.g. the association of pancytopenia and radio-ulnar synostosis (RUS) attributed to homeobox gene (*HOXA11*) mutations.[2] In the Paris-Trousseau syndrome, a decreased platelet production and a mild hemorrhagic tendency are associated mostly with *de novo* deletions at 11q23.3 and a heterozygous loss of *FLI1* (Friend leukemia virus integration 1).[2,12] Platelets are often large and feature giant α-granules formed by fusion after MK maturation. The phenotype is given by pathologically low Fli1 protein levels during what, in normal conditions, is transient monoallelic *FLI1* expression at an early stage of MK differentiation.[14] The result is a subpopulation of immature MKs that fail to reach the platelet production stage. Fli1 forms a complex with RUNX1 to regulate megakaryopoiesis. A characteristic of deficiencies of both genes is a persistance of MYH10 protein in platelets.[15] Paris-Trousseau is a variant of Jacobsen’s syndrome in which patients have congenital heart defects, trigonocephaly, facial dysmorphism, mental retardation and multiple organ malfunction.

X-linked familial thrombocytopenia (XLT) or XLT with thalassemia (XLTT) result from mutations in the *GATA1*

(GATA-binding protein 1 or globin transcription factor 1) gene.[2,13] The often enlarged platelets aggregate poorly to collagen. GATA-1 protein has domains that account for sequence-specific DNA binding and for the interaction of its co-factor, FOG-1 (Friend of GATA-1 protein). It coordinates hematopoietic cell differentiation by activating lineage specific genes. Erythrocytes are abnormal in size and shape. XLT without anemia is given by amino acid substitutions in GATA-1 (e.g. p.Asp218Tyr, p.Gly208Ser, p.Val205Met) that affect its interaction with FOG-1 but which allow GATA-1 binding to DNA. In contrast, a recurrent Arg216Gln substitution in the N-terminal finger of GATA-1 that destabilizes binding to DNA without affecting its interaction with FOG-1 gives red cell abnormalities and XLTT.[13] A low transcription of GATA-1 target genes, *GPIBB* and *GP9* is a characteristic of GATA-1 defects and platelets may have few α-granules. GATA-1 mutations are also seen in Down syndrome and related disorders.

Autosomal dominant mutations in the gene encoding the hematopoietic transcription factor RUNX1 (Runt related transcription factor) (previously known as CBFA2, AML1) give rise to haploinsufficiency and FT with a predisposition to hematological malignancies.[2,36] Inactivating (p. Arg139Ala) or dominant-negative p.Arg174Phe mutations interfering with DNA binding by RUNX1, lead to an arrest of MK maturation and an expanded population of progenitor cells. *RUNX1* is a master regulatory gene in hematopoiesis. The propensity to develop leukemia is accompanied by downregulation of *NR4A3*, a gene implicated in leukemia development; a process facilitated by the expanded population of progenitor cells susceptible to be hit by secondary pro-leukemic genetic events.[36] Impaired platelet aggregation and secretion are associated with a deficiency of protein kinase C-θ(PKC-θ) and defective phosphorylation of pleckstrin and myosin light chain.[37] Platelet expression profiling revealed a decreased expression of the genes encoding platelet 12-lipoxygenase (*ALOX12*) and myosin regulatory light chain polypeptide (*MYL9*) among other transcriptional targets of RUNX1.[37,38]

Defects in megakaryocyte production

In congenital amegakaryocytic thrombocytopenia (CAMT), severe thrombocytopenia at birth rapidly develops into a pancytopenia in most affected children. Patients have low numbers of MKs in their marrow; thrombopoietin (TPO) is unable to fulfill its normal thrombopoietic role due to homozygous or compound heterozygous mutations in the *MPL* (myeloproliferative leukemia virus oncogene) gene encoding the TPO receptor (c-MPL).[41] Only rarely are skeletal and central nervous system anomalies present.[93] Patients with early development into aplasia are more likely to have frameshift or nonsense mutations and a complete loss of c-MPL. Missense mutations leading to residual c-MPL result in a slower progression of the disease. Mutations in *MPL* giving rise to an activated form of c-MPL are seen in familial essential thrombocythemia with an overproduction of platelets.[2,4,41]

Thrombocytopenia with absent radii (TAR syndrome) associates CAMT-like thrombocytopenia and osteodysgenesis with shortened (or absent) forearms due to bilateral radial aplasia. Although other skeletal anomalies can be present, hands and fingers are unaffected. Serum TPO levels are elevated, and platelets of TAR patients fail to respond to recombinant TPO when added in combination with suboptimal amounts of platelet activators. A deletion at 1q21.1 was first associated with the disease.[33] Albers *et al*. then reported that TAR was given by compound inheritance of a null allele and one of two low-frequency SNPs in the regulatory regions of *RBM8A* encoding the Y14 subunit of the exon-junction complex (EJC) essential for RNA processing.[34] Platelet function can also be affected with an unexplained restoration of TPO signaling as patients age.[35]

Defects of the cytoskeleton and thrombocytopenia with increased platelet size

Macrothrombocytopenia occurs in *MYH9*-related disease (MYH9-RD; myosin heavy chain 9-related disease) affecting non-muscle myosin heavy-chain IIA (or myosin-9) (Table 1).[2,23] Platelets can be truly giant and ultrastructural modifications are seen in MKs taken from the marrow. Phenotypic heterogeneity extends to variable combinations of Döhle bodies in neutrophils, nephritis, hearing loss and cataracts. Diagnostic immunofluorescence patterns are seen for myosin-9 aggregates in leukocytes. Mutations in the head domain (affecting Ca^{2+}-ATPase activity) favor deafness and renal disease in later life, while those affecting the rod (and myosin-IIA assembly) may only have a hematologic consequence. Mutation hotspots characterize the disease. Haploinsufficiency and a dominant-negative effect can influence phenotype although other genetic or environmental factors can also intervene even in the same family. Decreased myosin light chain (MLC) phosphorylation and myosin-9 function in MKs slow MK migration towards the sinusoids as well as blurring the signaling mechanism for proplatelet formation.[24] Blebbistatin, an inhibitor of myosin-9 and inhibitors of Rho-associated kinase-1 (ROCK1) or MLC kinase rescue proplatelet formation in MKs in culture suggesting that treatment reducing myosin contractility, strangely augmented in MYH9-platelets, may offer therapeutic potential in MYH9-RD (N Debili *et al.,* submitted manuscript, 2013).

X-linked mutations in *FLNA* encoding filamin A give a variety of developmental defects with abnormal neuronal migration resulting in periventricular nodular heterotopia (PNH). Filamin A is an attachment site for GPIbα in the platelet cytoskeleton (see section on BSS above) and we have described that *FLNA* mutations can also give rise to bleeding and macrothrombocytopenia, including in a patient originally diagnosed as having ITP.[25] Significantly, *FLNA* mutation heterogeneity was associated with different functional impacts especially with regard to thrombus growth under flow.[26] *TBB1* mutations affecting β1-tubulin are also associated with thrombocytopenia and platelet anisocytosis (variable size) with enlarged forms having defects of microtubule assembly.[27]

Thrombocytopenia with small or normal-sized platelets

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disease combining thrombocytopenia and small platelets with eczema, recurrent infections, an increased risk for autoimmunity and malignancy.[2,42,43] A milder form without immune problems is known as hereditary X-linked thrombocytopenia (XLT). The small platelets aggregate poorly and have a low granule number. T lymphocytes show defective function. The *WAS* gene is composed of 12 exons, genetic defects result either in the decreased expression of WASP (WAS protein) or its absence, the latter being predictive of a more severe disease.[43] Missense mutations affecting the N-terminal Ena Vasp homology 1 domains predominate in hereditary XLT probably because of residual protein expression.[44] Deficiency in the WASP-interacting protein (WIP) also results in a block of WASP expression.[94] WASP is a key regulator of actin polymerization in hematopoietic cells; it is involved in signal transduction with tyrosine phosphorylation sites and adapter protein function. WASP induces premature proplatelet formation in the marrow where a lack of actin-rich podosomes retards MK migration to the vascular sinus. Mutations in *WAS* giving spontaneously activated WASP with increased actin polymerizing activity are responsible for an X-linked form of neutropenia.[42] One of the first disorders to be treated with hematopoietic stem cell transplantation, WAS is now the subject of several phase I/II gene therapy trials.[95]

ANKRD26-related thrombocytopenia (thrombocytopenia 2, THC2) is an autosomal dominant disease with mostly a moderate fall in platelet count, normal-sized platelets and mild bleeding.[12,51] Bone marrow examination revealed an evident dysmegakaryopoiesis. Platelets are often deficient in $\alpha 2\beta 1$ and have a reduced number of α-granules. A high incidence of leukemia is suspected. Recently discovered features are ubiquitin/proteasome-rich particulate cytoplasmic structures (PaCSs) in both platelets and MKs; their presence suggests a link with oncogenesis.[40]

Conclusions

We have provided an up-to-date assessment of the genetics of inherited disorders of platelets and MKs. Due to space restrictions, single case reports have mostly been omitted, as have historical reports with no recent update. We have also minimized discussion of disorders arising indirectly, such as the macrothrombocytopenia arising rapidly after birth in the lipid metabolic disorder linked to an inability to metabolize plant sterols (sitosterolemia) due to mutations in *ABCG5* or *ABCG8*.[12,32] A major question concerns the true abundance of inherited diseases with a low platelet count. Many patients with FTs are first falsely diagnosed as immune thrombocytopenic purpura (ITP).[96] Even now a high percentage of patients with a low platelet count do not fall into the categories covered by Tables 1 and 2 suggesting that other molecular causes are frequent. It may well be that ITP will require a new classification and that patients with MK gene mutations account for a significant number of cases, with perhaps the secondary formation of autoimmune antibodies aggravating the thrombocytopenia. Whole exome sequencing and other new generation technologies will help fill in the missing pieces of the puzzle.[16,34,46-48]

References

1. Bennett JS, Rao AK. Inherited disorders of platelet function. In: Marder VJ, Aird WC, Bennett JS, Schulman S, White GC 2nd (eds.) Hemostasis and Thrombosis, Basic Principles and Clinical Practice. 4th ed. Wolters Kluwer/Lippincott, Williams and Wilkins, Philadelphia; 2012:805-20.
2. Nurden P, Nurden AT. Inherited thrombocytopenias. In: Marder VJ, Aird WC, Bennett JS, Schulman S, White GC 2nd (eds.) Hemostasis and Thrombosis, Basic Principles and Clinical Practice. 4th ed. Wolters Kluwer/Lippincott, Williams and Wilkins, Philadelphia; 2012:785-95.
3. Goodeve AC, Perry DJ, Cumming T, Hill M, Jennings I, Kitchen S, et al. Genetics of haemostasis. Hemophilia. 2012;18(4 Suppl):73-80.
4. Nurden AT, Nurden P. Advances in our understanding of disorders of the molecular basis of platelet function. J Thromb Haemost. 2011;9(1 Suppl):76-91.
5. Nurden AT, Freson K, Seligsohn U. Inherited platelet disorders. Haemophilia. 2012;18(14 Suppl):154-60.
6. Berndt MC, Andrews RK. Bernard-Soulier syndrome. Haematologica. 2011;96(3):355-9.
7. McEwab P, Yang W, Carr KH, Mo X, Zheng X, Li R, et al. Quaternary organization of GPIb-IX complex and insights into Bernard-Soulier syndrome revealed by the structures of GPIbβ and a GPIbβ/GPIX chimera. Blood. 2011; 118(19):5292-301.
8. Noris P, Perrotta S, Bottega R, Pecci A, Melazzini F, Civaschi E, et al. Clinical and laboratory features of 103 patients from 42 Italian families with inherited thrombocytopenia derived from the monoallelic Ala156Val mutation of GPIbα (Bolzano mutation). Haematologica. 2012;97(1):82-8.
9. Othman M. Platelet-type von Willebrand disease: a rare, often misdiagnosed and underdiagnosed bleeding disorder. Semin Thromb Hemost. 2011;37(5):464-9.
10. Enayat S, Ravanbod S, Rassoulzadegan M, Jazebi M, Tarighat S, Ala F, et al. A novel D235Y mutation in the GPIBA gene enhances platelet interaction with von Willebrand factor in an Iranian family with platelet-type von Willebrand disease. Thromb Haemost. 2012;108(5):946-54.
11. Nurden P, Gobbi G, Nurden A, Enouf J, Youlyouz-Marfak I, Carubbi C, et al. Abnormal VWF modifies megakaryocytopoiesis: studies of platelets and megakaryocyte cultures from patients with von Willebrand disease type 2B. Blood. 2010;115(13):2649-56.
12. Balduini CL, Savoia A. Genetics of familial forms of thrombocytopenia. Hum Genet. 2012;131:1821-32.
13. Ciovacco WA, Raskind WH, Kacena MA. Human phenotypes associated with GATA-1 mutations. Gene. 2008;427(1-2):1-6.
14. Raslova H, Komura E, Le Couédic JP, Larbret F, Debili N, Feunteun J, et al. FLI1 monoallelic expression combined with its hemizygous loss underlies Paris-Trusseau/Jacobsen thrombopenia. J Clin Invest 2004;114(1):77-84.
15. Anthony-Debré I, Bluteau D, Itzyksson R, Baccini V, Renneville A, Boehlen F, et al. MYH10 protein expression in platelets as a biomarker of RUNX1 and FLI1 alterations. Blood. 2012;120(13):2719-22.
16. Albers CA, Cvejic A, Favier R, Bouwmans EE, Alessi MC, Bertone P, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. Nat Genet. 2011;43(8):735-7.
17. Villeneuve J, Block A, Le Bousse-Kerdilés MC, Lepreux S, Nurden P, Ripoche J, et al. Tissue inhibitors of metalloproteinases in platelets and megakaryocytes: a novel organization for these secreted proteins. Exp Hematol. 2009;37(7):849-56.
18. Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, Zivony-Elboum Y, Gumruk F, Cetin M, et al. NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet α-granules. Nat Genet. 2011;43(8):732-4.
19. Kahr WH, Hinckley J, Li L, Schwertz H, Christensen H, Rowley JW, et al. Mutations in NBEAL2 encoding a beach protein, cause gray platelet syndrome. Nat Genet. 2011;43(8):738-40.
20. Bottega R, Pecci A, De Candia E, Pujol-Moix N, Heller PG, Noris P, et al. Correlation between platelet phenotype and NBEAL2 genotype in patients with congenital thrombocytopenia and α-granule deficiency. Haematologica. 2012 Nov 9 [Epub ahead of print]
21. Blavignac J, Bunimov N, Rivard GE, Hayward CP. Quebec

platelet disorder: update on pathogenesis, diagnosis, and treatment. Semin Thromb Haemost. 2011;37(6):713-20.
22. Paterson AD, Rommens JM, Bharaj B, Blavignac J, Wong I, Diamondis M, et al. Persons with Quebec platelet disorder have a tandem duplication of PLAU, the urokinase plasminogen activator gene. Blood. 2010;115(6):1264-6.
23. Balduini CL, Pecci A, Savoia A. Recent advances in the understanding and management of MYH9-related inherited thrombocytopenias. Br J Haematol. 2011;154(2):161-74.
24. Pecci A, Bozzi V, Panza E, Barozzi S, Gruppi C, Seri M, et al. Mutations responsible for MYH9-related thrombocytopenia impair SDF-1 driven migration of megakaryoblastic cells. Thromb Haemost. 2011;106(4):693-704.
25. Nurden P, Debili N, Coupry I, Bryckaert M, Youlyouz-Marfak I, Solé G, et al. Thrombocytopenia resulting from mutations in filamin A can be expressed as an isolated syndrome. Blood. 2011;118(22):5928-37.
26. Berrou E, Adam F, Lebret M, Fergelot P, Kauskot A, Coupry I, et al. Heterogeneity of platelet functional alterations in patients with filamin A mutations. Arterioscler Thromb Vasc Biol. 2013;33(1):e11-8.
27. Kunishima S, Kobayashi R, Itoh TJ, Hamaguchi M, Saito H. Mutation of the beta1-tubulin gene associated with congenital macrothrombocytopenia affecting microtubule assembly. Blood. 2009;113(2):458-61.
28. Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on phenotypic variability, and mouse models. Blood. 2011;118(23):5996-6005.
29. Nurden AT, Pillois X, Nurden P. Understanding the genetic basis of Glanzmann thrombasthenia: implications for treatment. Expert Rev Hematol. 2012;5(5):487-503.
30. Nurden AT, Pillois X, Fiore M, Heilig R, Nurden P. Glanzmann thrombasthenia-like syndromes associated with macrothrombocytopenia and mutations in the genes encoding the αIIbβ3 integrin. Semin Thromb Hemost. 2011;37(6):698-706.
31. Bury L, Malara A, Gresele P, Balduini A. Outside-in signaling generated by a constitutively activated integrin αIIbβ3 impairs proplatelet formation in human megakaryocytes. PLoS One. 2012;7(4):e3449.
32. Chase TH, Lyons BL, Bronson RT, Foreman O, Donahue LR, Burzenski LM, et al. The mouse mutation "thrombocytopenia and cardiomyopathy" (trac) disrupts Abcg5: a spontaneous single gene model for human hereditary phytosterolemia/sitosterolemia. Blood. 2010;115(6):1267-76.
33. Houeijeh A, Andrieux J, Saugier-Veber P, David A, Goldenberg A, Bonneau D, et al. Thrombocytopenia-absent radius (TAR) syndrome: a clinical genetic series of 14 further cases. Impact of the associated 1q21.1 deletion on the genetic counseling. Eur J Med Genet. 2011;54(5):e471-7.
34. Albers CA, Paul DS, Schulze H, Freson K, Stephens JC, Smethurst PA, et al. Compound inheritance of a low-frequency regulatory SNP and a rare null mutation in exon-junction complex subunit RBM8A causes TAR syndrome. Nat Genet. 2012;44(4):435-9.
35. Fiedler J, Strauβ G, Wannack M, Schwiebert S, Siedal K, Henning K et al. Two patterns of thrombopoietin signaling suggest no coupling between platelet production and thrombopoietin reactivity in thrombocytopenia-absent radii syndrome. Haematologica. 2012;97(1):73-81.
36. Bluteau D, Gilles L, Hilpert M, Anthony-Debré I, James C, Debili N, et al. Down-regulation of the RUNX1-target gene NR4A3 contributes to hematopoiesis deregulation in familial platelet disorder/acute myelogenous leukemia. Blood. 2011;118(24):6310-20.
37. Kaur G, Jalagadugula G, Mao G, Rao AK. RUNX1/core binding factor A2 regulates platelet 12-lipoxygenase gene (ALOX12): studies in human RUNX1 haplodeficiency. Blood. 2010;115(15):3128-35.
38. Jalagadugula G, Mao G, Kaur G, Goldfinger LE, Dhanasekaran DN, Rao AK. Regulation of platelet myosin light chain (MYL9) by RUNX1: implications for thrombocytopenia and platelet dysfunction in RUNX1 haplodeficiency. Blood. 2010;116(26):6037-45.
39. Rayes J, Hollestelle MJ, Legendre P, Marx I, de Groot P, Christophe OD et al. Mutation and ADAMTS13-dependent modulation of disease severity in a mouse model for von Willebrand disease type 2B. Blood. 2010;115(23):4870-7.
40. Necchi V, Balduini A, Noris P, Barozzi S, Sommi P, di Buduo C, et al. Ubiquitin/proteasome-rich particulate cytoplasmic structures (PaCSs) in the platelets and megakaryocytes of ANKRD26-related thrombocytopenia. Thromb Haemost. 2013;109(2):263-71.
41. Ballmeier M, Germeshausen M. Congenital amegakaryocytic thrombocytopenia: clinical presentation, diagnosis, and treatment. Semin Thromb Haemost. 2011;37(6):673-81.
42. Thrasher AJ, Burns SO. WASP: A key immunological multitasker. Nat Rev Immunol. 2010;10(3):182-92.
43. Mahlaoui N, Pellier I, Mignot C, Jais JP, Bilhou-Nabéro C, Moshous D, et al. Characteristics and outcome of early onset, severe forms of Wiskott-Aldrich syndrome. Blood. 20013;121(9):1510-6.
44. Worth AJ, Metelo J, Bouma G, Moulding D, Fritzsche M, Vernay B, et al. Disease-associated missense mutations in the EVH1 domain disrupt intrinsic WASp function causing dysregulated actin dynamics and impaired dendritic cell migration. Blood. 2013;121(1):72-84.
45. Kunicki TJ, Williams SA, Nugent DJ. Genetic variants that affect platelet function. Curr Opin Hematol. 2012;9(5):371-9.
46. Edelstein LC, Luna EJ, Gibson IB, Bray M, Jin Y, Kondkar A, et al. Human genome-wide association and mouse knockout approaches identify platelet supervillin as an inhibitor of thrombus formation under shear stress. Circulation. 2012;125(22):2762-71.
47. Faraday N, Yanek LR, Yang XP, Mathias R, Herrera-Galeano JE, Suktitipat B, et al. Identification of a specific intronic PEAR1 gene variant associated with greater platelet aggregability and protein expression. Blood. 2011;118(12):3367-75.
48. Nürnberg ST, Rendon A, Smethurst PA, Paul DS, Voss K, Thon JN, et al. A GWAS sequence variant for platelet volume marks an alternative DNM3 promoter in megakaryocytes near a MEIS1 binding site. Blood. 2012;120(24):4859-88.
49. Kanaji S, Kuether EL, Fahs SA, Schroeder JA, Ware J, Montgomery RR, et al. Correction of murine Bernard-Soulier syndrome by lentivirus-mediated gene therapy. Mol Ther. 2012;20(3):625-32.
50. Golder M, Pruss CM, Hegadorm C, Mewburn J, Laverty K, Sponagle K, et al. Mutation-specific hemostatic variability in mice expressing common type 2B von Willebrand disease substitutions. Blood. 2010;115(23):4862-9.
51. Noris P, Perrotta S, Seri M, Pecci A, Gnan C, Loffredo G, et al. Mutations in ANKRD26 are responsible for a frequent form of inherited thrombocytopenia: Analysis of 78 patients from 21 families. Blood. 2011;117(24):6673-80.
52. Dumont B, Lasne D, Rothschild C, Bouabdelli M, Olivier V, Oudin C, et al. Absence of collagen-induced platelet activation caused by compound heterozygous GPVI mutations. Blood. 2009;114(9):1900-3.
53. Hermans C, Wittevrongel C, Thys C, Smethurst PA, Van Geet C, Freson K. A. Compound heterozygous mutation in glycoprotein VI in a patient with a bleeding disorder. J Thromb Haemost. 2009;7(8):1356-63.
54. Bender M, Hoffmann S, Stegner D, Chalaris A, Bosi M, Braun A, et al. Differentially regulated GPVI ectodomain shedding by multiple platelet-expressed proteinases. Blood. 2010; 116(17):3347-55.
55. Dawood B, Lowe GC, Lordkipanidzé M, Bern D, Daly ME, Makris M, et al. Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel. Blood. 2012;120(25):5041-9.
56. Cattaneo M. Molecular defects of the platelet P2 receptors. Purinergic Signalling. 2011;7(3):333-9.
57. Hollopeter G, Jantzen H-M, Vincent D, Li G, England L, Ramakrishnan V, et al. Molecular identification of the platelet receptor targeted by antithrombotic drugs. Nature. 2001; 409(6817):202-7.
58. Nisar S, Daly ME, Federici AB, Artoni A, Mumford AD, Watson SP, et al. An intact PDZ motif is essential for correct P2Y12 purinoceptor traffic in human platelets. Blood. 2011;118(20):5641-51.
59. Hirata T, Ushikubi F, Kakizuka A, Okuma M, Narumiya S. Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylate cyclase with different sensitivity to Arg60 to Leu mutation. J Clin Invest. 1996;97(4):949-56.
60. Van Geet C, Izzi B, Labarque V, Freson K. Human platelet pathology related to defects in the G-protein signaling cascade. J Thromb Haemost. 2009;7(1 Suppl):282-6.
61. Noé L, Di Michele M, Giets E, Thys C, Wittevrongel C, De Vos R, et al. Platelet Gs hypofunction and abnormal morphology resulting from a heterozygous RGS2 mutation. J Thromb Haemost. 2010;8(7):1594-603.
62. Izzi B, Francois I, Labarque V, Thys C, Wittevrongel C, Devriendt K, et al. Methylation defect in imprinted genes detected in patients with an Albright's hereditary osteodystrophy-like phenotype and platelet Gs hypofunction. PLoS One.

2012;7(6):e38579.
63. Pionatelli P, Carnevale R, Di Santo S, Bartimoccia S, Sanguigni V, Lenti L, et al. Inherited human gp91phox deficiency is associated with impaired isoprostane formation and platelet dysfunction. Arterioscler Thromb Vasc Biol. 2011; 31(2):423-34.
64. Lasne D, Baujat G, Mirault T, Liunardi J, Grelac F, Egot M, et al. Bleeding disorders in Lowe syndrome patients: evidence for a link between OCRL mutations and primary haemostasis disorders. Br J Haematol. 2010;150(6):685-8.
65. Nurden AT, Nurden P, Sanchez M, Andia I, Anitua E. Platelets and wound healing. Front Biosci. 2008;13:3532-48.
66. Smyth SS, McEver RP, Weyrich AS, Morrell CN, Hoffman MR, Arepally GM, et al. Platelet functions beyond hemostasis. J Thromb Haemost. 2009;7(11):1759-66.
67. Peters CG, Michelson AD, Flaumenhaft R. Granule exocytosis is required for platelet spreading: differential sorting of a-granules expressing VAMP-7. Blood. 2012;120(1):199-206.
68. Thon JN, Peters CG, Machlus KR, Aslam R, Rowley J, Macleod H, et al. T granules in human platelets function in TLR9 organization and signaling. J Cell Biol. 2012;198(4):561-74.
69. Smith H, Galmes R, Gogolina E, Straatman-Iwanowska A, Reay K, Banushi B, et al. Associations among genotype, clinical phenotype, and intracellular localization of trafficking proteins in ARC syndrome. Hum Mutat. 2012;33(12):1656-64.
70. Urban D, Li L, Christensen H, Pluthero FG, Chen SZ, Pulhacz M, et al. The VPS33B-binding protein, VPS16B is required in megakaryocyte and platelet α-granule biogenesis. Blood. 2012;120(25):5032-40.
71. Huizing M, Helip-Wooley A, Westbroek W, Gunay-Aygun M, Gahl WA. Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. Annu Rev Genomics Hum Genet. 2008;9:359-86.
72. Cullinane AR, Curry JA, Carmona-Rivera C, Summers CG, Ciccone C, Cardillo ND, et al. A BLOC-1 mutation screen reveals that PLDN is mutated in Hermansky-Pudlak syndrome type 9. Am J Hum Genet. 2011;88(6):778-87.
73. Meng R, Wang Y, Yao Y, Zhang Z, Harper DC, Heijnen HF, et al. SLC35D3 delivery from megakaryocyte early endosomes is required for platelet dense granule biogenesis and is differentially defective in Hermansky-Pudlak syndrome models. Blood. 2012;120(2);404-14.
74. Cullinane AR, Vilboux T, O'Brien K, Curry JA, Maynard DM, Carlson-Donohoe H, et al. Homozygosity mapping and whole exome sequencing to detect SLC45A2 and G6PC3 mutations in a single patient with oculocutaneous albinism and neutropenia. J Invest Dermatol. 2011;131(10):2017-25.
75. Jones ML, Murden SL, Berm D, Mundell SJ, Gissen P, Daly ME, et al. Rapid genetic diagnosis of heritable platelet function disorders with next-generation sequencing: proof-of-principle with Hermansky-Pudlak syndrome. J Thromb Haemost. 2012;10(2):306-9.
76. Enders A, Zieger B, Schwartz K, Yshimi A, Speckmann C, Knoepfle EM, et al. Lethal hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type II. Blood. 2006;108(1):81-7.
77. Ren Q, Wimmer C, Chicka MC, Ye SE, Ren Y, Hughson FM, et al. Munc 13-4 is a limiting factor in the pathway required for platelet granule release and hemostasis. Blood. 2010;116(6):869-77.
78. Ye S, Karim ZA, Al Hawas R, Pessin JE, Fillipovich AH, Whiteheart SW. Syntaxin-11, but not syntaxin-2 or syntaxin-4, is required for platelet secretion. Blood. 2012;120(12):2484-92.
79. Al Hawas R, Ren Q, Ye S, Karim ZA, Filipovich AH, Whiteheart SW. Munc18b/STXBP2 is required for platelet secretion. Blood. 2012;120:2493-500.
80. Fiore M, Pillois X, Nurden P, Nurden AT, Austerlitz F. Founder effect and estimation of the age of the French gypsy mutation associated with Glanzmann thrombasthenia in Manouche families. Eur J Hum Genet. 2011;19(9):981-7.
81. Mansour W, Einay Y, Hauschner H, Koren A, Seligsohn U, Rosenberg N. An aIIb mutation in patients with Glanzmann thrombasthenia located in the N-terminus of blade 1 of the β-propeller (Asn2Asp) disrupts a calcium binding site in blade 6. J Thromb Haemost. 2011;9(1):192-200.
82. Mor-Cohen R, Rosenberg N, Einav Y, Zelzion E, Landau M, Mansour W, et al. Unique disulfides in epidermal growth factor (EGF) domains of β3 affect structure and function of αIIbβ3 and αIIbβ3 integrins in different manner. J Biol Chem. 2012;287(12):8879-91.
83. Kannan M, Ahmad F, Yadav BK, Kumar R, Choudhry VP, Saxena R. Molecular defects in ITGA2B and ITGB3 genes in patients with Glanzmann thrombasthenia. J Thromb Haemost. 2009;7(11):1878-85.
84. Jallu V, Dusseaux M, Panzer S, Torchet MF, Hezard N, Goudemand J, et al. AlphaIIbbeta3 integrin: new allelic variants in Glanzmann thrombasthenia, effects on ITGA2B and ITGB3 mRNA splicing, expression, and structure function. Hum Mutat. 2010;31(3):237-46.
85. Nurden AT, Mercié P, Zely P, Nurden P. Deep vein thrombosis, Raynaud's phenomenon, and Prinzmetal angina in a patient with Glanzmann thrombasthenia. Case Rep. Hematol 2012;2012:156290.
86. Fang J, Jensen ES, Boudreaux MK, Du LM, Hawkins TB, Koukouritaki SB, et al. Platelet gene therapy improves hemostatic function for integrin αIIbβ3-deficient dogs. Proc Natl Acad Sci USA. 2011;108(23):9583-8.
87. Svensson L, Howarth K, McDowall A, Patzak I, Evans R, Ussar S, et al. Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. Nat Med. 2009;15(3):306-12.
88. Bialkowska K, Ma YQ, Bledzka K, Sossey-Alaoui K, Izem I, Zhang X, et al. The integrin co-activator kindlin-3 is expressed and functional in a non-hematopoietic cell, the endothelial cell. J Biol Chem. 2010;285(24):18640-9.
89. Van de Vijver E, De Cuyper IM, Gerrits AJ, Verhoeven AJ, Seeger K, Gutiérrez L, et al. Defects in Glanzmann thrombasthenia and LAD-III (LAD-1/v) syndrome: the role of integrin β1 and β3 in platelet adhesion to collagen. Blood. 2012;119(2):583-6.
90. Lhermusier T, Chap H, Payrastre B. Platelet membrane phospholipid asymmetry: from the characterization of a scramblase activity to the identification of an essential protein mutated in Scott syndrome. J Thromb Haemost. 2011;9(10):1883-91.
91. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. Nature. 2010;468 (7325):834-8.
92. Yang H, Kim A, David T, Palmer D, Jin T, Tien J, et al. TMEM16F forms a Ca2+-activated cation channel required for lipid scrambling in platelets during blood coagulation. Cell. 2012;151(1):111-22.
93. Martinon-Torres N, Vasquez-Donsion M, Loidi L, Couselo JM. CAMT in a female with developmental delay, facial malformations and central nervous system anomalies. Pediatr Blood Cancer. 2011;56(3):452-3.
94. Lanzi G, Moratto D, Vairo D, et al. A novel primary human immunodeficiency due to deficiency in the WASP-interacting protein WIP. J Exp Med. 2012;209(1):29-34.
95. Galy A, Thrasher AJ. Gene therapy for the Wiskott-Aldrich syndrome. Curr Opin Allergy Clin Immunol. 2011;11(6):545-50.
96. Nurden AT, Viallard JF, Nurden P. New-generation drugs that stimulate platelet production in chronic immune thrombocytopenic purpura. Lancet. 2009;373(9674):1562-9.

Inhibitor development in mild hemophilia A

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A B S T R A C T

The most challenging complication in the treatment of hemophilia is the formation of inhibiting antibodies (inhibitors). In contrast to severe hemophilia A, many patients with mild hemophilia A (MHA) will have a lifelong risk of new inhibitor formation. Inhibitors may change the clinical phenotype dramatically, as the inhibitor frequently cross-reacts with the patient's endogenous FVIII, reducing the endogenous FVIII plasma levels below 0.01 IU/mL. Specific *F8* missense mutations predispose for inhibitor development. Inhibitors are frequently provoked by intensive treatment with therapeutic FVIII concentrates (more than 5 consecutive exposure days). Specific immunological characteristics, associated with the underlying F8 missense mutation, have been demonstrated in experimental studies. The lifelong inhibitor risk in MHA requires lifelong vigilance by the hemophilia team to minimize risk of inhibitor formation and to ensure early detection to optimize subsequent management.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe the morbidity and risk factor for inhibitors in mild hemophilia A;
- describe the immunological characteristics of inhibitors in mild hemophilia A;
- discuss treatment options for mild hemophilia inhibitor patients, both for the treatment of bleeding episodes and for inhibitor eradication.

What is mild hemophilia and how is it treated?

Hemophilia A is an inherited clotting disorder that occurs in 1:4000 men, caused by a deficiency of clotting factor VIII (FVIII), due to molecular genetic defects in the *F8* gene.[1;2] The severity of bleeding symptoms in patients with hemophilia depends on the residual, functional FVIII plasma concentration, which is expressed in international units (IU). The FVIII plasma concentration varies between 0.50 and 1.50 IU/mL in healthy individuals. Patients with hemophilia are classified into three severity groups according to the residual FVIII plasma concentration: severe (<0.01 IU/mL), moderate (0.02-0.05 IU/mL) and mild (0.06-0.40 IU/mL). Patients with severe hemophilia may bleed spontaneously in their joints, muscles or other locations without preceding trauma. Spontaneous bleeding is less likely in moderate hemophilia and increasingly unusual in mild hemophilia. In patients with moderate and mild hemophilia, excessive bleeding rather takes place after minor trauma, dental or surgical procedures.

Bleeding is treated by intravenous administration of FVIII concentrates.[3] FVIII concentrates are dosed in IU/kg body weight; 1.0 IU/kg body weight of factor VIII concentrate increases the plasma concentration by about 0.02 IU/mL. In case of a life threatening bleed, the target plasma concentration is 1.00 IU/mL. This can be achieved by infusion of 50 IU/kg FVIII concentrate. The short half-life of FVIII (6-12 hours) requires repeated administration of FVIII concentrate to maintain plasma concentrations of FVIII above the hemostatic threshold of 0.40-0.50 IU/mL. For joint bleeds, one or two infusions are often enough, whereas intracranial bleeds may need treatment for up to two weeks.

In mild hemophilia A (MHA) small bleeds may be managed by infusion of DDAVP (desmopressin).[4;5] This analog of the physiological pituitary hormone vasopressin increases the FVIII plasma concentration 3-5 fold by releasing FVIII from an unidentified releasable pool and von Willebrand Factor (VWF) from the Weibel-Palade bodies where it is stored in endothelial cells. VWF serves as a carrier protein for FVIII in plasma and protects it from proteolytic degradation.[6] The effect of DDAVP decreases after 2-3 consecutive administrations, due to depletion of storage pools for VWF in the endothelium.

In patients with severe hemophilia, DDAVP cannot be used since there is none or extremely little endogenous FVIII synthesis. Patients with severe hemophilia A need frequent treatment with FVIII concentrates. They receive regular prophylactic treatment to prevent joint bleedings, as recurrent joint bleeds may lead to arthropathy, i.e. painful joint deformation with limited function, especially located in elbows,

knees and ankles.[7] By prophylactic administration of FVIII concentrate 3-4 times per week, FVIII plasma levels are kept above 0.01 IU/mL. This is generally sufficient to prevent spontaneous joint hemorrhage.

Incidence of inhibitors in mild hemophilia

The most challenging complication in the treatment of hemophilia is the formation of inhibiting antibodies (inhibitors) directed against active parts of the FVIII protein. FVIII concentrates are no longer effective in patients with inhibitors, as the administered FVIII is swiftly inactivated (neutralized) by the circulating inhibitors. Bypassing therapies such as recombinant FVIIa (Novoseven®, NovoNordisk) or activated prothrombin complex concentrate (FEIBA®, Baxter) are necessary to control bleeds in these patients, with accompanying clinical challenges and financial expense. The management of bleeding in patients with inhibitors will be discussed further on in this article. The cumulative incidence of inhibitor development is approximately 30% in severe hemophilia A[8] and historically reported at lower incidences of 3%-13% in patients with moderate or mild hemophilia A.[9-12] As inhibitor development is elicited by exposure to therapeutic FVIII concentrates, the risk increases with every new exposure day (ED). Therefore, the incidence of inhibitors in a specific population is dependent on the cumulative number of EDs that these patients have received. From studies in severe hemophilia A, it has become clear that the risk of inhibitor development is highest during the first 10-20 ED and decreases to less than 1% after 50 ED.[13] In contrast, data on MHA inhibitor rates have been derived from cross-sectional studies and do not take the cumulative number of ED into account. Moreover, information about FVIII concentrate exposure in MHA patients is especially important as they receive factor VIII replacement therapy on an irregular, 'on demand' basis and much less frequently than severe hemophilia A patients, as their bleeding phenotypes are milder. Some adult patients with MHA may still have had less than 50 EDs to therapeutic factor VIII and be at risk of developing inhibitors, in contrast to patients with severe hemophilia who generally reach 50 ED within the first years of life.

In order to account for EDs in the analysis of incidence and risk factors for inhibitor development in MHA, the INSIGHT study (International Study on etiology of inhibitors in patients with moderate/mild hemophilia A: influences of Immuno Genetic and Hemophilia Treatment factors) was initiated. This observational study included 2711 moderate and MHA patients (FVIII 0.02-0.40 IU/mL) from 34 hemophilia treatment centers in Europe and Australia who received at least one exposure to factor VIII concentrate between 1980 and 2011. The risk of inhibitor development was calculated adjusting for the number of ED and appeared to be 6.7% (95%CI: 4.5-8.9) at 50ED and further increased to 13.3% (95%CI: 9.6-17.0) at 100 ED, with greater risk for particular genotypes.[14] Thus, in contrast to severe hemophilia A, the risk of inhibitor development in MHA does not seem to decrease below 1% after 50 ED.

Presenting symptoms and morbidity of inhibitors in mild hemophilia

In the first case series of 26 moderate and MHA inhibitor patients described by Hay *et al.*, the median age at inhibitor development was 33 years and inhibitors were detected after a median of 5.5 bleeding episodes.[15] In the INSIGHT cohort, inhibitors developed at a median age of 37 years (interquartile range, IQR, 15-60) after a median of 29 ED (IQR 14-70).[16] An even higher median age at inhibitor development of 66 years was reported in a recent study of 14 MHA inhibitor patients from a single center.[17]

In patients with MHA, inhibitor development may change the clinical phenotype dramatically, as the inhibitor frequently cross-reacts with the patient's endogenous FVIII, reducing the endogenous FVIII plasma levels below 0.01 IU/mL. This occurred in 23 of the 26 MHA inhibitor patients described by Hay and was associated with spontaneous bleeding.[15] The bleeding pattern was similar to acquired hemophilia in 17 patients, often severe and caused deaths in 2 patients due to uncontrollable GI and retroperitoneal hemorrhage. Extensive mucocutaneous bleeding was common, whereas joint bleeding occurred relatively rarely. A reduction in FVIII plasma levels was confirmed in the unselected cohort of MHA inhibitor patients from in the INSIGHT study. In 58 of 101 (57%) inhibitor patients, FVIII plasma level fell below 0.01 IU/mL.[16] In those patients who maintain their endogenous FVIII level despite the presence of an inhibitor, the inhibitor seems to be exclusively directed against the exogenous therapeutic FVIII concentrate.

What causes inhibitors in mild hemophilia?

Risk factors for inhibitor development have been extensively studied in severe hemophilia A and may be environmental or genetic.[18-20] Most studies on risk factors for inhibitors were conducted in severe hemophilia A patients. These studies indicated the following host-related factors to increase the risk of inhibitor development in severe hemophilia A: null mutations and large deletions in the *F8* gene, non-Caucasian ethnicity, positive family history for inhibitor development.[19-24] Treatment-related factors reported to increase the risk of inhibitor development in severe hemophilia A are: intensive exposure (5 or more EDs) to FVIII concentrates at first treatment and surgery combined with an intensive first exposure (> 4 ED).[23;25-27]

Clinical risk factors

Relatively few studies have addressed risk factors for inhibitor development in MHA. Most of our knowledge on risk factors for inhibitor development in patients with MHA is derived from small, uncontrolled observational studies or case series. Inhibitors in patients with MHA frequently arise following a period of intensive treatment when therapeutic FVIII concentrates are given for several consecutive days, e.g. for surgery. In the largest case series of MHA inhibitor patients reported up to now, 16 of 26 inhibitors arose following intensive treatment.[15] In a Canadian study, four of seven inhibitors arose within six weeks of FVIII administration for at least six consecutive

days.[28] In a recent single center cohort study of 14 MHA inhibitor patients, the inhibitor arose in 13 patients following intensive treatment (at least 5 consecutive ED).[17] Similarly, in a Dutch cohort, inhibitor development was preceded by intensive treatment with therapeutic FVIII concentrates in 7 of 10 inhibitor patients.[29] The latter cohort study included 138 MHA patients of whom 41 received perioperative FVIII replacement. Surgery as the reason for first intensive exposure was associated with a 186-fold (95%CI: 25-1403) increased risk of inhibitor development in the three months after the surgical procedure as compared to any other 3-month period during the observation period of the study. This extremely high relative risk is explained by the low exposure to therapeutic factor concentrates during the time period to which the post-operative period of three months was compared. Patients with MHA do not need frequent therapeutic FVIII concentrates and months may pass without any exposure to FVIII concentrate. Thus, the analysis of clinical risk factors in MHA inhibitor development requires a careful methodological approach. The difference between the groups to be compared should be carefully defined (e.g. intensive FVIII treatment for surgery *vs.* FVIII treatment for other reasons), and efforts should be made to compare patients with a similar likelihood of developing inhibitors. Especially the number of previous FVIII exposure days should be as similar as possible. In a case-control study of 36 inhibitor cases and 62 controls, half of the inhibitor cases (18 of 36; 50%) and 18% (11 of 62) of the controls received intensive FVIII treatment (defined as 6 or more consecutive days of FVIII replacement) during the prior year.[30] Intensive treatment in the prior year was only associated with inhibitor development in those 30 years of age or older (OR 12.6; 95%CI: 2.8-57.8), when multivariate analysis was adjusted for less than 50 previous days of FVIII, the p.Arg612Cys genotype, Caucasian ethnicity and baseline FVIII from 0.01 to less than 0.02 IU/mL. The association between surgery and inhibitor development could not be further analyzed because information on surgery was only available in subjects who received intensive treatment. The authors observed an interaction between intensive treatment and age that persisted after adjustment for a cumulative lifetime exposure to FVIII of less than 50 days. Therefore, the authors concluded that the impact of intensive treatment in adults does not appear to be the result of less FVIII exposure prior to adulthood. This seems to contrast with the findings of Mauser-Bunschoten *et al.* who found in the above-mentioned cohort study of 14 MHA inhibitor patients that age at first treatment (43 *vs.* 6.7 years) and age at intensive treatment (53 *vs.* 21 years) were significantly higher in the inhibitor patients than in the patients who did not develop an inhibitor.[17] The interesting observations of these studies should be interpreted with caution, as not all potential confounders may have been addressed. The most important of these is prior exposure to FVIII. In the study by Kempton *et al.,* this was accounted for by classifying patients into two categories: those with and without a cumulative lifetime exposure to FVIII of less than 50 days. However, this classification does not take into account differences that may exist within these groups. Both among patients with fewer than 50 ED and among patients with more than 50 ED there remains substantial variation in the numbers of EDs and consequently the likelihood to develop inhibitors. Ideally, control patients should be matched to the case patients based on their cumulative number of EDs. Without this matching on EDs, the study result may be confounded by differences in exposure histories.

The effect of continuous infusion on inhibitor development has been the subject of intense debate, as inhibitors frequently occur following intensive treatment administered by continuous infusion. In the Canadian study mentioned above, four inhibitors occurred after continuous infusion.[28] In the Dutch cohort study, an adjusted RR of 13 (95%CI: 1.9-86) was calculated for the three months following surgery covered by continuous infusion.[29] However, as stated above, this was inadvertently compared to periods in which exposure to FVIII concentrates may have been very low. In the studies by Kempton and Mauser-Bunschoten, no significant association was found between continuous infusion and inhibitor development.[17;30] A large European cohort study analyzing a total of 1079 continuous infusions given peri-operatively or for major bleeds in 742 patients with severe, moderate or mild hemophilia A, confirmed that the absolute inhibitor risk of continuous infusion is limited, as only 9 patients (1.2%) developed an inhibitor.[31] In a cohort study of 46 consecutive surgical procedures in MHA patients, of whom 57% received continuous infusion, the inhibitor incidence was 4% (95%CI: 0.5-14.8). Both inhibitors that occurred in this study were of low titer (< 5 BU/mL).[32]

Genetic risk factors

Inhibitor development in MHA is associated with a positive family history for inhibitor development, pointing at a genetic predisposition for this. The genetic susceptibility may be largely due to the underlying *F8* genotype. MHA is generally caused by missense mutations of which there are over 500 reported causative mutations on the Haemophilia A database (http://hadb.org.uk/).[33] Missense mutations confer a low risk of inhibitor development in comparison to null mutations or large deletions of the *F8* gene, which are associated with a complete absence of FVIII protein. In patients with missense mutations, the presence of circulating endogenous, albeit aberrant FVIII protein maintains a state of immunological tolerance towards FVIII. In contrast, in patients without any circulating endogenous F VIII, the complete therapeutic FVIII protein that is administered will be seen as 'foreign' by the immune system. However, there are certain missense mutations that predispose to inhibitor development in MHA. An overview of reported associations between specific missense mutations and inhibitor development is available from the Haemophilia A Mutation Structure, Test and Resource Site (HAMSTeRs) database[33] (http://hadb.org.uk/) or on the CDC Hemophilia A Mutation Project (CHAMP) database (http://www.cdc.gov/ncbddd/hemophilia/champs.html).[34] Missense mutations associated with inhibitor development are clustered in the A2 domain and the C1-C2 domains. Mutations in the C1-C2 domains may lead to changes in the 3-dimensional structure of FVIII, influencing binding to VWF and phospholipid membranes. The change in 3-dimensional structure may also affect the antigenic characteristics of the endogenous FVIII protein. Specific missense mutations that are associated with inhibitors in MHA are: p.Arg612Cys, p.Tyr2124Cys, p.Arg2169Cys, p.Trp2248Cys, p.Pro2319Leu.[19] Amino acid numbering

for point mutations is given in the format recommended by the Human Genome Variation Society, HGVS (http://www.hgvs.org), as this format is now recognized as the standard way to report mutations. HGVS universally uses the first A of the initiation ATG codon to start amino acid numbering; however, most original FVIII studies started numbering from the beginning of the mature processed protein. To convert to the classical-type numbering found in the literature, subtract 19 from the HGVS number (e.g. p.Arg612Cys used to be referred to as Arg593Cys).[35] Not only does the position of the missense mutation determine inhibitor risk, but the risk is also influenced by the type of substitution. Amino acids can be divided into 4 classes according to their chemico-physical properties: small/hydrophobic, neutral, acidic and basic. A recent study in 36 inhibitors that occurred in 720 patients with missense mutations (46% mild and 22% moderate hemophilia A) found that the risk of inhibitor formation is significantly higher if the substituted amino acid in FVIII belongs to another physical-chemical class than the original residue.[36]

Immunological characteristics of antibody formation in MHA patients

In patients with severe hemophilia, the characteristics of inhibitory antibodies have been extensively characterized.[37;38] Immuno-dominant B-cell epitopes are located in specific regions of the FVIII protein: the A2 domain, A3 and C2 domain.[39-42] In contrast to patients with severe hemophilia A, the epitopes of inhibitory antibodies in patients with MHA may also be associated with the missense mutation underlying MHA. An early study by Santagostino and co-workers reported a discrepant response to DDAVP and therapeutic FVIII concentrate in 2 MHA patients carrying the p.Arg2169His mutation.[43] Despite the presence of the inhibitor, a rise in endogenous FVIII was observed after DDAVP administration, whereas plasma FVIII levels did not increase in response to infused therapeutic FVIII concentrate. This suggested that anti-FVIII antibodies bound to infused wild-type FVIII, but not to the patient's endogenously synthesized FVIII. Characterization of anti-FVIII antibodies in a patient with the missense mutation p.Arg612Cys, located in the A2 domain, unambiguously demonstrated this phenomenon: anti-FVIII antibodies bound specifically to wild-type recombinant A2 domain, but not to a recombinant A2 domain containing the p.Arg612Cys substitution.[44] Similarly, anti-FVIII antibodies from patients with the p.Arg2169His mutation were shown to bind exclusively to wild-type p.Arg2169-FVIII and not to p.His2169-FVIII.[45;46] Together these findings support the concept that the immunological response to FVIII in MHA is linked to the underlying *F8* missense mutation.

The immunological knowledge on the development of tolerance to endogenous or 'self' proteins provides an explanation for the differential response to wild-type and endogenous FVIII in MHA patients with inhibitors. CD4+ T-cell responses play a central role in the regulation of the immune response through their ability to support the generation of B cells producing high affinity antibodies. The repertoire of circulating T cells is shaped by positive and negative selection in the thymus.[47] Positive selection processes control the propensity of T cells to recognize foreign antigen-derived peptides in the context of MHC class I or II. Negative selection ensures that T cells that bind with high affinity to peptides derived from self-antigens are efficiently eliminated in the thymus. [47] In severe hemophilia A, potential FVIII-reactive CD4+ T cells are not efficiently eliminated due to the absence of FVIII. Exposure to FVIII concentrate may then readily elicit CD4+ T-cell responses, explaining the high frequency of FVIII inhibitors in patients with severe hemophilia A. In contrast, in patients with MHA, the endogenously expressed aberrant FVIII protein is expected to promote elimination of potential FVIII-reactive CD4+ T cells. This tolerance is only established for peptides derived from the endogenously expressed FVIII protein and not for sequence-mismatched wild-type FVIII. Therefore, CD4+ T-cell responses directed towards peptides that do not contain the amino acid substitutions dictated by the underlying missense mutation will provoke an immune response after exposure to infused 'wild-type' FVIII. The proof of this concept was demonstrated in a pioneering study by Jacquemin and co-workers.[48] From an MHA inhibitor patient with the p.Arg2169His substitution, they isolated three FVIII specific CD4+ T-cell clones that were directed towards a synthetic peptide encompassing p.Arg2169. Thus, a CD4+ T-cell response directed towards a single amino acid mismatch with endogenously expressed FVIII can provoke a CD4+ T-cell response in patients with MHA. Subsequent analyses of CD4+ T-cell responses in patients with p.Arg612Cys and p.Ala2220Pro substitutions have confirmed that CD4+ T-cell responses in MHA patients are directed towards wild-type factor VIII-derived peptides containing a single amino acid mismatch with endogenously expressed FVIII (Figure 1).[49-51]

Interestingly, synthetic p.Arg612, p.Arg2169 and p.Ala2220-containing peptides can bind to multiple MHC class II molecules,[48-51] suggesting that inhibitor development in mild hemophilia A is not strictly dependent on HLA class II profile. Indeed, a study in a small group of patients with the p.Arg612Cys mutation did not yield a clear association with a particular MHC class II allele.[52] Future studies using large numbers of patients are needed to further substantiate these findings but our current data suggest that at least for patients with the p.Arg612Cys, p.Arg2169His and p.Ala2220Pro mutations inhibitor formation is unlikely to be strongly linked to HLA class II profile.

Further studies are needed to obtain greater insight into genetic risk factors and immunological mechanisms leading to inhibitor development in MHA. The following critical questions need to be addressed. Which specific *F8* missense mutations predispose to inhibitor development? By what mechanism do patients with these missense mutations confer an increased susceptibility for inhibitor development? Why does a minority of patients develop antibodies that do not cross-react with their endogenous FVIII? Further analyses need to confirm whether restricted CD4+ T-cell responses to mismatched FVIII are a common pathogenic mechanism for inhibitor formation in MHA.

Prevention of inhibitors

Treatment intent in MHA should primarily aim at secur-

ing hemostasis and, secondly, at minimizing risk of inhibitor formation if possible. The majority of treatment episodes are likely to be 'on demand' in response to trauma or for a surgical procedure. In addition to the type of injury or surgery, the personal and family history of the patient should be taken into account when the treatment regimen is decided on. Particular information is needed on previous treatment episodes, follow up and timing of inhibitor screening, and any personal or family history of inhibitor detection. Knowledge of the causative *F8* genotype is of increasing interest to pre-emptively identify those patients with a mutation that carries an increased inhibitor risk. The recent UKHCDO inhibitor guidelines advise clinicians to consult existing databases in order to check if a specific *F8* genotype has been reported to be associated with an inhibitors.[21] Recommended frequency of inhibitor testing is annual in MHA (if they have been exposed to FVIII), after intensive exposure (>5 EDs), or after surgery. Patients with MHA and a *F8* genotype with high inhibitor prevalence and/or a family history of inhibitors should undergo inhibitor testing after all exposures.

In the context of significant trauma or major surgery, particularly involving the head, the decision to treat with FVIII concentrate is uncontroversial in the context of a patient without an inhibitor history, regardless of family history of inhibitors or *F8* genotype. Vigilance is required, however, for a re-emerging (but previously missed) or *de novo* inhibitor, both during the treatment and in a 'convalescent' inhibitor screen a number of weeks post exposure. Prevention of inhibitor development assumes greater significance in the context of a mild injury or minor surgical episode in which hemostatic compromise would be neither life nor limb threatening. As such, treatment with DDAVP should always be considered, with tranexamic acid used as an adjunct (except in the context of urogenital bleeding). By maximizing the availability of endogenous FVIII without exposure to allogeneic therapeutic FVIII concentrates,

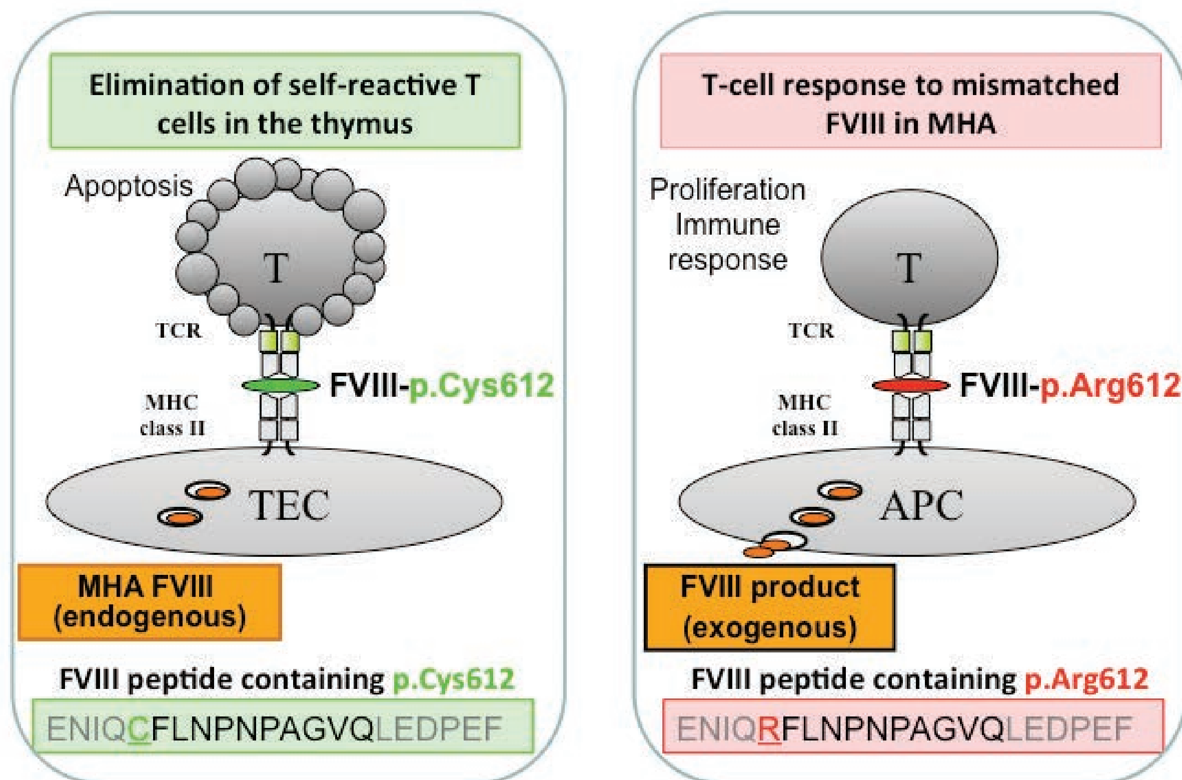

Figure 1. T-cell responses to mismatched FVIII in MHA. (A) T cells that bind with high affinity to peptides derived of self-antigens are efficiently eliminated in the thymus. Peptides derived from endogenously expressed FVIII in patients with MHA are presented on MHC class II on the surface of thymic epithelial cells (TEC). T cells that bind with high affinity to the presented peptides undergo apoptosis and are eliminated from the T-cell repertoire. MHA patients carrying an p.Arg612Cys substitution can present a p.Cys612 containing peptide (indicated in green) on MHC class II. T cells reactive with this peptide are eliminated from the repertoire. (B) Following treatment, peptides derived from the infused exogenous FVIII are presented on the surface of antigen presenting cells (APC). Peptides harboring p.Arg612 (indicated in red) can be recognized by CD4$^+$ T cells that specifically recognize this p.Arg612 containing peptide in the context of MHC class II. Subsequent T-cell proliferation provides the initial event in the development of an immune response. The sequence of the two peptides is shown below the panels. MHC class II binding motifs are depicted in bold. The left peptide is derived from endogenous FVIII and contains an Cys at position 612 (indicated in green). The right peptide is derived from infused therapeutic FVIII and contains an Arg at position 612 (indicated in red). A single mismatch between these peptides results in CD4 T-cell response to the red peptide that originates from infused FVIII. This figure is based on data reported by James *et al.*[49]

DDAVP carries no risk of inhibitor formation. Other hemostatic adjuncts, e.g. fibrin glue, topical tranexamic acid, vasoconstrictive local anesthetic agents and fine bore needles in dental work should also enhance a conservative approach when appropriate, further minimizing FVIII concentrate exposure. In case of musculoskeletal injury, protection, rest, ice, compression and elevation (PRICE) maneuvers should be re-emphasized to the patient as these contribute to optimal recovery, whilst minimizing FVIII concentrate use.

How are bleeds in inhibitor patients treated?

Once inhibitory antibodies are detected, treatment can be challenging depending on the personal circumstances of the patient, inhibitor titer and whether the inhibitor cross-reacts with endogenous FVIII. In general, bleeding episodes in inhibitor patients can be prevented or treated with FVIII bypassing agents, such as recombinant FVIIa (Novoseven®) or activated prothrombin complex concentrate (FEIBA®). A recent randomized cross-over trial in 26 inhibitor patients with severe hemophilia A demonstrated a 61% reduction in hemarthrosis during prophylactic treatment with activated prothrombin complex as compared with 'on demand' therapy.[53]

Detection of an inhibitor within an ongoing treatment episode with hemostatic requirement will either need escalation of FVIII concentrate dosing and frequency to overcome the inhibitory activity in the case of a low titer inhibitor or conversion to conventional bypassing agent treatment for higher titer antibodies. The former strategy risks provoking an anamnestic response and eliciting a cross-reacting antibody that compromises the patient's endogenous baseline FVIII level with a worsening bleeding phenotype that may then persist. For new bleeding episodes in MHA inhibitor patients, bypassing agents are the first choice for significant trauma or major surgery.

More challenging is the situation when hemostatic treatment is needed for significant trauma or major surgery in an individual with a history of an inhibitor that has become undetectable since the last treatment episode. If time allows, the patient should be counseled about the risks of the various therapeutic options. Indeed, if major surgery is truly elective, one such option would be not to proceed with the surgery at all. In an emergency situation, FVIII concentrate may be used for major bleeding for its optimal efficacy during the window of opportunity of no detectable inhibitory activity. In that case, surveillance for re-emergence of the inhibitor should be robust with a low threshold for change to bypassing agents. For mild trauma or minor surgery in MHA inhibitor patients that have circulating endogenous FVIII levels, desmopressin (DDAVP) is an important treatment option. The treatment decision becomes more complex in the aging individual with emergent co-morbidities (e.g. cardiac) and a past history but currently undetectable inhibitor (particularly if there had been cross-reactive anti-FVIII activity). Each scenario that might require hemostatic cover then needs careful assessment of the necessity for treatment at all, the likely intensity of required treatment, the likelihood of more intense treatment being required if immediate treatment is avoided (e.g. dental complications), and the potential risks of either re-challenge with FVIII concentrate, or use of DDAVP or a bypassing agent.

The complexities of treating aging MHA inhibitor patients exemplify the need to maintain regular contact with all individuals with hemophilia, regardless of severity. The annual review is an important opportunity to provide educational reminders that might change behavior to minimize future bleed risk or prevent avoidable surgical interventions e.g. optimizing primary dental care, avoidance of aspirin and non-steroid anti-inflammatory drugs (NSAIDs), counseling adolescents about avoidance of violence/moderation of high-risk activities, cardiovascular risk stratification in the middle-aged and risk reduction strategies including smoking cessation and weight reduction measures.

Inhibitor eradication

In contrast to the recently published International immune tolerance induction (ITI) data for severe hemophilia A,[54] there is a recognized lack of equivalent data to support any evidence-based guidance in MHA.[55;56] In MHA patients, avoidance of FVIII concentrate will often allow the inhibitor titer to decline. However, tolerance is not likely and re-exposure to FVIII concentrates will often result in a return of the inhibitor.

Small ITI case series reporting high-risk MHA patients with severe bleeding suggest traditional ITI regimens used in severe HA are less efficacious in mild HA, with a less than 30% 'success' rate.[15;17;57] In the recently reported, largest single-center experience,[17] only a minority of patients (3 of 14) with inhibitors actually proceeded to attempt ITI eradication, with only one reported success. A single patient was treated with rituximab alone with success, although the authors do not confirm that he was successfully re-challenged with FVIII without anamnesis occurring. Of the remaining 10 patients, 3 died of unrelated causes and the remaining 7 all experienced anamnesis.

Further case reports of rituximab use alone have been reported[58;59] and a literature review[60] suggests higher than expected success rates (12 of 16, 75%) with rituximab either alone or with/after other immunomodulatory therapy. This figure should be treated with caution as there may be reporting bias of successful cases and there is no analysis of the number of cases re-challenged with FVIII to establish true tolerance. However, it does illustrate an important difference between MHA and severe hemophilia A. This was recently supported by the case series of 36 MHA inhibitor patients reported by Kempton.[61] The inhibitor persisted in 11 patients and 8 subjects cleared the inhibitor spontaneously. The other 17 subjects cleared their inhibitor following eradication treatment. Rituximab alone (n=6) and other immunomodulating treatments alone (n=2) were associated with an increased likelihood of inhibitor clearance (adjusted HR 4.4; 95%CI: 1.06-20.03 and 10.21; 95%CI: 1.17-78.28), whereas ITI alone (n=9) was not (adjusted HR 1.35; 95%CI: 0.44-4.07) in a multivariate analysis adjusted for race, age, base-line FVIII and inhibitor peak titer. Unfortunately, it is not clear how many of the patients that were classified as 'inhibitor clearance' withstood a re-challenge with FVIII. Preliminary results from a French-Belgium study suggest that immune tolerance induction could be more effective than no specific treatment or immunomodulating drugs in

preventing further risk of anamnesis.[5]

In the face of a reduced baseline FVIII and severe bleeding phenotype, it is an acceptable and clinically important goal in MHA to reduce the antibody titer more rapidly than might occur naturally, as re-achieving baseline FVIII levels mitigates the bleeding phenotype without recourse to treatment with a bypassing agent or prophylaxis. Importantly, unless re-challenged with FVIII, investigators should avoid denoting cases as 'complete remissions' or 'successful tolerization' as is often the case in the literature. Equally, perceived 'spontaneous disappearance' of antibody should not necessarily be reassuring to the clinician. A high proportion experience anamnesis upon rechallenge[15;17] and, as alluded to, 'spontaneous disappearance' of antibody coincident with an attempt to tolerize an individual, e.g. with immunosuppression, may lead to the potentially false assumption of success or tolerization. Other case reports describe use of other immunosuppressive regimens used in acquired hemophilia A.[62-64]

In conclusion, in contrast to severe hemophilia A, many patients with MHA will have a lifelong risk of new inhibitor formation, with life changing consequences to the patients in whom they occur, adding a substantial risk of morbidity and mortality. This lifelong risk requires lifelong vigilance by the hemophilia team to minimize risk of inhibitor formation and to ensure early detection to optimize subsequent management. Further understanding of the causative missense *F8* mutations and underlying immunological processes will help personalize inhibitor-risk prediction. In high-risk patients who need surgery or intensive treatment, alternative treatment options such as DDAVP or bypassing agents may be considered.

References

1. Fijnvandraat K, Cnossen MH, Leebeek FW, Peters M. Diagnosis and management of haemophilia. Brit Med J. 2012;344:e2707.
2. Berntorp E, Shapiro AD. Modern haemophilia care. Lancet. 2012;379(9824):1447-56.
3. Keeling D, Tait C, Makris M. Guideline on the selection and use of therapeutic products to treat haemophilia and other hereditary bleeding disorders. A United Kingdom Haemophilia Center Doctors' Organisation (UKHCDO) guideline approved by the British Committee for Standards in Haematology. Haemophilia. 2008;14(4):671-84.
4. Franchini M, Favaloro EJ, Lippi G. Mild hemophilia A. J Thromb Haemost. 2010;8(3):421-32.
5. d'Oiron R, Pipe SW, Jacquemin M. Mild/moderate haemophilia A: new insights into molecular mechanisms and inhibitor development. Haemophilia. 2008;14(3 Suppl):138-46.
6. Lenting PJ, van Mourik JA, Mertens K. The life cycle of coagulation factor VIII in view of its structure and function. Blood. 1998;92(11):3983-96.
7. Manco-Johnson MJ, Abshire TC, Shapiro AD, Riske B, Hacker MR, Kilcoyne R, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. N Engl J Med. 2007;357(6):535-44.
8. Gouw SC, van der Bom JG, Ljung R, Escuriola C, Cid AR, Claeyssens-Donadel S, et al. Factor VIII products and inhibitor development in severe hemophilia A. N Engl J Med. 2013;368(3):231-9.
9. McMillan CW, Shapiro SS, Whitehurst D, Hoyer LW, Rao AV, Lazerson J. The natural history of factor VIII:C inhibitors in patients with hemophilia A: a national cooperative study. II. Observations on the initial development of factor VIII:C inhibitors. Blood. 1988;71(2):344-8.
10. Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, et al. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. Lancet. 1992;339(8793):594-8.
11. Addiego J, Kasper C, Abildgaard C, Hilgartner M, Lusher J, Glader B, et al. Frequency of inhibitor development in haemophiliacs treated with low-purity factor VIII. Lancet. 1993;342(8869):462-4.
12. Sultan Y. Prevalence of inhibitors in a population of 3435 hemophilia patients in France. French Hemophilia Study Group. Thromb Haemost. 1992;67(6):600-2.
13. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. Haemophilia. 2003;9(4):418-35.
14. Eckhardt CL, van Velzen AS, Peters M, Peerlinck K, Oldenburg J, Santagostino E, et al. Identification of 18 high risk mutations for inhibitor development in 2,700 non-severe hemophilia A patients. Haemophilia. 2012;18(3 Suppl):96.
15. Hay CR, Ludlam CA, Colvin BT, Hill FG, Preston FE, Wasseem N, et al. Factor VIII inhibitors in mild and moderate-severity haemophilia A. UK Haemophilia Centre Directors Organisation. Thromb Haemost. 1998;79(4):762-6.
16. Van Velzen AS, Eckhardt CL, Peters M, Yee T, Nijziel M, Kruijt C, et al. The Challenging Treatment of Bleeding Episodes in Non-Severe Hemophilia A Patients with Inhibitors. Haemophilia. 2012;18(3 Suppl):96.
17. Mauser-Bunschoten EP, Den Uijl IE, Schutgens RE, Roosendaal G, Fischer K. Risk of inhibitor development in mild haemophilia A increases with age. Haemophilia. 2012;18(2):263-7.
18. Astermark J, Altisent C, Batorova A, Diniz MJ, Gringeri A, Holme PA, et al. Non-genetic risk factors and the development of inhibitors in haemophilia: a comprehensive review and consensus report. Haemophilia. 2010;16(5):747-66.
19. Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factors VIII and IX. Haemophilia. 2006;12(6 Suppl):15-22.
20. Gouw SC, van den Berg HM, Oldenburg J, Astermark J, de Groot PG, Margaglione M, et al. F8 gene mutation type and inhibitor development in patients with severe hemophilia A: systematic review and meta-analysis. Blood. 2012;119(12):2922-34.
21. Collins PW, Chalmers E, Hart DP, Liesner R, Rangarajan S, Talks K, et al. Diagnosis and treatment of factor VIII and IX inhibitors in congenital haemophilia. (4th ed.) Br J Haematol. 2013;160(2):153-70.
22. Viel KR, Ameri A, Abshire TC, Iyer RV, Watts RG, Lutcher C, et al. Inhibitors of factor VIII in black patients with hemophilia. N Engl J Med. 2009;360(16):1618-27.
23. Maclean PS, Richards M, Williams M, Collins P, Liesner R, Keeling DM, et al. Treatment related factors and inhibitor development in children with severe haemophilia A. Haemophilia. 2011;17(2):282-7.
24. Astermark J, Berntorp E, White GC, Kroner BL. The Malmo International Brother Study (MIBS): further support for genetic predisposition to inhibitor development in hemophilia patients. Haemophilia. 2001;7(3):267-72.
25. Gouw SC, van der Bom JG, van den Berg M. Treatment-related risk factors of inhibitor development in previously untreated patients with hemophilia A: the CANAL cohort study. Blood. 2007;109(11):4648-54.
26. Gouw SC, van den Berg HM, le Cessie S, van der Bom JG. Treatment characteristics and the risk of inhibitor development: a multicenter cohort study among previously untreated patients with severe hemophilia A. J Thromb Haemost. 2007;5(7):1383-90.
27. Eckhardt CL, van der Bom JG, van der Naald M, Peters M, Kamphuisen PW, Fijnvandraat K. Surgery and inhibitor development in hemophilia A: a systematic review. J Thromb Haemost. 2011;9(10):1948-58.
28. Sharathkumar A, Lillicrap D, Blanchette VS, Kern M, Leggo J, Stain AM, et al. Intensive exposure to factor VIII is a risk factor for inhibitor development in mild hemophilia A. J Thromb Haemost. 2003;1(6):1228-36.
29. Eckhardt CL, Menke LA, van Ommen CH, van der Lee JH, Geskus RB, Kamphuisen PW, et al. Intensive peri-operative use of factor VIII and the Arg593-->Cys mutation are risk factors for inhibitor development in mild/moderate hemophilia A. J Thromb Haemost. 2009;7(6):930-7.
30. Kempton CL, Soucie JM, Miller CH, Hooper C, Escobar MA, Cohen AJ, et al. In non-severe hemophilia A the risk of inhibitor after intensive factor treatment is greater in older patients: a case-control study. J Thromb Haemost. 2010;8(10):2224-31.
31. Batorova A, Holme P, Gringeri A, Richards M, Hermans C, Altisent C, et al. Continuous infusion in haemophilia: current practice in Europe. Haemophilia. 2012;18(5):753-9.
32. Eckhardt CL, Mauser-Bunschoten EP, Peters M, Leebeek FW, van der Meer FJ, Fijnvandraat K. Inhibitor incidence after intensive FVIII replacement for surgery in mild and moderate

haemophilia A: a prospective national study in the Netherlands. Br J Haematol. 2012;157(6):747-52.
33. Haemophilia A Mutation, Structure, Test and Resource Site. [Internet] Available from: http://hadb.org.uk
34. CDC Hemophilia A Mutation Project. [Internet] Available from: http://www.cdc.gov/ncbddd/hemophilia/champs.html
35. Human Genome Variation Society. [Internet] Available from: http://www.hgvs.org
36. Schwaab R, Pavlova A, Albert T, Caspers M, Oldenburg J. Significance of F8 missense mutations with respect to inhibitor formation. Thromb Haemost. 2013;109(3):464-70.
37. Lollar P. Molecular characterization of the immune response to factor VIII. Vox Sang. 2002;83(1 Suppl):403-8.
38. Pratt KP. Inhibitory antibodies in hemophilia A. Curr Opin Hematol. 2012;19(5):399-405.
39. Healey JF, Lubin IM, Nakai H, Saenko EL, Hoyer LW, Scandella D, et al. Residues 484-508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. J Biol Chem. 1995;270(24):14505-9.
40. Healey JF, Barrow RT, Tamim HM, Lubin IM, Shima M, Scandella D, et al. Residues Glu2181-Val2243 contain a major determinant of the inhibitory epitope in the C2 domain of human factor VIII. Blood. 1998;92(10):3701-9.
41. Fijnvandraat K, Celie PH, Turenhout EA, ten Cate JW, van Mourik JA, Mertens K, et al. A human alloantibody interferes with binding of factor IXa to the factor VIII light chain. Blood. 1998;91(7):2347-52.
42. Zhong D, Saenko EL, Shima M, Felch M, Scandella D. Some human inhibitor antibodies interfere with factor VIII binding to factor IX. Blood. 1998;92(1):136-42.
43. Santagostino E, Gringeri A, Tagliavacca L, Mannucci PM. Inhibitors to factor VIII in a family with mild hemophilia: molecular characterization and response to factor VIII and desmopressin. Thromb Haemost. 1995;74(2):619-21.
44. Fijnvandraat K, Turenhout EA, van den Brink EN, ten Cate JW, van Mourik JA, Peters M, et al. The missense mutation Arg593 --> Cys is related to antibody formation in a patient with mild hemophilia A. Blood. 1997;89(12):4371-7.
45. Peerlinck K, Jacquemin MG, Arnout J, Hoylaerts MF, Gilles JG, Lavend'homme R, et al. Antifactor VIII antibody inhibiting allogeneic but not autologous factor VIII in patients with mild hemophilia A. Blood. 1999;93(7):2267-73.
46. Jacquemin M, Lavend'homme R, Benhida A, Vanzieleghem B, d'Oiron R, Lavergne JM, et al. A novel cause of mild/moderate hemophilia A: mutations scattered in the factor VIII C1 domain reduce factor VIII binding to von Willebrand factor. Blood. 2000;96(3):958-65.
47. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. Annu Rev Immunol. 2003;21:139-76.
48. Jacquemin M, Vantomme V, Buhot C, Lavend'homme R, Burny W, Demotte N, et al. CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. Blood. 2003;101(4):1351-8.
49. James EA, van Haren SD, Ettinger RA, Fijnvandraat K, Liberman JA, Kwok WW, et al. T-cell responses in two unrelated hemophilia A inhibitor subjects include an epitope at the factor VIII R593C missense site. J Thromb Haemost. 2011;9(4):689-99.
50. James EA, Kwok WW, Ettinger RA, Thompson AR, Pratt KP. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. J Thromb Haemost. 2007;5(12):2399-407.
51. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. HLA-DR-restricted T-cell responses to factor VIII epitopes in a mild haemophilia A family with missense substitution A2201P. Haemophilia. 2010;16(102):44-55.
52. Bril WS, MacLean PE, Kaijen PH, van den Brink EN, Lardy NM, Fijnvandraat K, et al. HLA class II genotype and factor VIII inhibitors in mild haemophilia A patients with an Arg593 to Cys mutation. Haemophilia. 2004;10(5):509-14.
53. Leissinger C, Gringeri A, Antmen B, Berntorp E, Biasoli C, Carpenter S, et al. Anti-inhibitor coagulant complex prophylaxis in hemophilia with inhibitors. N Engl J Med. 2011;365(18):1684-92.
54. Hay CR, DiMichele DM. The principal results of the International Immune Tolerance Study: a randomized dose comparison. Blood. 2012;119(6):1335-44.
55. DiMichele D. Immune tolerance therapy for factor VIII inhibitors: moving from empiricism to an evidence-based approach. J Thromb Haemost. 2007;(1 Suppl):143-50.
56. DiMichele DM. Immune tolerance in haemophilia: the long journey to the fork in the road. Br J Haematol. 2012;159 (2):123-34.
57. Giuffrida AC, Genesini S, Franchini M, De GM, Aprili G, Gandini G. Inhibitors in mild/moderate haemophilia A: two case reports and a literature review. Blood Transfus. 2008;6(3):163-8.
58. Carcao M, St Louis J, Poon MC, Grunebaum E, Lacroix S, Stain AM, et al. Rituximab for congenital haemophiliacs with inhibitors: a Canadian experience. Haemophilia. 2006;12(1):7-18.
59. Dunkley S, Kershaw G, Young G, Warburton P, Lindeman R, Matthews S, et al. Rituximab treatment of mild haemophilia A with inhibitors: a proposed treatment protocol. Haemophilia. 2006;12(6):663-7.
60. Franchini M, Mengoli C, Lippi G, Targher G, Montagnana M, Salvagno GL, et al. Immune tolerance with rituximab in congenital haemophilia with inhibitors: a systematic literature review based on individual patients' analysis. Haemophilia. 2008;14(5):903-12.
61. Kempton CL, Allen G, Hord J, Kruse-Jarres R, Pruthi RK, Walsh C, et al. Eradication of factor VIII inhibitors in patients with mild and moderate hemophilia A. Am J Hematol. 2012;87(9):933-6.
62. Holstein K, Marx G, Lentz B, Bokemeyer C, Langer F. Successful eradication of a FVIII inhibitor in a 60-year-old patient with mild haemophilia A using single-agent prednisolone. Hamostaseologie. 2012;32(1 Suppl):S48-S51.
63. Robbins D, Kulkarni R, Gera R, Scott-Emuakpor AB, Bosma K, Penner JA. Successful treatment of high titer inhibitors in mild hemophilia A with avoidance of factor VIII and immunosuppressive therapy. Am J Hematol. 2001;68(3):184-8.
64. Vlot AJ, Wittebol S, Strengers PF, Turenhout EA, Voorberg J, van den Berg HM, et al. Factor VIII inhibitor in a patient with mild haemophilia A and an Asn618-->Ser mutation responsive to immune tolerance induction and cyclophosphamide. Br J Haematol. 2002;117(1):136-40.

Immune tolerance induction: current status

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A B S T R A C T

Factor VIII or IX inhibitors render patients with hemophilia A and B resistant to conventional replacement therapy and therefore cause considerable morbidity and increase treatment cost. All such patients should be considered for some form of immune tolerance (ITI) regimen to attempt inhibitor eradication. Established predictors of successful ITI include severe hemophilia A, peak historical inhibitor titer less than 200 and starting titer less than 10 BU/mL, young age at the start of ITI and minimum delay in starting ITI. Interruption in ITI and infection reduce the chance of achieving tolerance, especially in poor risk patients. Tolerance is defined by restoration of the patient's normal factor VIII pharmacokinetics. Since this will usually be unknown, population norms and high-sensitivity inhibitor assay tests must be used.High-dose ITI is associated with reduced intercurrent bleeding and may achieve tolerance more rapidly compared with low-dose ITI, but has not been shown to have a superior ITI response-rate, except perhaps in poor risk patients. There is no conclusive evidence that the response to ITI is affected by the type of factor VIII concentrate used. Immunosuppression has a very limited place in rescue therapy for patients with severe hemophilia failing to respond to ITI. Immunosuppression may be more helpful as part of an ITI regimen for patients with mild or moderate severity hemophilia A and in hemophilia B, groups that have a very disappointing response to conventional ITI.

Learning goals

At the end of this activity, the participant will be able to:
- identify patients suitable for immune tolerance induction (ITI);
- evaluate the likelihood of successful ITI;
- know how to select the most appropriate regimen of ITI;
- know how to conduct ITI and monitor response to treatment;
- manage the morbidity associated with ITI.

Introduction

Factor VIII or IX inhibitors are now the commonest treatment-related complication of hemophilia. Given the therapeutic limitations of the hemostatic treatment options for patients with persistent inhibitors, and the increased morbidity and treatment cost associated with persistent inhibitors, inhibitor elimination through immune tolerance induction is the ideal solution for many inhibitor patients. Although described first over 30 years ago,[1] and widely practiced since, our knowledge of immune tolerance induction (ITI) is largely derived from case histories, single center case series[2-7] and retrospective registries.[8-11] Commonly used regimens are summarized in Table 1. Due to the rarity of the condition and prohibitive logistical difficulties involved, only a single randomized study of ITI has been published.[12] This International Immune tolerance Study (I-ITI study) was a randomized controlled comparison of high (200 IU/kg/day) and low-dose ITI (50 IU/kg/day). These studies have identified what are probably the most important host factors affecting the outcome of ITI. Treatment factors are still hotly debated however, and the optimal approach to ITI is unresolved,

The conduct and outcome of ITI

Immune tolerance induction for factor VIII inhibitors is generally conducted by the administration of factor VIII regularly in doses ranging from 50 IU/kg three times weekly to 100 IU/kg administered twice daily (Table 1).[2-11] Progress of ITI is monitored by checking the inhibitor titer monthly until the titer has fallen below the level of detection and then checking factor VIII recovery until this has normalized (>66%), and then confirming tolerance by demonstrating a normal factor VIII half-life (>7 h) after a 3-day washout.[12,13-16] An alternative, pragmatic equivalent of a more than 7 h half-life has recently been proposed, which is a measurable FVIII trough level 48 h after administration of standard prophylaxis (20-50 IU/kg).[16] The attraction of such an approach is

that it avoids the need for difficult pharmacokinetic (PK) studies in very small children.

Normal half-life (taken as ≥7h) is considered an indicator of tolerance because, up to now, it has been the most sensitive indicator available. This end point has serious limitations, however, since 7 h is the lower limit of the normal range in a pediatric population rather than the individual's normal half-life, which is generally unknown. Normal factor VIII half-life varies considerably between individuals[17,18] so that a patient considered tolerant with a half-life of 7 h may still have a persistent low-level inhibitor and a normal half-life significantly in excess of 7 h. This suspicion derives support from the ITI study, in which it has been shown that at least 4 patients with a factor VIII half-life of more than 7 h had persisting inhibitors measured using a low titer inhibitor assay with a sensitivity of 0.04 BU/mL (M Dardikh *et al*., submitted manuscript, 2013).[19] Furthermore, patients with a past inhibitor history, but thought to be inhibitor free, use significantly more (P=0.005) factor VIII than patients with no such history implying that low level residual inhibitor activity may commonly persist following ITI.[20] This low level inhibitor activity may be abolished by prolonged ITI or by regular prophylaxis with doses of factor VIII similar to those employed in low-dose ITI.[20] It is not known whether patients with residual low level inhibitor activity are more prone to frank relapse. A proportion of patients will suffer obvious relapse with recurrence of an inhibitor measurable using the Bethesda assay and loss of clinical responsiveness to factor VIII. Estimates of the risk of relapse following ITI vary, depending on the length of follow up and the definitions used. Mariani reported gross relapse following ITI in 15% after 25 years follow up[10] and DiMichele reported 12% relapse after eight months.[11] The I-ITI study reported 8% relapse within 12 months, using a sensitive pharmacokinetic definition of success but none of these patients had gross relapse with loss of responsiveness to factor VIII.[12] Most patients achieve normal PK within 6-12 months though a minority may take 1-3 years or more.[2-4,9-11] The International ITI Study, however, found that, in good risk patients, the median time on ITI in the low-dose arm was 16.4 months and in the high-dose arm 14.2 months.[12] Response rates to ITI have been variously reported at 50-90% and depend on patient selection, the end points used, and whether an intention to treat analysis is used.[2-12] The use of intenion to treat analysis will result in lower reported response rates will tend to have lower response rates because they include patients who stopped early for logistical reasons and could be judged not to have had a fair trial of therapy. The omission of such patients from many reports amounts to reporting bias. Although not immunological failures, these patients are therapeutic failures and should always be included. ITI is demanding and may be discontinued because of line infection, loss of venous access, lack of commitment, and a host of reasons not directly related to poor clinical response. The immunological response is determined by a number of host and treatment factors and these are described below.

Host factors and the outcome of ITI

Factors that potentially affect the outcome of ITI are listed in Table 2. The starting inhibitor titer is the most powerful predictor of ITI success.[10,11,21,22] These include: patient age at the start of ITI, peak historical inhibitor titer, titre at the start of ITI and peak titer after starting ITI, ethnic origin, and factor VIII genotype.[23]

Inhibitor titer

Both the North American and International Immune Tolerance registries identified a starting inhibitor titer less than 10 Bu/mL as the most powerful predictor of successful ITI.[11,12] Peak historical titer, reflecting the strength of the secondary immune response to factor VIII, also correlated with success, though less powerfully.[10,11] Based on these data and a meta-analysis of the two registries, good risk patients are commonly defined as having an inhibitor titer of more than 10 BU/mL at the start of ITI and a historic peak titer of less than 200 BU/mL.[12,20,24] The peak

Table 1. Commonly used ITI protocols.

Protocol	Terapeutic regimen
Bonn[1,4]	Phase 1: VIII:C 100 IU/Kg twice daily. FEIBA 100 IU/Kg twice daily. Until the inhibitor is undetectable by the Bethesda method. Phase 2: VIII:C 100 IU/kg twice daily. Tail off over 3 months when VIII half-life is normal.
Van Creveld[2]	VIIIC 25-50 IU/kg twice daily for 1-2 weeks. VIIIC 25-50 IU/kg every 2nd day or 3 times/week until tolerant.
Other variants[7,10,11]	VIIIC 100-200 IU/kg once daily is commonly used.
Malmö[5]	Neutralizing continuous infusion of VIII:C to maintain 0.3 IU/mL VIIIC for 10-14 days. Cyclophosphamide 12-15 mg/kg iv on Days 1 and 2. Cyclophosphamide 2-3 mg/kg orally on Days 3-10. IV immunoglobulin 2.5-5g on Day 1 and 0.4 g/kg/day on days 4 and 5. Protein A adsorption if the inhibitor titer is >10 BU/mL before the start of treatment to reduce to <10 BU/mL.

Table 2. Determinants of successful ITI.

Host factors	Treatment factors
Historical peak inhibitor titer.	>5 years delay starting ITI
Inhibitor titer at start of ITI.	Interruption of ITI
Peak inhibitor titer on ITI.	Dose of VIII/IX
Age at start of ITI.	CVAD infection
Hemophilia A or B.	Type of concentrate?
Hemophilia A severity.	
Ethnicity?	
Factor VIII genotype?	

inhibitor titer after starting immune tolerance correlated more strongly than historical titer both in data from the North American Immune Tolerance registry and the International Immune Tolerance Study (I-ITI study).[11,12] Indeed, patients who are super-high responders (inhibitor rises rapidly to >500 BU/mL after starting ITI) usually have a poor outcome.[11,12] It has, therefore, been argued that ITI can be abandoned in such patients after 6-9 months and rescue therapy considered unless there is evidence of a significant ongoing decline in inhibitor titer.[13,16]

Ethnic origin, factor VIII genotype and patient age

Although ethnic origin has a very significant influence on the risk of developing an inhibitor, no convincing evidence has so far emerged that it influences the outcome of ITI. Attempts to investigate this have been hampered by low subject numbers and so the possibility that ethnicity may affect the outcome of ITI cannot be excluded.[10,12,23,24]

The effect of factor VIII genotype on the outcome of ITI has been investigated in the PROFIT study.[25] A higher success rate of 81% was observed amongst 16 of 86 high responders with low inhibitor risk mutations when compared with the 47% success rate observed in the 70 of 86 subjects with a high inhibitor risk mutation (P=0.01).

Older patients are less successfully tolerized.[2,3,10,11,26] This is probably partly because delay in starting ITI has an adverse effect on the outcome of ITI,[2,3,11] but is also because inhibitors arising later in life may be more difficult to tolerize, however quickly ITI is started.[2,26] For most patients presenting with inhibitors early in life, this is no longer a practical problem. The universal use of prophylaxis causes inhibitors to present very early in life and ITI is generally considered immediately. Even after deferring the start of ITI until the inhibitor titer had fallen below 10 BU/mL, the average age at which ITI started in the I-ITI study was 24 months.[12] For that reason, we were unable to demonstrate an effect of age on outcome in the very restricted age group recruited to the I-ITI study.[12]

Treatment factors and the outcome of ITI

Immediate or deferred start to ITI?

Opinion is divided on whether ITI should start as soon as the inhibitor is detected or whether it should be deferred until the titer has fallen below 10 BU/mL. Many clinicians start immediately because they argue that this avoids a further anamnestic increase in inhibitor and minimizes the risk of morbidity whilst waiting to start ITI. Anamnesis can be avoided, however, by the use of recombinant activator factor VII (NovoSeven, Novo Nordisk, Denmark) to treat bleeding whilst waiting to start ITI.[13-16] They also argue that a delayed start may adversely affect the outcome of ITI. This seems unlikely, however, since the response to ITI did not decline for five years from the time of diagnosis in the NAITR[11] and it took a median of only five months from inhibitor diagnosis for the titer to decline to less than 10 BU/mL in the I-ITI study.[12]

However, a starting titer less than 10 BU is the most powerful predictor of a good outcome and deliberately deferring the start of ITI until the titer has fallen to this level may improve the outcome. Certainly, case-series where ITI was deferred until the inhibitor has fallen below 10 BU/mL report very high success-rates[2,6,7] and ITI does not usually have to be deferred for very long. This hypothesis has not been tested in a clinical trial, however, and so, although deferring the start of ITI is recommended by some, there is no consensus.[13,16] Inhibitors that fail to fall to less than 10 BU/mL over 12-24 months have usually been observed to respond poorly to ITI.

The ITI regimen

The choice of ITI regimen remains problematic. Common regimens are summarized in Table 1.

Patients with very low titer inhibitors peaking at less than 5 BU/mL are usually successfully tolerized using low-dose regimens (50 IU/kg three times weekly.[16,27] Even very low titer inhibitors should be eliminated by ITI, if possible, since they may prevent effective prophylaxis and be associated with increased morbidity and treatment cost even if the patient remains responsive to factor VIII to some degree.

At the other extreme, the IITR and NAITR and various case-series suggest that poor-risk patients (peak titer >200, starting titer >10 BU/mL) are best tolerized using a high-dose regimen.[3,4,10,11,21,22,28] High level high-responders have a lower overall response rate but appear to respond better to high-dose ITI using doses of 100-200 IU/kg/day.

Meta-analysis of these registries suggested, and the I-ITI study showed, that high-dose and low-dose (50 IU/Kg 3 times/week) regimens are equally effective in inducing tolerance in good risk patients.[2,10-12,28] By implication, therefore, 200 IU/Kg/day and the commonly used 100 IU/kg/day can assumed to be equally efficacious for inducing tolerance in good risk patients.[12,15]

Low-dose ITI (50 IU/kg three times/week) takes longer to achieve a negative Bethesda titer[11,12] and slightly longer overall to achieve tolerance than high-dose regimens (200 IU/kg/day). Low-dose ITI was also associated with more intercurrent bleeding than high-dose ITI (Odds Ratio 2.2; P=0.0019). Eighty-five percent of bleeds occurred in the early phase of ITI, before the Bethesda titer became negative. There were 72 hospitalizations for bleeding in the low-dose arm and 39 in the high-dose arm. Almost one-third of subjects remained bleed-free throughout ITI (8 of 58 low-dose and 21 of 57 high-dose).[12] There was a suggestion that low-dose patients may have developed more arthropathy as a consequence of their relative excess of intercurrent bleeding on ITI, since their bleed rate was still greater than high-dose patients even after they had become tolerant and when patients in both arms were using the same regimen of prophylaxis.[12]

Developing countries with limited resources have derived reassurance that low-dose and high-dose ITI appear to be equally effective in inducing tolerance and have adopted low-dose therapy. Where resources are less limited, clinicians have tended to opt for high-dose ITI to minimize intercurrent bleeding. Although high-dose ITI is associated with less morbidity, the difference is not dramatic and high-dose may not be very cost-efficient. A preliminary pharmaco-economic analysis of the I-ITI study shows a median of 6 bleeds in high-dose *versus*12 in low-dose patients and estimated a cost of more than £100,000 per averted bleed using the high-dose regimen. Overall, high-dose ITI costs four times as much per ITI success than low-dose ITI. The bleeding phenotype varies between

patients, with bleed-free patients in both treatment arms. Furthermore, 85% of bleeding occurred in the first phase of ITI, before the Bethesda titer became negative. Perhaps the optimal regimen is that tailored to the patient and the natural history of ITI itself.

The choice of FVIII

Uncontrolled data have been used to suggest that tolerance may be more readily achieved using low-purity pdFVIII than with rFVIII.[22,29,30] Kreuz observed that 6 patients regarded as failing to respond to ITI using rFVIII responded when changed to intermediate-purity plasma derived concentrate rich in von Willebrand factor.[22] This group also reported a far lower (29%) response to high-purity factor VIII concentrate when compared with the 91% success rate observed in a historical group treated with intermediate purity plasma derived factor VIII. Gringeri also reported a surprisingly good response-rate amongst a series of poor risk patients treated with von Willebrand containing concentrate.[30] Neither the IITR nor the NAITR could corroborate this observation, due to the skewed distribution of products used in the reported patients,[10,11] though data from the NAITR appeared to show greater success using monoclonally purified factor VIII than observed with rFVIII.[31] Other case series using a variety of products report much higher ITI success-rates amongst patients using high purity products than those reported by the Frankfurt group and success rates for ITI apparently unaffected by product type.[2,3,6,7,31-33] Although the I-ITI study showed no difference in ITI success rate between patients using plasma-derived or recombinant factor VIII, this study was underpowered for this comparison and most patients using pdFVIII were also using high purity products.[12] A randomized comparison of the efficacy of high-dose pdFVIII or rFVIII for ITI in poor risk patients is in progress.[34] However, product choice was left to the discretion of the managing clinician in the I-ITI study and 102 of 115 randomized patients used recombinant products.[12] Clearly, the current consensus is that first-line ITI should be conducted using rFVIII usually with the product used by the patient at the time of inhibitor development, unless as part of a clinical trial.

Venous access

A central venous access device (CVAD) is commonly inserted to facilitate ITI though the frequency with which CVADs are used varies from country to country. Some authorities attempt to avoid the use of CVADs during ITI to minimize the risk of infection. CVAD infection is significantly more common in inhibitor than non-inhibitor patients (RR3.5; P=0.00).[35] Infection has been observed to have a very marked adverse affect on the outcome of ITI, especially in poor risk patients. The I-ITI study observed that, in good risk patients, infection or CVAD placement had no effect on either the proportion achieving tolerance or the time taken to achieve tolerance.[12] Implantable CVADs are significantly less likely to become infected during ITI than external lines such as Hickman or Broviac catheter.[12,35] Careful attention to the details of optimal line management are important to minimize the risk of infection.[36] An alternative approach to facilitate venous access, which is largely free from the risk of infection, is the surgical creation of an arteriovenous fistula.[37] Very careful attention to surgical detail and deliberate restriction of the flow rate through the fistula is required, however, if increased growth of the limb used for the fistula is to be avoided.[37]

Dose adjustment during ITI

Most published ITI regimens, with occasional exceptions,[7] maintain the same dose of FVIII until the patient is considered tolerant. Dose tailoring, however, has been used on an empirical basis by some clinicians, and the pattern of response and morbidity in the I-ITI study suggests a dose tailoring regimen that may be suitable for further study. High-dose ITI is associated with a statistically significant reduction in bleeding only in the early phase of ITI, following which factor VIII dose could be reduced without seriously affecting the rate of intercurrent bleeding.[12] Furthermore; although high-dose patients achieve a negative Bethesda titer three times faster than low-dose, the time taken to achieve the subsequent milestones of normal recovery and half-life were similar.[12] This implies that it may be possible, having started with high-dose ITI, to reduce the dose of FVIII during the course of ITI without affecting the time taken to achieve tolerance. This may be associated with a risk that some patients may lose ground when the dose is reduced. This risk may be higher in poor risk patients, since these patients are more dependent on high-dose therapy than good risk patients to achieve successful ITI. It is planned to investigate this approach further in the UK.[16]

It is important to avoid interruption to ITI because interruptions of a few weeks to several months markedly reduce the likelihood of successful ITI.[9]

Inadequate response to ITI, failed first-line therapy and relapse

If first-line therapy is considered not to be effective or to have failed, the strategy should be reviewed without interrupting ITI. Options to be considered include abandoning ITI, increasing the dose of FVIII to 200 IU/kg/day, changing to a pdFVIII of high VWF content, adding immunosuppression or both. pdFVIII with a high VWF content has been associated with anecdotal reports of success and the risks associated with the presence of a long-term inhibitor are likely to outweigh the very small potential risk of transfusion-transmitted disease. Rituximab has also been used in patients who have failed conventional ITI, but with mixed responses.[38-40] A consecutive cohort of 15 patients treated with rituximab as rescue therapy demonstrated that it probably can be used in combination with standard ITI regimens. Few (14%) achieved a complete and stable remission with rituximab alone but 58% of those treated with rituximab combined with FVIII obtained at least a stable partial response.[40] A systematic review from Franchini reported similar findings and durable remission in 53% of cases.[41]

Inhibitor eradication in mild hemophilia

About 25% of factor VIII inhibitors are reported from kindreds with mild hemophilia, often with high-risk-mutations.[26] The appearance of an inhibitor in mild hemophilia

is usually accompanied by a severe decline in endogenous factor VIII level and the development of a pattern of soft tissue bleeding similar to that observed in acquired hemophilia. These inhibitors disappeared in approximately 50% of patients after a median nine months. If factor VIII therapy can be avoided, though, they often return when the patient is challenged again. The distinctive bleeding pattern and the poor, 25% response reported to conventional approaches to ITI[26] has encouraged a number of investigators to attempt ITI using rituximab or other immunosuppressive agents alone or in combination with factor VIII.[41-43] This appears to be successful in at least 50% of such patients, although the number of subjects is small and the response rate was, in some cases, no different from the spontaneous disappearance time, making an accurate evaluation of the efficacy of this approach impossible. Masterly inactivity may still be the first-line approach to inhibitors in mild hemophilia, unless recurrent spontaneous bleeding makes this impossible.

Immune tolerance induction in hemophilia B

Conventional ITI using regimens analogous to those used in severe hemophilia A are notably unsuccessful in hemophilia B, particularly in those patients whose inhibitors present with severe reactions to factor IX. The NAITR reported 31% success (5 of 16) success using a median dose of 100 U/kg/day.[11] There were insufficient data to differentiate outcome between differing dose regimens, but patients with an allergic phenotype and a family history of inhibitors had a poorer outcome.[11,14] ITI in hemophilia B may also be complicated by anaphylaxis and, sometimes irreversible, nephrotic syndrome.[11,44-46]

Successful ITI using the Malmo protocol has been reported in 6 of 7 patients, although 2 required two or more ITI courses and one relapsed after six months.[5] The Malmo ITI regimen has been used historically for both factor VIII and IX inhibitors, and involves intensive factor VIII or IX infusion to maintain a circulating level of 0.3 IU/mL combined with immunosuppression using cyclophosphamide, high-dose immunoglobulin and extracorporeal immunoadsorption (for details see Table 1).[5] Although very intensive, the regimen had the advantage that when it was successful, tolerance was achieved within as little as three weeks. It has largely fallen into disuse, however, because the overall success rate was relatively low (62.5%) in hemophilia A and there was a growing reluctance to administer cyclophosphamide to small children. A number of individual case reports offer further support for a role for immunosuppression as a component of the ITI regimen used in patients with hemophilia B. Success was reported in a patient with an allergic phenotype using initial desensitization with steroids, intravenous immunoglobulin and escalating doses of FIX, followed by the Malmo regimen.[5,47] Others have reported successful desensitization to FIX using a combination of rituximab and factor XI infusions as part of ITI.[48,49] Mycophenolate combined with dexamethasone, intravenous immunoglobulin and high-dose FIX has also been used in a few patients with success[50] and rituximab has been used as part of the treatment regimen with variable outcomes in single case reports or small series.[38,48,49,51-53]

Conclusion

Progress in the practice of ITI has been hampered by the small number of patients available for study and the difficulty in conducting well designed clinical trials. Nevertheless the principle determinants of successful ITI are reasonably well understood, though further investigation of the effect of ethnicity and factor VIII genotype are required.

All patients with factor VIII/IX inhibitors should be considered for ITI although the family's ability to comply with this demanding treatment and the likelihood of a successful outcome and morbidity should all be evaluated before a final decision is taken. The relative merits of high- and low-dose ITI appear reasonably well established and clinicians will choose one or the other based on the patient's inhibitor titer, age, an evaluation of the importance of intercurrent bleeding, and the resources available to them.

The optimal approach to patients with severe hemophilia who are resistant to ITI has not been established. Von Willebrand containing concentrates may have a part to play here, though the evidence for this is not strong and the combination of high-dose ITI with immunosuppression with rituximab appears more promising. Similarly, there is growing, though fragmentary evidence that immunosuppression is an important modality of ITI in mild hemophilia and hemophilia B, improving the dismal success rates observed for conventional ITI in those conditions.

References

1. Brackmann HH, Gormsen J. Massive factor VIII infusion in haemophiliac with factor VIII inhibitor, high responder. Lancet. 1977;2:933.
2. Mauser-Bunschoten E.P, Niewenhuis HK, Roosendaal G, van den Berg HM. Low-dose immune tolerance induction in haemophilia A patients with inhibitors. Blood 1995;86:983-8.
3. Kreuz W, Ehrenforth, S, Funk M, Auerswald D, Mentzer J, Joseph-Steiner J, et al. Immune tolerance therapy in paediatric haemophiliacs with factor VIII inhibitors: 14 years follow-up. Haemophilia 1995;1:24-32.
4. Brackmann HH, Oldenburg J, Swaab R. Immune tolerance for the treatment of factor VIII inhibitors - twenty years of the Bonn protocol. Vox Sanguinis. 1996;70:30-5.
5. Freiburghaus C, Berntorp E, Ekman M, Gunnarsson M, Kjellberg B, Nilsson IM. Tolerance induction using the Malmö treatment model 1982-1995. Haemophilia 1999;5:32-9.
6. Rocino A, de Biasi R. Successful immune tolerance treatment with monoclonal or recombinant factor VIII concentrates in high responding inhibitor patients. Vox Sanguinis. 1999; 81:35-8.
7. Smith MP, Spence KJ, Waters EL, Berresford-Webb R, Mitchell MJ, Cuttler J, et al. Immune tolerance therapy for haemophilia A patients with acquired factor VIII antibodies: comprehensive analysis of experience at a single institution. Thrombosis and Haemostasis. 1999;81:35-8.
8. Kroner BL. Comparison of the international immune tolerance registry and the North American Immune tolerance registry. Vox Sanguinis. 1999;77:33-7.
9. Lenk H. The German National Immune Tolerance Registry 1997 update. Vox Sanguinis. 1999;77(Suppl 1):28-30.
10. Mariani G, Ghirardini A, Belloco R. Immunetolerance in hemophilia. Principal results from the international registry. Thrombosis and Haemostasis. 1994;72:155-8.
11. DiMichele DM, Kroner B; and the North American Immune Tolerance Study Group. The North American Immune Tolerance registry: practices, outcomes, outcome predictors.

Thrombosis and Haemostasis. 2002;87:52-7.
12. Hay CRM, DiMichele DM; the ITI Study Group. The principal Results of the International Immune Tolerance Study. Blood. 2012;119(6):1335-44.
13. Hay CRM, Brown S, Collins PW, Keeling DM, Liesner R. The diagnosis and management of factor VIII and IX inhibitors: a guideline from the UKHCDO. Brit J Haematol. 2006;133: 591-605.
14. DiMichele DM, Hoots WK, Pipe SW, Rivar GE, Santagostino E. International Workshop on immune tolerance induction: consensus recommendations. Haemophilia. 2007;13 (Suppl 1):1-22.
15. Astermark J, Morado M, Rocino A, Vandenberg HM, von Depka M, Gringeri A, et al. Current European practice in immune tolerance induction: consensus recommendations. Haemophilia. 2006;12:363-71.
16. Collins P, Liesner Ri, Chalmers E, Hart DP, Liesner R, Rangarajan S, et al. Guidelines on the diagnosis and management of factor VIII and IX inhibitors 4th ed. Brit J Haematol. 2013;160:153-70.
17. Björkman S, Oh M, Spotts G, Schroth P, Fritsch S, Ewenstein BM, et al. Population pharmacokinetics of recombinant factor VIII: the relationships of pharmacokinetics to age and body weight. Blood. 2012;119:612-8.
18. Blanchette VS, Shapiro AD, Liesner RJ, Hernández Navarro F, Warrier I, Schroth PC, et al; rAHF-PFM Clinical Study Group. Plasma and albumin-free recombinant factor VIII: pharmacokinetics, efficacy and safety in previously treated pediatric patients. J Thromb Haemost. 2008;6:1319-26.
19. Dardikh M, Albert T, Masereeuw R, Oldenburg J, Novakova I, van Heerde WL, et al. Low-titre inhibitors, undetectable by the Nijmegen assay, reduce factor VIII half-life after immune tolerance induction. J Thromb Haemost. 2012;10(4):706-8.
20. Hay CRM. UKHCDO Annual Report 2012 and Bleeding Disorder Statistics for the financial year 2011/12. UKHCDO 2012. ISBN 978-1-901787-16-0. Available from: WWW.UKHCDO.org
21. Mariani G, Kroner B. Immune tolerance in hemophilia with factor VIII inhibitors: predictors of success. Haematologica. 2001;86:1186-993.
22. Kreuz, W, Mentzer D, Auerswald G, Becker S. Joseph-Steiner J. Successful immunetolerance therapy of FVIII inhibitor in children after changing from high to intermediate purity FVIII concentrate. Haemophilia. 1996;2(Supple 1):19.
23. DiMichele DM. Immune tolerance induction in haemophilia: evidence and the way forward. J Thrombosis and Haemostasis. 2011;9(Suppl 1):216-25.
24. Callaghan MU, Rajpurkar M, Chitlur M, Warrier I, Lusher J. Immune Tolerance in 31 children with haemophilia a: is ITI less successful in African Americans? Haemophilia. 2011;17:483-9.
25. Coppola A, Margaglione M, Santagostino E, Rocino A, Grandone E, Mannucci PM, et al. Factor VIII gene mutations as predictors of outcome in immune tolerance induction of haemophilia A patients with high responding inhibitors. J Thrombosis and Haemostasis. 2009;7:1809-15.
26. Hay CRM, Ludlam CA, Colvin BT, Hill FGH, Preston FE; for the UKHCDO Inhibitor Working Party. Factor VIII inhibitors in mild and moderate-severity haemophilia A. Thrombosis and Haemostasis. 1998;79:762-6.
27. Ter Avest PC, Fischer K, Gouw SC, Van Dijk K, Mauser-Bunschoten EP. Successful low dose immune tolerance induction in severe haemophilia A with inhibitors below 40 Bethesda Units. Haemophilia. 2010;16:71-9.
28. Kroner BL. Comparison of the International Immune Tolerance Registry and the North American Immune Tolerance Registry. Vox Sang. 1999;77(Suppl.1):33-37.
29. Ettinhausen CE, Kreuz W. Role of von Willebrand factor in immune tolerance induction. Blood Coagulation Fibrinolysis. 2005;16:27-31.
30. Gringeri A, Musso R, Mazzucconi MG, Piseddu G, Schiavoni M, Pignoloni P, et al; RITS-FITNHES Study Group. Immune tolerance induction with a high purity von Willebrand factor VIII complex concentrate in haemophilia A patients with inhibitors at high risk of poor response. Haemophilia. 2007;13:373-9.
31. DiMichele DM, Kroner B. Higher immune tolerance success rate with monoclonal FVIII (mFVIII) when compared with recombinant (rFVIII): an analysis of the North American Immune Tolerance Registry (NAITR). Blood. 2000;96: 266a(Abstract).
32. Franchini M, Lippi G. Von Willebrand factor factor VIII concentrates and inhibitors in haemophilia A: a critical literature review. Thrombosis and Haemostasis. 2010;104:931-40.
33. Batlle J, Lopez MF, Brackmann HH. Induction of immune tolerance with recombinant factor VIII in haemophilia A patients with inhibitors. Haemophilia. 1999;5:31-5.
34. Gringeri A. VWF/FVIII concentrates in high-risk immunotolerance: the RESIST Study. Haemophilia. 2007;13(Suppl 5):73-7.
35. Valentino LA, Ewenstein B, Navickis RJ, Wilkes MM. Central venous access devices in haemophilia. Haemophilia. 2004;10(2):134-46.
36. Ewenstein BM, Valentino LA, Journeycake JM, Tarantino MD, Shapiro AD, Blanchette VS, et al. Conmsensus recommendations for the use of central venous access devices in hemophilia. Haemophilia. 2004;10:629-48.
37. Mancuso ME, Berardinelli L. Arteriovenous fistula as stable venous access in children with severe haemophilia. Haemophilia. 2010;16(Suppl 1):25-8.
38. Mathias M, Khair K, Hann I, Liesner R. Rituximab in the treatment of alloimmune factor VIII and IX antibodies in two children with severe haemophilia. Br J Haematol. 2004;125 (3):366-8.
39. Carcao M, St Louis J, Poon MC, Grunebaum E, Lacroix S, Stain AM, et al; Inhibitor Subcommittee of Association of Hemophilia Clinic Directors of Canada. Rituximab for congenital haemophiliacs with inhibitors: a Canadian experience. Haemophilia. 2006;12:7-18.
40. Collins PW, Mathias M, Hanley J, Keeling D, Keenan R, Laffan M, et al; UK Haemophilia Centre Doctors' Organisation. Rituximab and immune tolerance in severe hemophilia A: a consecutive national cohort. J Thromb Haemost. 2009;7:787-94.
41. Franchini M, Mengoli C, Lippi G, Targher G, Montagnana M, Salvagno GL, et al. Immune tolerance with Rituximab in congenital haemophilia with inhibitors: a systematic review based on individual patients analysis. Haemophilia. 2008;14(5):903-12.
42. Dunkley S, Kershaw G, Young G, Warburton P, Lindeman R, Mathews S, et al. Rituximab treatment of mild haemophilia A with inhibitors: a proposed treatment protocol. Haemophilia. 2006;12(6):663-7.
43. Kempton CL, Allen G, Hord J, Krus-Jarres R, Pruthi RK, Walsh C, et al. Am J Hematol. 2012;87(9):933-6.
44. Ewenstein BM, Takemoto C, Warrier I, Lusher J, Saidi P, Eisele J, et al. Nephrotic syndrome as a complication of immune tolerance in hemophilia B. Blood. 1997;89;1115-6.
45. Warrier I, Ewenstein BM, Koerper MA, Shapiro A, Key N, DiMichele D, et al. Factor IX inhibitors and anaphylaxis in hemophilia B. J Pediatr Hematol Oncol. 1997;19:23-7.
46. Warrier I, Lusher JM. Development of anaphylactic shock in haemophilia B patients with inhibitors. Blood Coagul Fibrinolysis. 1998;9(Suppl 1):S125-8.
47. Curry NS, Misbah SA, Giangrande PL, Keeling DM. Achievement of immune tolerance in a patient with haemophilia B and inhibitory antibodies, complicated by an anaphylactoid reaction. Haemophilia. 2007;13:328-30.
48. Alexander S, Hopewell S, Hunter S, Chouksey A. Rituximab and desensitization for a patient with severe factor IX deficiency, inhibitors, and history of anaphylaxis. J Pediatr Hematol Oncol. 2008;30:93-5.
49. Chuansumrit A, Moonsup Y, Sirachainan N, Benjaponpitak S, Suebsangad A, Wongwerawattanakoon P. The use of rituximab as an adjuvant for immune tolerance therapy in a hemophilia B boy with inhibitor and anaphylaxis to factor IX concentrate. Blood Coagulation Fibrinolysis. 2008;19:208-11.
50. Klarmann D, Martinez Saguer I, Funk MB, Knoefler R, von Hentig N, Heller C, et al. Immune tolerance induction with mycophenolate-mofetil in two children with haemophilia B and inhibitor. Haemophilia. 2008;14(1):44-9.
51. Beutel K, Hauch H, Rischewski J, Kordes U, Schneppenheim J, Schneppenheim R. ITI with high-dose FIX and combined immunosuppressive therapy in a patient with severe haemophilia B and inhibitor. Hamostaseologie. 2009;29:155-7.
52. Barnes C, Davis A, Furmedge J, Egan B, Donnan L, Monagle P. Induction of immune tolerance using rituximab in a child with severe haemophilia B with inhibitors and anaphylaxis to factor IX. Haemophilia. 1995;16:840-1.
53. Fox RA, Neufeld EJ, Bennett CM. Rituximab for adolescents with haemophilia and high titre inhibitors. Haemophilia 2006; 12:218-22.

Pathogenesis of bone marrow failure and leukemia progression in Fanconi anemia

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A B S T R A C T

Fanconi anemia (FA) is the most frequent inherited cause of bone marrow failure (BMF), myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). Cells deficient in the FA DNA repair pathways, i.e. the FA cells, are highly sensitive to DNA damage, especially DNA interstrand crosslinks that can be triggered by naturally produced reactive aldehydes. Hematopoietic progenitor cells (HPCs) are impaired in patients, largely due to p53/p21 activation, cell cycle arrest and cell death related to the accumulation of damaged DNA and cell stress. Loss of quiescence of the hematopoietic stem cells (HSCs) has been evidenced in young Fanc-deficient mice and likely contributes to the accelerated aging and BM attrition. Remarkably, the hematopoietic alterations begin during prenatal life and the HSC pool can be already limited at birth. While many patients develop an overt BMF in childhood, the stem cell defect and genomic instability favor clonal evolution and frequent emergence of MDS or AML with a specific pattern of somatic lesions, e.g. unbalanced chromosomal translocations resulting in 1q+, 3q+, 7q-, and 21q/RUNX1 alterations. A better understanding of the multistep progression towards MDS/AML in FA patients should be relevant for complex-karyotype or secondary MDS/AML in older, non-FA, patients, for whom FA represents a model genetic condition.

Learning goals

At the conclusion of this activity, participants should:
- be aware that FA is a major predisposing cause of MDS and AML in young patients;
- be able to describe the natural history of the disease through life;
- be able to describe the main characteristics of MDS and AML occurring in FA patients;
- understand the pathogenesis bases of a DNA damage repair syndrome that leads to HSC deficiency and clonal evolution.

Fanconi anemia: clinical signs throughout life

Fanconi anemia (FA) is the most frequent inherited cause of bone marrow failure (BMF), myelodysplastic syndrome (MDS) and, beside Down syndrome, of acute myeloid leukemia (AML).[1-3] There are typically several clinical stages in FA that are related to age.[1,4-6] At birth and early childhood, only physical signs are present, and these range from discreet to extensive. Many patients then experience bone marrow failure (BMF) between 5 and 15 years of age, and the diagnosis is often made at this stage. Still later, during their teens or young adulthood, the risk of AML and MDS/AML becomes very high. Still later, in adult patients, a range of solid cancers can be seen, especially oropharyngeal cancer.[7] A significant and probably underestimated fraction of older patients have no family history of the disease nor any physical signs, and they do not develop bone marrow failure; but they still have an increased risk of malignancies. Throughout life, the hematopoietic situation can change spontaneously by genetic reversion or by clonal evolution including progression to MDS or AML.[8-13] New insights have recently emerged regarding disease pathophysiology from new mouse models and from clinical studies in patients. These help provide a better understanding of the natural history of the disease.

FA clinical and laboratory diagnosis

Except for the very rare *FANCB,* which is located on the X chromosome,[14] all other *FANC* genes are autosomic and the disease is recessive. FA patients often, but not always, present with a combination of various congenital abnormalities such as a short stature, Fanconi facies and microphthalmia, thumb and radius deformities, and/or other malformations (reviewed in Shimamura and Alter[1]). Many FA patients develop a progressive BMF during the course of the disease, usually during the first and second decades of life,[4,5] and, for the majority of patients, the suspicion of FA will only be made after the onset of pancytopenia. In some patients, the underlying diagnosis of FA is not known until MDS/AML occurs, and it is safe to screen children and young adults with MDS or AML with physical

signs and/or FA-associated chromosomal abnormalities in the bone marrow such as 1q+ or 3q+ (see below). Increases in HbF, serum alpha-fetoprotein and macrocytosis are commonly noted in FA but their absence does not rule out the disease; although not specific for FA, they may help to distinguish an inherited from an acquired BMF.[1] The biological diagnosis of FA is primarily based on the hypersensitivity of FA cells to DNA interstrand crosslink (ICL) chemicals such as diepoxybutane (DEB) or mitomycin C (MMC). The chromosomal breakage test with these agents is the technique of reference for diagnosing FA.[15,16] In the majority of cases, a precise diagnosis can be made with careful analysis of case history, physical examination and a positive chromosomal breakage blood test (breaks and radials). Other blood tests, available in research laboratories, include cell-cycle analysis[17] and evaluation of FANCD2 mono-ubiquitination, which can positively diagnose FA core patients.[18] However, all these tests can be ambiguous or even give false negative results in patients who develop hematopoietic reversion and somatic mosaicism. Hematopoietic reversion occurs when, after a spontaneous genetic event in a hematopoietic stem cell (i.e. a reverse point mutation or intragenic recombination), one FA allele is corrected, with a consequent recovery of a normal or subnormal protein activity and cellular phenotype.[8,19] Because there has been no evidence that this same phenomenon could occur in primary skin fibroblasts, these cells have been used to overcome misleading results in blood due to somatic mosaicism.[10,20-23] Once the FA diagnosis is established at the cellular level, *FANC* gene mutations can be screened. To date, 15 *FANC* genes (genes that have been found to be mutated in FA patients) have been identified (*FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1, FANCL, FANCM, FANCN/PALB2, FANCO/RAD51C,* and *FANCP/SLX4*), the most frequent in patients being *FANCA, FANCC, FANCG* and *FANCD2*.[24-26] FA mutations are listed in the Fanconi Anemia Mutation Database (www.rockefeller.edu/fanconi). There are few clear genetic-phenotype correlations in the classical 'FA core' patients, although 'hypomorphic' mutations might be associated with milder phenotypes and could give time to develop to clonal evolution and late onset solid cancer.[27-29] In contrast, FA-D2 patients usually experience a more severe phenotype,[30] and FA-D1/BRCA2 patients develop an early and rapidly lethal cancer-prone syndrome.[31-33]

The FA pathway is involved in DNA repair and counteracts the genotoxic effects of naturally produced aldehydes

Products of the 15 FA genes function in a common DNA repair signaling pathway, the FA pathway, which closely co-operates with other DNA repair proteins for resolving DNA ICLs during replication (reviewed by Kee *et al.*[25] and Kottemann *et al.*[26]). Until recently, the nature of the endogenous cross-linking agent remained unknown, but a breakthrough came when Patel's group discovered that endogenous reactive aldehydes are the genotoxins that are probably largely responsible for the pathophysiology of FA.[34-36] Reactive aldehydes, such as acetaldehyde, are naturally produced by-products of metabolism that can trigger protein and DNA lesions, particularly ICLs. Therefore, an intact FA pathway is necessary to counteract the genotoxic effects of reactive aldehydes.[34] Upon DNA damage, a central event in the FA pathway is the mono-ubiquitination of FANCD2 and FANCI, which is mediated by a group of upstream FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) that are assembled into a large nuclear E3 ubiquitin ligase complex called the FA core complex. The mono-ubiquitinated FANCD2 and FANCI heterodimer functionally interacts with downstream FA proteins such as FANCD1/BRCA2, FANCN/PALB2, FANCJ/BRIP1, FANCP/SLX4, RAD51C and their associated protein, BRCA1. FAN1, an FA-associated protein, provides a nuclease activity during the ICL repair. The FA pathway is also involved in the regulation of mitosis and cytokinesis to prevent micro-nucleation and chromosome abnormalities.[37,38] Moreover, FA cells are uniquely hypersensitive to oxidative stress and apoptotic cytokine cues including IFN-γ and TNF-α that could be related to additional function of the FA proteins.[39-43]

Pathogenesis of the bone marrow failure: genotoxicity impairs HSCs

The efficiency of allogeneic HSC transplant in FA patients shows that BMF is primarily related to an intrinsic defect of hematopoietic cells. Progressive medullary hypoplasia during childhood suggests cumulative deleterious effects in HSCs. Attempts to uncover the mechanisms leading to BMF have been hampered by practical difficulties associated with studying a rare human disorder with low bone marrow cells in patients, and because the initial murine Fanc$^{-/-}$ models, although extremely useful, did not fully recapitulate the phenotype of human FA (reviewed by Parmar *et al.*[44]). Indeed, mice with a single genetic deficiency in the FA pathway are generally small, have reduced fertility with abnormal germ cell development, and exhibit cellular hypersensitivity to ICL agents; but they do not develop spontaneous BMF despite stem cell dysfunction upon transplantation,[44-49] with the exception of the Slx4$^{-/-}$ and double-mutant Fancc$^{-/-}$Fancg$^{-/-}$.[50,51]

Using new genetic mouse models, the Patel team recently showed that the deficiency in the Aldehyde dehydrogenase gene *Aldh2* (leading to impaired aldehyde detoxification) in combination with *Fancd2* deficiency severely impacts the hematopoietic cells.[35] While most of the double KO animals succumbed to T-cell leukemia as they aged, the mice which did not develop leukemia spontaneously developed aplastic anemia, with the concomitant accumulation of damaged DNA and apoptosis within the HSPC pool. Moreover, the analysis of the bone marrow cells in young Fancd2$^{-/-}$Aldh2$^{-/-}$ mice before the onset of leukemia or aplasia showed a marked reduction in the HSPC and long-term HSC (LT-HSC) populations, along with less quiescent and more actively cycling LT-HSCs. Interestingly, only the HSPCs, but not the more mature blood precursors, require Aldh2 for protection against acetaldehyde toxicity. These studies suggest that the emergence of BMF in Fanconi anemia is probably due to aldehyde-mediated genotoxicity restricted to the HSPC pool.[34,35]

By analyzing a large series of primary bone marrow

samples from FA patients, in collaboration with the D'Andrea team, we observed a strong reduction in CD34+ BM cells that worsened with age, along with an accumulation of DNA damage (marked by H2AX foci), induction of p53 and p21, and cell cycle arrest in G0/G1 with a senescence gene expression profile.[52] The depletion of p53 or p21 in CD34+ BM cells using short hairpin RNAs resulted in a dramatic increase in the number of clonogenic progenitors *in vitro*. Moreover, the HSPC defects and clonogenic ability were rescued in several *in vivo* models, including Fancd2$^{-/-}$p53$^{-/-}$ mice, and in a new xenograft model involving transfer of human FA-like cord blood cells into NOD/scid/IL2R$\gamma^{-/-}$ (NSG) immunodeficient mice.[52] Taken together, these data show that an exaggerated physiological stress response results from the accumulation of DNA damage, underlying progressive aging and HSC depletion in FA patients (Figure 1). Interestingly, such response to accumulation of DNA damage and cellular stress has been previously involved in the hematopoietic aging occurring throughout life, both under physiological conditions in healthy subjects and in some DNA repair defects in mice.[53-56] Further analyses are needed to decipher precisely how protein and DNA damage accumulates in the HSCs and in the more differentiated progenitor cells, how each cell type responds to damage, and how this response changes in the several stages of the disease throughout the patient's life. Collectively, these and previous studies[41,44-49,57-59] support the view that DNA damage and cell stress in the HSPCs trigger cell cycle abnormalities, senescence and cell death, leading to impaired HSPCs, accelerated aging and BM exhaustion. Interestingly, common signaling downstream mechanisms including p53/p21 activation and senescence could participate in the pathogenesis of several inherited BMF syndromes, triggered by unresolved cellular conflicts, i.e. DNA damage accumulation in Fanconi anemia, abnormal telomeres in dyskeratosis congenita, and defective ribosomal function in Diamond-Blackfan anemia.[52,60,61]

Prenatal beginning of the HSC defect

Hematologic signs are not present at birth in FA subjects; instead, they generally occur during childhood.[1-3] Intriguingly, we observed low CD34+ BM counts in very young FA patients who were diagnosed soon after birth, before the onset of any peripheral hematologic signs.[52] This raised the possibility of an early beginning of the HSC defect in FA, i.e. during fetal life. Consistent with this hypothesis, the analysis of human FA fetal liver samples obtained from medical abortions showed increased expression of the cell cycle inhibitor CDKN1A/p21, at a developmental stage (14-18 weeks) at which the liver is mainly hematopoietic, suggesting the early onset of cell cycle abnormalities and stress response in fetal hematopoiesis.[52] In Fancc$^{-/-}$ mouse, quantitative and qualitative deficiencies were observed in the fetal HSPCs pool.[62] Moreover, FA pathway silencing in human embryonic stem cells (hESCs) leads to early hematopoietic development defects *in vitro*.[63] Collectively, these data suggest that the hematopoietic development is impaired early in life in FA, during the expansion of the HSC pool (a stage with high replication stress), leading to an altered HSC pool at birth (Figure 2).

Clonal evolution towards myelodysplastic syndromes and acute myeloid leukemia

In such a context of deficient HSPCs, bone marrow cells experience a strong selective advantage upon clonal evolution, probably also facilitated by the constitutive genomic instability of FA (Figure 3).[9,64,65] Clinically, FA patients develop MDS and AML with the highest frequency during their teens or young adulthood.[1,5,66] These are often, but not always, preceded by a hypoplastic or aplastic phase. MDS in FA often presents as refractory cytopenia with multilineage dysplasia (RCMD, according to the World Health Organization (WHO) 2008 classification), with or without excess of blasts (RAEB).[11,67] A certain level of dyserythropoiesis is almost constant in FA, and a mild dyserythropoiesis is not considered as an MDS criteria in this population. Acute leukemia can be diagnosed primarily (in approx. 30% of the AML in FA patients) or after an MDS phase with an increasing fraction of blast cells in the bone marrow. Karyotypic abnormalities are frequently found, and translocations of chromosome 1q, monosomy 7, and gains of 3q have been reported.[11,66-70] In contrast, classic *de*

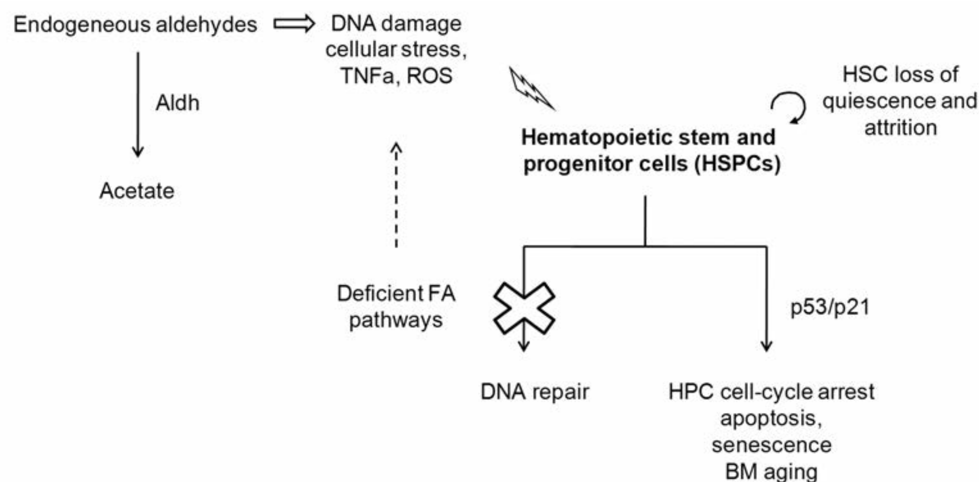

Figure 1. Accumulation of cell stress and DNA damage impairs HSPCs in FA.

novo translocations like t(8;21), inv(16) and 11q23-t/MLL are not seen in FA. Using array-based high-density chromosomal profiling and oncogene sequencing, we analyzed bone marrow samples from a large series of FA patients at various stages of the disease (hypoplasia/aplastic anemia, MDS and AML, normal BM).[11] We found a highly recurrent pattern of somatic abnormalities that were related to unbalanced chromosomal translocations and led to partial chromosomal arm duplications or losses (Figure 4). In contrast to what is seen in non-FA MDS/AML patients,[71] somatic copy-neutral loss of heterozygosity (uniparental disomy, UPD) were rarely found in FA, which is consistent with a constitutive defect of homologous recombination repair in FA. Regions of homozygosity were found using SNP arrays in some patients but these were related to consanguinity, as was demonstrated by paired fibroblast analysis. The most frequent somatic lesion was partial duplication of chromosome 1q (1q+, 44.8% of 29 MDS/AML), following by 3q+ (41.3%), RUNX1/21q- (20.7%), monosomy 7/7q- (17.2%), and 11q- (13.8%). Mutations of MDS/AML oncogenes and tumor suppressor genes were rarely found (isolated *FLT3-ITD*, *NRAS*, *MLL-PTD*, but no *TP53*, *CBL*, *TET2*, *CEBPα*, *NPM1*, and *FLT3-TKD* mutations in this series).

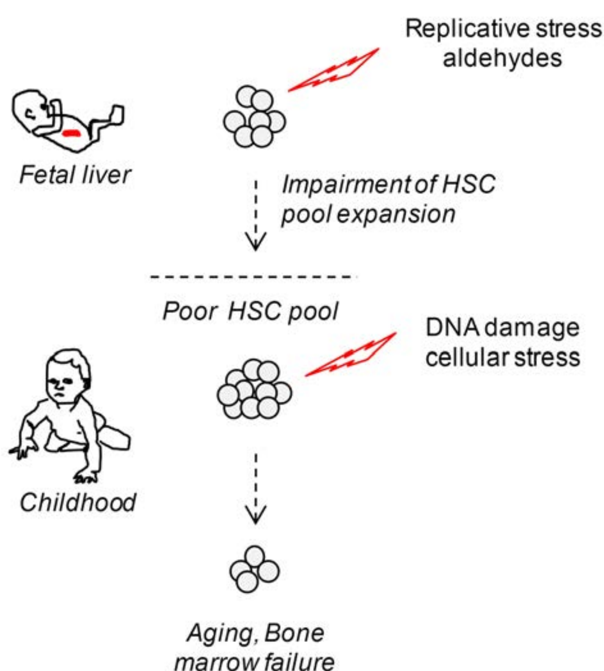

Figure 2. Prenatal beginning of the hematopoietic involvement in FA.

Therefore, it appears that myeloid oncogenesis in FA shares common lesions with non-FA patients (7q-, 5q-, 21q-/*RUNX1* lesions, *PRDM16* translocations, *MLL-PTD*), but lacks frequent *TP53* deletion/mutation, maybe suggesting alternative inactivation of tumor suppressor pathways. In addition, FA-specific chromosomal lesions are very often present, especially 1q+ and 3q+. The molecular targets of these two lesions are not known, although the *EVI1* oncogene at 3q26 is a strong candidate and, indeed, is strongly expressed in 3q+ cases.[72] It might be that EVI1 is preferentially deregulated in FA through unbalanced chromosomal translocations resulting in copy-number gain, rather than by direct balanced translocation as usually seen in a subset of non-FA MDS/AML. Whereas the lesions 7q, 3q+, and *RUNX1* abnormalities were found at the MDS and AML stages only, translocation/duplication 1q+ can be seen at all stages in the bone marrow, including 'normal' or hypoplastic bone marrow without apparent transformation signs (Figure 4), suggesting that 1q+ could rescue FA cells without necessarily transforming them into MDS/AML.[11] Clonal evolution with acquired attenuation of the FA-prototypical G2 checkpoint and resistance to TNFα have been described in human and mouse, respectively.[12,64,73] It is likely that these and other cellular phenotypes rescue FA HSPC by conferring them a selective advantage, but also predispose patients to develop malignancies. Thorough longitudinal characterization of the molecular and cellular features associated with bone marrow progression should lead to a better understanding of the step-wise mechanisms of transformation. With this aim in mind, chromosomal profiling and next-generation sequencing (NGS) in sequential BM samples will allow information to be obtained as to the architecture of clonal evolution and tumor progression in FA, as was performed in secondary or relapsing AML in non-FA patients or in inherited severe congenital neutropenia progressing to AML.[74-76]

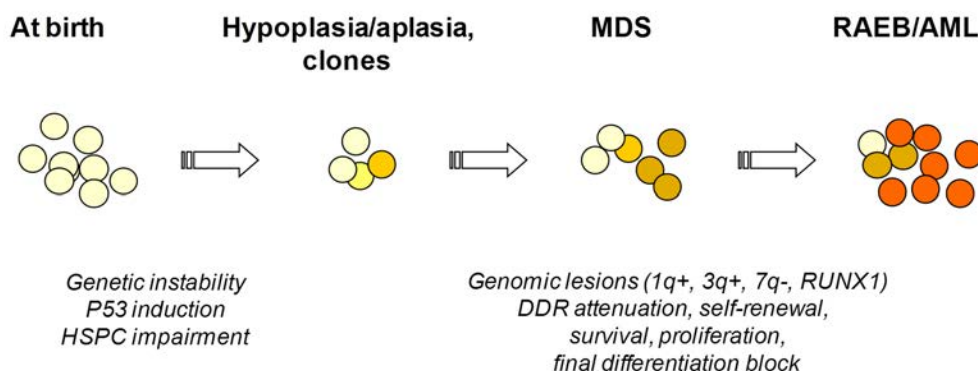

Figure 3. Frequent evolution in the bone marrow of FA patients.

Myelodysplastic syndromes and acute myeloid leukemia monitoring in FA patients

Hematopoietic stem cell transplantation (HSCT) is currently the best treatment to cure FA aplastic anemia or MDS/AML.[77] However, great care has to be taken when considering HSCT, and decision-making should be based upon clinical and biological criteria including age, severity of the cytopenia, significant bone marrow dysplasia, excess of blast cells, cytogenetic/molecular abnormalities, and immunological compatibility with the donor. Because MDS/AML is a frequent and severe occurrence in FA, it is necessary to follow up patients with regular bone marrow aspirate tests with expert morphological and karyotype evaluation to detect transformation before the onset of an overt MDS/AML.[1,15] Conventional karyotype appears to be sensitive for the early detection of discreet subclones, due to the possibility of observing large unbalanced translocations in individual cells and probably a clonal advantage in culture. Systematic interphase fluorescence *in situ* hybridization (FISH) screening using probes for chromosome 7q, 3q, and break-apart RUNX1 might increase the sensitivity of detection of clonal cells. MDS/AML cases can have an apparently normal kary-

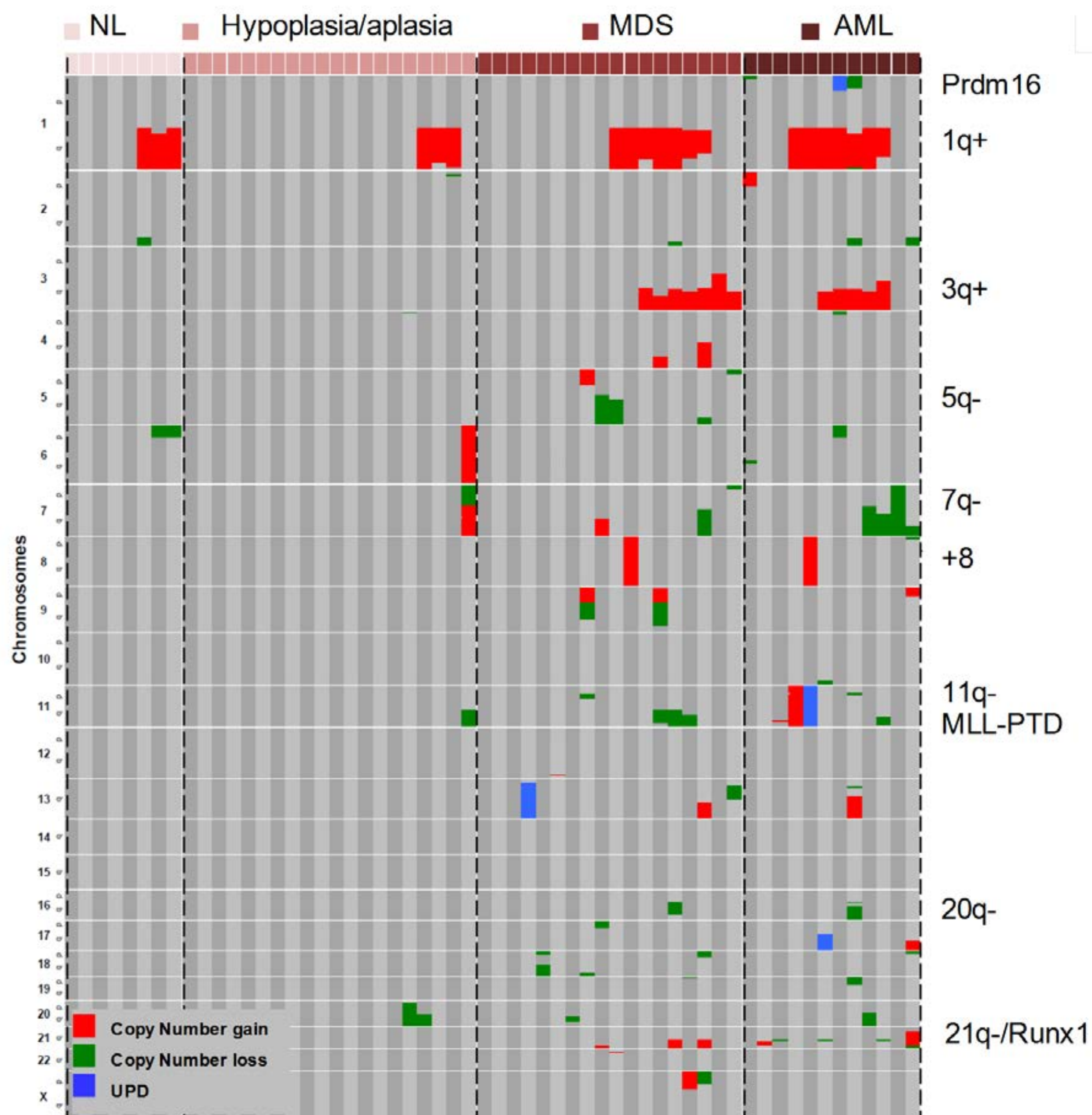

Figure 4. A recurrent profile of chromosomal abnormalities in bone marrow samples of FA patients. Samples are grouped by bone marrow stages (normal, medullary hypoplasia/aplasia, MDS, AML). The 1q+ and 3q+ lesions are specific from FA compared to MDS/AML of non-FA patients, other abnormalities including 7q- and RUNX1 mutations can be found in non-FA patients. 1q+ is found at all stages of the bone marrow progression, whereas most other lesions are found at most advanced MDS/AML stages. (This research was originally published in Blood. Quentin S, Cuccuini W, Ceccaldi R, et al. Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic RUNX1/AML1 lesions. Blood. 2011;117(15):e161-70. ©the American Society of Hematology[11]).

otype but cryptic chromosomal or genomic abnormalities detected by FISH and/or array analysis.[11] Therefore, when abnormal cells are observed with a bone marrow smear in FA patients, we now implement the karyotype analysis using high-density DNA arrays and *RUNX1* FISH. This approach is useful in detecting short cryptic lesions and in characterizing the abnormal karyotypes precisely, frequently highlighting meaningful chromosomal lesions.[11] Whether a systematic screen by high-throughput genomic tools could usefully improve the early detection of genome abnormalities is under investigation. The predictive value of the various chromosomal/genomic abnormalities in patients with or without MDS/AML will have to be carefully evaluated in the long term in large cohorts of FA patients with respect to the therapeutic options and clinical benefits. For example, a sole clonal abnormality like 1q+ can be present in a 'normal' or non-MDS hypoplastic bone marrow, and may not necessarily predict a progression into AML in the following years. By contrast, abnormalities like 7q-, 3q+, *RUNX1* or complex karyotype may encourage a decision to proceed to HSCT.

Conclusions

Recent studies have revealed insights in the pathophysiology of the BMF and MDS/AML in Fanconi anemia, drawing a picture from which emerge genomic instability, cellular stress, balance of HSC quiescence *versus* proliferation/differentiation and senescence, accelerated aging, and predisposition to clonal evolution. A better understanding of the multistep progression towards MDS/AML in FA patients should be relevant for complex-karyotype or secondary MDS/AML in older, non-FA patients, for whom close physiopathological cellular mechanisms are likely involved.[78,79]

At the therapeutic level, in addition to prevention from exposure to exogenous DNA insults, FA patients might benefit from new treatments aiming to enhance patients' capacity for detoxifying aldehydes,[34] or to inhibit proinflammatory cytokines or oxidative stress.[59,80] While development of gene therapy and IPS cells in FA are still hampered by practical issues,[80] the recently acquired insights into disease pathogenesis also reinforce the view that allogeneic HSCT is a radical and curative therapy for severe BMF and predisposition to MDS/AML in FA patients by replacing defective cells.[77,81] The utility of transplant is increasing with the use of unrelated and mismatched donors and improvements in the management of complications.[7,77,82]

References

1. Shimamura A, Alter BP. Pathophysiology and management of inherited bone marrow failure syndromes. Blood Rev. 2010;24(3):101-22.
2. Dokal I, Vulliamy T. Inherited aplastic anaemias/bone marrow failure syndromes. Blood Rev. 2008;22(3):141-53.
3. Liew E, Owen C. Familial myelodysplastic syndromes: a review of the literature. Haematologica. 2011;96(10):1536-42.
4. Kutler DI, Singh B, Satagopan J, et al. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood. 2003;101(4):1249-56.
5. Rosenberg PS, Huang Y, Alter BP. Individualized risks of first adverse events in patients with Fanconi anemia. Blood. 2004;104(2):350-5.
6. Rosenberg PS, Alter BP, Ebell W. Cancer risks in Fanconi anemia: findings from the German Fanconi Anemia Registry. Haematologica. 2008;93(4):511-7.
7. Rosenberg PS, Socie G, Alter BP, Gluckman E. Risk of head and neck squamous cell cancer and death in patients with Fanconi anemia who did and did not receive transplants. Blood. 2005;105(1):67-73.
8. Lo Ten Foe JR, Kwee ML, Rooimans MA, et al. Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet. 1997;5(3):137-48.
9. Lensch MW, Rathbun RK, Olson SB, Jones GR, Bagby GC Jr. Selective pressure as an essential force in molecular evolution of myeloid leukemic clones: a view from the window of Fanconi anemia. Leukemia. 1999;13(11):1784-9.
10. Gross M, Hanenberg H, Lobitz S, et al. Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. Cytogenet Genome Res. 2002;98(2-3):126-35.
11. Quentin S, Cuccuini W, Ceccaldi R, et al. Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic RUNX1/AML1 lesions. Blood. 2011;117(15):e161-70.
12. Ceccaldi R, Briot D, Larghero J, et al. Spontaneous abrogation of the G(2)DNA damage checkpoint has clinical benefits but promotes leukemogenesis in Fanconi anemia patients. J Clin Invest. 2011;121(1):184-94.
13. Soulier J. Fanconi anemia. Hematology Am Soc Hematol Educ Program. 2011;2011:492-7.
14. Meetei AR, Levitus M, Xue Y, et al. X-linked inheritance of Fanconi anemia complementation group B. Nat Genet. 2004;36(11):1219-24.
15. Auerbach AD. Fanconi anemia and its diagnosis. Mutat Res. 2009;668(1-2):4-10.
16. Oostra AB, Nieuwint AW, Joenje H, de Winter JP. Diagnosis of fanconi anemia: chromosomal breakage analysis. Anemia. 2012;2012:238731.
17. Seyschab H, Friedl R, Sun Y, et al. Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood. 1995;85(8):2233-7.
18. Shimamura A, de Oca RM, Svenson JL, et al. A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood. 2002;100(13):4649-54.
19. Waisfisz Q, Morgan NV, Savino M, et al. Spontaneous functional correction of homozygous fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat Genet. 1999;22(4):379-83.
20. Auerbach AD. Diagnosis of fanconi anemia by diepoxybutane analysis. Curr Protoc Hum Genet. 2003;Chapter 8:Unit 8.7.
21. Soulier J, Leblanc T, Larghero J, et al. Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood. 2005;105(3):1329-36.
22. Mankad A, Taniguchi T, Cox B, et al. Natural gene therapy in monozygotic twins with Fanconi anemia. Blood. 2006;107(8):3084-90.
23. Pinto FO, Leblanc T, Chamousset D, et al. Diagnosis of Fanconi anemia in patients with bone marrow failure. Haematologica. 2009;94(4):487-95.
24. de Winter JP, Joenje H. The genetic and molecular basis of Fanconi anemia. Mutat Res. 2009;668(1-2):11-9.
25. Kee Y, D'Andrea AD. Molecular pathogenesis and clinical management of Fanconi anemia. J Clin Invest. 2012;122(11):3799-806.
26. Kottemann MC, Smogorzewska A. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. Nature. 2013;493 (7432):356-63.
27. Faivre L, Guardiola P, Lewis C, et al. Association of complementation group and mutation type with clinical outcome in fanconi anemia. European Fanconi Anemia Research Group. Blood. 2000;96(13):4064-70.
28. Neveling K, Endt D, Hoehn H, Schindler D. Genotype-phenotype correlations in Fanconi anemia. Mutat Res. 2009;668(1-2):73-91.
29. Castella M, Pujol R, Callen E, et al. Origin, functional role, and clinical impact of Fanconi anemia FANCA mutations. Blood. 2011;117(14):3759-69.
30. Kalb R, Neveling K, Hoehn H, et al. Hypomorphic mutations in the gene encoding a key Fanconi anemia protein, FANCD2, sustain a significant group of FA-D2 patients with severe phenotype. Am J Hum Genet. 2007;80(5):895-910.
31. Alter BP, Rosenberg PS, Brody LC. Clinical and molecular features associated with biallelic mutations in FANCD1/BRCA2. J Med Genet. 2007;44(1):1-9.
32. Hirsch B, Shimamura A, Moreau L, et al. Biallelic BRCA2/FANCD1 mutations: association with spontaneous chromosomal instability and solid tumors of childhood. Blood. 2004;103:2554-9.

33. Wagner JE, Tolar J, Levran O, et al. Germline mutations in BRCA2: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. Blood. 2004;103(8):3226-9.
34. Langevin F, Crossan GP, Rosado IV, Arends MJ, Patel KJ. Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. Nature. 2011;475(7354):53-8.
35. Garaycoechea JI, Crossan GP, Langevin F, Daly M, Arends MJ, Patel KJ. Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. Nature. 2012;489(7417):571-5.
36. Parmar K, D'Andrea AD. Stressed out: endogenous aldehydes damage hematopoietic stem cells. Cell Stem Cell. 2012;11(5):583-4.
37. Naim V, Rosselli F. The FANC pathway and BLM collaborate during mitosis to prevent micro-nucleation and chromosome abnormalities. Nat Cell Biol. 2009;11(6):761-8.
38. Vinciguerra P, Godinho SA, Parmar K, Pellman D, D'Andrea AD. Cytokinesis failure occurs in Fanconi anemia pathway-deficient murine and human bone marrow hematopoietic cells. J Clin Invest. 2010;120(11):3834-42.
39. Pang Q, Andreassen PR. Fanconi anemia proteins and endogenous stresses. Mutat Res. 2009;668(1-2):42-53.
40. Pang Q, Fagerlie S, Christianson TA, et al. The Fanconi anemia protein FANCC binds to and facilitates the activation of STAT1 by gamma interferon and hematopoietic growth factors. Mol Cell Biol. 2000;20(13):4724-35.
41. Zhang X, Sejas DP, Qiu Y, Williams DA, Pang Q. Inflammatory ROS promote and cooperate with the Fanconi anemia mutation for hematopoietic senescence. J Cell Sci. 2007;120(Pt 9):1572-83.
42. Dufour C, Corcione A, Svahn J, et al. TNF-alpha and IFN-gamma are overexpressed in the bone marrow of Fanconi anemia patients and TNF-alpha suppresses erythropoiesis in vitro. Blood. 2003;102(6):2053-9.
43. Briot D, Mace-Aime G, Subra F, Rosselli F. Aberrant activation of stress-response pathways leads to TNF-alpha oversecretion in Fanconi anemia. Blood. 2008;111(4):1913-23.
44. Parmar K, D'Andrea A, Niedernhofer LJ. Mouse models of Fanconi anemia. Mutat Res. 2009;668(1-2):133-40.
45. Carreau M, Gan OI, Liu L, Doedens M, Dick JE, Buchwald M. Hematopoietic compartment of Fanconi anemia group C null mice contains fewer lineage-negative CD34+ primitive hematopoietic cells and shows reduced reconstruction ability. Exp Hematol. 1999;27(11):1667-74.
46. Haneline LS, Gobbett TA, Ramani R, et al. Loss of FancC function results in decreased hematopoietic stem cell repopulating ability. Blood. 1999;94(1):1-8.
47. Carreau M, Gan OI, Liu L, et al. Bone marrow failure in the Fanconi anemia group C mouse model after DNA damage. Blood. 1998;91(8):2737-44.
48. Parmar K, Kim J, Sykes SM, et al. Hematopoietic stem cell defects in mice with deficiency of Fancd2 or Usp1. Stem Cells. 2010;28(7):1186-95.
49. Barroca V, Mouthon MA, Lewandowski D, et al. Impaired functionality and homing of Fancg-deficient hematopoietic stem cells. Hum Mol Genet. 2012;21(1):121-35.
50. Crossan GP, van der Weyden L, Rosado IV, et al. Disruption of mouse Slx4, a regulator of structure-specific nucleases, phenocopies Fanconi anemia. Nat Genet. 2011;43(2):147-52.
51. Pulliam-Leath AC, Ciccone SL, Nalepa G, et al. Genetic disruption of both Fancc and Fancg in mice recapitulates the hematopoietic manifestations of Fanconi anemia. Blood. 2010;116(16):2915-20.
52. Ceccaldi R, Parmar K, Mouly E, et al. Bone marrow failure in Fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells. Cell Stem Cell. 2012;11(1):36-49.
53. Blanpain C, Mohrin M, Sotiropoulou PA, Passegue E. DNA-damage response in tissue-specific and cancer stem cells. Cell Stem Cell. 2011;8(1):16-29.
54. Niedernhofer LJ. DNA repair is crucial for maintaining hematopoietic stem cell function. DNA Repair (Amst). 2008;7(3):523-9.
55. Rossi DJ, Jamieson CH, Weissman IL. Stems cells and the pathways to aging and cancer. Cell. 2008;132(4):681-96.
56. Nijnik A, Woodbine L, Marchetti C, et al. DNA repair is limiting for haematopoietic stem cells during ageing. Nature. 2007;447(7145):686-90.
57. Rani R, Li J, Pang Q. Differential p53 engagement in response to oxidative and oncogenic stresses in Fanconi anemia mice. Cancer Res. 2008;68(23):9693-702.
58. Geiselhart A, Lier A, Walter D, Milsom MD. Disrupted Signaling through the Fanconi Anemia Pathway Leads to Dysfunctional Hematopoietic Stem Cell Biology: Underlying Mechanisms and Potential Therapeutic Strategies. Anemia. 2012;2012:265790.
59. Zhang QS, Marquez-Loza L, Eaton L, et al. Fancd2-/- mice have hematopoietic defects that can be partially corrected by resveratrol. Blood. 2010;116(24):5140-8.
60. Narla A, Ebert BL. Ribosomopathies: human disorders of ribosome dysfunction. Blood. 2010;115(16):3196-205.
61. Calado RT, Young NS. Telomere diseases. N Engl J Med. 2009;361(24):2353-65.
62. Kamimae-Lanning AN, Goloviznina NA, Kurre P. Fetal origins of hematopoietic failure in a murine model of Fanconi anemia. Blood. 2013;121(11):2008-12.
63. Tulpule A, Lensch MW, Miller JD, et al. Knockdown of Fanconi anemia genes in human embryonic stem cells reveals early developmental defects in the hematopoietic lineage. Blood. 2010;115(17):3453-62.
64. Li X, Le Beau MM, Ciccone S, et al. Ex vivo culture of Fancc-/- stem/progenitor cells predisposes cells to undergo apoptosis, and surviving stem/progenitor cells display cytogenetic abnormalities and an increased risk of malignancy. Blood. 2005;105(9):3465-71.
65. Bagby GC, Meyers G. Bone marrow failure as a risk factor for clonal evolution: prospects for leukemia prevention. Hematology Am Soc Hematol Educ Program. 2007:40-6.
66. Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio AP, Auerbach AD. Hematologic abnormalities in Fanconi anemia: an International Fanconi Anemia Registry study. Blood. 1994;84(5):1650-5.
67. Cioc AM, Wagner JE, MacMillan ML, DeFor T, Hirsch B. Diagnosis of myelodysplastic syndrome among a cohort of 119 patients with fanconi anemia: morphologic and cytogenetic characteristics. Am J Clin Pathol. 2010;133(1):92-100.
68. Alter BP, Caruso JP, Drachtman RA, Uchida T, Velagaleti GV, Elghetany MT. Fanconi anemia: myelodysplasia as a predictor of outcome. Cancer Genet Cytogenet. 2000;117(2):125-31.
69. Maarek O, Jonveaux P, Le Coniat M, Derre J, Berger R. Fanconi anemia and bone marrow clonal chromosome abnormalities. Leukemia. 1996;10(11):1700-4.
70. Tonnies H, Huber S, Kuhl JS, Gerlach A, Ebell W, Neitzel H. Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. Blood. 2003;101(10):3872-4.
71. O'Keefe C, McDevitt MA, Maciejewski JP. Copy neutral loss of heterozygosity: a novel chromosomal lesion in myeloid malignancies. Blood. 2010;115(14):2731-9.
72. Meyer S, Bristow C, Wappett M, et al. Fanconi anemia (FA)-associated 3q gains in leukemic transformation consistently target EVI1, but do not affect low TERC expression in FA. Blood. 2011;117(22):6047-50.
73. Li J, Sejas DP, Zhang X, et al. TNF-alpha induces leukemic clonal evolution ex vivo in Fanconi anemia group C murine stem cells. J Clin Invest. 2007;117(11):3283-95.
74. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. Cell. 2012;150(2):264-78.
75. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-10.
76. Beekman R, Valkhof MG, Sanders MA, et al. Sequential gain of mutations in severe congenital neutropenia progressing to acute myeloid leukemia. Blood. 2012;119(22):5071-7.
77. Gluckman E, Wagner JE. Hematopoietic stem cell transplantation in childhood inherited bone marrow failure syndrome. Bone Marrow Transplant. 2008;41(2):127-32.
78. Nimer SD. MDS: a stem cell disorder—but what exactly is wrong with the primitive hematopoietic cells in this disease? Hematology Am Soc Hematol Educ Program. 2008:43-51.
79. Bejar R, Levine R, Ebert BL. Unraveling the molecular pathophysiology of myelodysplastic syndromes. J Clin Oncol. 2011;29(5): 504-15.
80. Dufour C, Svahn J. Fanconi anaemia: new strategies. Bone Marrow Transplant. 2008;41(2 Suppl):S90-5.
81. Ayas M, Saber W, Davies SM, et al. Allogeneic Hematopoietic Cell Transplantation for Fanconi Anemia in Patients With Pretransplantation Cytogenetic Abnormalities, Myelodysplastic Syndrome, or Acute Leukemia. J Clin Oncol. 2013 April 1. [Epub ahead of print]
82. Peffault de Latour R, Porcher R, Dalle JH, et al. Long-Term Outcome After Matched Allogeneic Hematopoietic Stem Cell Transplantation for Fanconi Anemia On Behalf of the FA Committee of the Severe Aplastic Anemia Working Party (SAA WP) and the Pediatric Working Party of the European Group for Blood and Marrow Transplantation (EBMT). ASH Annual Meeting Abstracts. 2011.

Diamond-Blackfan anemia: pathogenesis, management and development of future therapies

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A B S T R A C T

Diamond-Blackfan anemia (DBA) is an inherited bone marrow failure syndrome characterized by a macrocytic anemia, reticulocytopenia and reduction in erythroid precursors in an otherwise normocellular bone marrow. The disease usually presents before one year of age. Fifty percent of the patients have congenital anomalies. The mainstay of current therapy is corticosteroids and if the patients do not respond to steroids, chronic blood transfusion therapy is needed. The disease can be cured by allogeneic bone marrow transplantation. DBA is a genetic disorder and is inherited in an autosomal dominant manner with variable penetrance in 50% of cases while the remainders represent new mutations. To date, mutations have been identified in 60-70% of DBA patients. Practically all of these patients have a mutation in or a deletion of, a ribosomal protein gene. Ten different ribosomal protein genes have now been identified as DBA genes and recently a handful of patients have been found with mutations in *GATA1*. Abnormal ribosome biogenesis and ribosomal stress leads to activation of the tumor suppressor p53. The p53 response appears to be particularly prominent in erythroid progenitors and may explain many features of the DBA phenotype and symptoms.

Learning goals

At the conclusion of this activity, participants should have:
- learnt the key clinical manifestations, diagnostic criteria and current treatment options for Diamond-Blackfan anemia;
- got an insight into the molecular and cellular pathogenesis of Diamond-Blackfan anemia and how mechanism-based therapies may be developed to reduce side effects or cure the disease.

Introduction

Bone marrow failure syndromes consist of diverse disorders characterized by the dysfunction of bone marrow to produce cells of one or more blood lineages. In one-third of the pediatric marrow failure cases the disease is inherited involving a genetic component causing the bone marrow dysfunction[1]. Inherited bone marrow failure syndromes (IBMFS) usually present in childhood and are associated with physical abnormalities and cancer predisposition. Recent progress in genetics and molecular biology investigations has revolutionized the understanding of IBMFS pathophysiology. Many of the genes mutated in these disorders encode components of fundamental cellular processes such as DNA damage repair (Fanconi anemia) or telomere maintenance (Dyskeratosis congenita). Diamond-Blackfan anemia (DBA) is a congenital bone marrow failure syndrome that is emerging as a paradigm for diseases associated with defects in ribosome biogenesis and function. Similarly to other IBMFS, physical abnormalities and cancer predisposition are both characteristic for DBA. However, why defects in ribosome biogenesis result in anemia, a relatively tissue-specific phenotype, is intriguing and not perfectly understood.

Clinical symptoms and diagnosis

Diamond-Blackfan anemia is a congenital bone marrow failure syndrome that manifests early in life. It classically presents at 2-3 months of age, and the majority of patients (approx. 90%) are diagnosed during their first year of life. However, in some rare cases DBA may present in adulthood.[2,3] The main hematology findings at presentation include macrocytic anemia, reticulocytopenia and selective absence of erythroid precursors in an otherwise normocellular bone marrow.[4] Together with the early onset of symptoms (<1 year), these criteria have remained the accepted standard for DBA diagnosis. As a supporting hematologic feature, the vast majority of patients have elevated erythrocyte adenosine deaminase (eADA) activity.[5,6] Elevated fetal hemoglobin is also often observed. Although DBA is sometimes referred to as pure red cell aplasia, this term may be misleading since other hematopoietic lineages may be affected. Some patients present with a modest neutropenia, thrombocytosis or thrombocytopenia.[2] Furthermore, neutropenia and thrombocytopenia become increasingly common during the course of the disease.[7]

Similarly to other IBMFS, physical defects and cancer predisposition are characteristic of

DBA. Congenital abnormalities are present in approximately 40-50% of the patients.[2,3,8,9] The majority of these involve head and eyes, upper limbs, heart and the genitourinary system. Furthermore, one-third of cases show retarded growth. Patients with DBA have an increased risk of developing cancer.[10] The mechanism of increased carcinogenesis is unknown. The observed-to-expected ratio of all cancers combined is 5.4-fold higher than in the general population with the highest risk for myelodysplastic syndrome (MDS, 287-fold), acute myeloid leukemia (AML, 28-fold), colon carcinoma (36-fold) and osteogenic sarcoma (33-fold). The cancer risks appear lower than in Fanconi anemia and dyskeratosis congenital.[11] Specific cancer screening approaches may be difficult to design in practice due to diversity of the cancers that develop in DBA.

For diagnosis, laboratory blood analysis, bone marrow analysis (aspiration and biopsy) and genotyping are required (Table 1). The differential diagnosis of DBA includes other IBMFS and several acquired disorders, for example, transient erythroblastopenia of childhood and infections by parvovirus B19.[12] Findings from National Patient Registries in North America and Europe have provided extensive clinical data and, together with the recent advances in gene discovery, have provided key clinical insights.[2,3,8,9,13] Detailed and extensive descriptions of the recommended approach to clinical diagnosis and management of DBA have recently been described in the report from the DBA Clinical Consensus Conference and a scholarly written "How I Treat Diamond Blackfan anemia" overview.[12,13]

Current treatment

Corticosteroids form the main therapeutic regimen in DBA and approximately 80% of the patients initially respond to this treatment. However, because of the progressive loss of response or unacceptable side effects, only half of these patients (40% of total) can be sustained on corticosteroids.[3,13] If the patient responds to corticosteroids, an attempt is made to reduce the dose gradually to reduce side effects that include slow growth rate, cataracts and demineralization of bone leading to pathological fractures. It is recommended to treat congenital anomalies by surgery before steroid treatment starts to facilitate wound healing.[13] The remaining patients require chronic transfusion therapy every 3-5 weeks to maintain sufficient hemoglobin levels (>8 g/dL) that allows for adequate growth and development, while not suppressing the endogenous red blood cell production. Chronic transfusion therapy must be combined with iron chelation to avoid the accumulation of iron in the liver, heart and other organs. Approximately 20% of the patients enter spontaneous remission in which physiologically acceptable hemoglobin level is maintained without therapeutic interventions.

Allogeneic bone marrow transplantation is the only curative treatment for the hematopoietic manifestation of DBA, and it is normally considered among the young patients (<10 years) who are transfusion-dependent and have access to a matched sibling donor.[3,12] However, although matched sibling donor bone marrow transplantations have been reported with satisfactory results, transplantation using a matched alternative donor is associated with a poor outcome.

Numerous alternative therapies (growth factors, prolactin, immunosuppressants) have been applied in the treatment of DBA but these are not routinely used since they have either been ineffective or only found to be effective in rare cases.[12,13] Of special interest is the recent case report demonstrating a complete remission in response the amino acid L-leucine.[14] Supporting this report, therapeutic experiments with L-leucine improved the erythroid defect in zebrafish and mouse models for DBA.[15,16] With the current therapies, the overall survival at over 40 years is 75.1%.[3] A high proportion of deaths are treatment-related and corticosteroid-responsive patients have a significant survival advantage compared to transfusion-dependent patients.

Inheritance and genetics of DBA

The incidence of DBA is estimated to be 5-7 cases per million live births without ethnic predilection or biased sex ratio.[2,8,9] Almost 50% of DBA cases are familial and inherited as an autosomal dominant trait with variable penetrance.[6] Family members who share a common genetic alteration may show dramatic variation in the severity of anemia and treatment response.

Mutations in or deletions of genes encoding ribosomal protein (RP) S19, RPS24, RPS17, RPL35a, RPL5, RPL11, RPS7, RPS10, RPS26 and RPL26 collectively explain the genetic basis for approximately 60-70% of DBA cases[17-25] (Figure 1). Furthermore, alterations in additional RP genes have been identified in isolated patients, although the pathogenic significance of these rare variants is not clear.[21-23] All reported mutations are heterozygous, which

Table 1. Diagnostic criteria, genetic analysis and current therapeutic approaches for Diamond-Blackfan anemia. This is a simplified overview based on the report from the DBA Clinical Consensus Conference[12] and a recent clinical review.[13]

Main diagnostic criteria
Age less than one year
Macrocytic anemia with no significant cytopenias
Reticulocytopenia
Normal marrow cellularity with a relatively low number of erythroid precursors
Minor diagnostic criteria
Elevated erythrocyte adenosine deaminase activity
Elevated fetal hemoglobin (HbF)
Congenital anomalies described in classical DBA
Inheritance and genetic analysis
Gene mutation in one of the ribosomal protein genes described in classical DBA
Positive family history (found in 50% of cases)
Differential diagnosis
Other IBMFS: Fanconi anemia, Schwachman Diamond Syndrome, Dyskeratosis congenita
Acquired disorders: transient erythroblastopenia of childhood, Pearson syndrome
Viral infections, *e.g.* B19 parvovirus
Current therapies
The natural therapy: remission (20%)
Corticosteroids
Blood transfusion
Allogeneic transplantation (relatively rare, see text)

is consistent with the dominant inheritance pattern.

Twenty-five percent of the patients have mutations in the gene coding for RPS19 making it the most common DBA gene. More than 120 unique alterations have been identified (Available from: www.dbagenes.unito.it Accessed January 2013[26]). The mutations may completely disrupt the expression of *RPS19*, or interfere with the folding of RPS19 or its assembly into the 40S ribosomal subunit, and thus result in a functional haploinsufficiency.

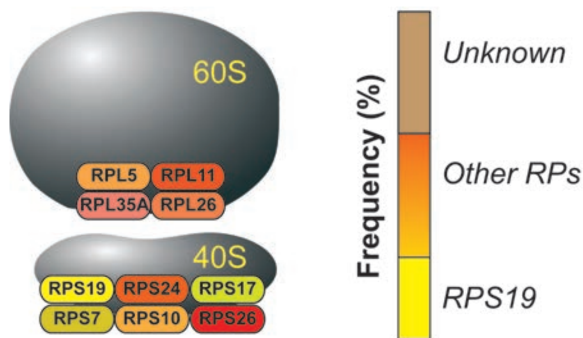

Figure 1. The ribosomal proteins mutated in DBA, their frequency and distribution within the ribosomal subunits. The figure shows the two ribosomal subunits, the large 60S and the small 40S subunit. Approximately half of the ribosomal mass consists of the ribosomal RNA and the other half of the ribosomal proteins, which are referred to as the RPL when they are found in the large 60S subunit and RPS in the small 40S subunit. In approximately 30-40% of patients the mutation is unknown. *RPS19* is by far the most common disease gene and it is found mutated in 25% of patients. *GATA1* mutations are not shown here since GATA1 is a transcription factor and these mutations are rare.

Nearly all mutations in the other DBA genes are predicted to cause premature termination, splicing disruption, frame shifting or complete deletion of one allele, supporting functional haploinsufficiency as the basis for the disease pathology.[27-29]

Recently, patients with *GATA1* mutations were identified in two unrelated families.[30] However, the identification and phenotypic characterization of additional DBA patients with *GATA1* mutations will eventually determine whether these patients present ‘classical’ DBA.

Erythropoiesis

The erythrocyte is the most common cell type in blood. Mature erythrocytes have a limited life span, approximately 120 days in humans and 40 days in mice, and they must be continuously produced in order to renew the red cell mass. The erythroid lineage consists of erythroid progenitor and precursor cell compartments (Figure 2). Erythroid progenitor cells are relatively infrequent and can be divided into the early and late progenitor cells based on their colony-forming potential *in vitro*. The early progenitor cells (burst-forming unit-erythroid, BFU-E) are the first solely erythroid-restricted cells and give rise to large multi-clustered colonies.[32] BFU-Es also possess a limited self-renewal capacity. The late progenitor cells (colony-forming unit-erythroid, CFU-E) give rise to smaller colonies than BFU-Es. The proliferation and survival of BFU-Es is mainly dependent on stem cell factor (SCF) and interleukin-3 (IL-3) signaling, while erythropoietin (Epo) alone is sufficient to support CFU-Es. CFU-Es differentiate into morphologically distinguishable erythroid precursor cells. The first recognizable precursor, proerythroblast, undergoes 3-5 cell divisions giving rise to basophilic, polychromatic and orthochromatic erythroblasts. These differentiation divisions are characterized by a

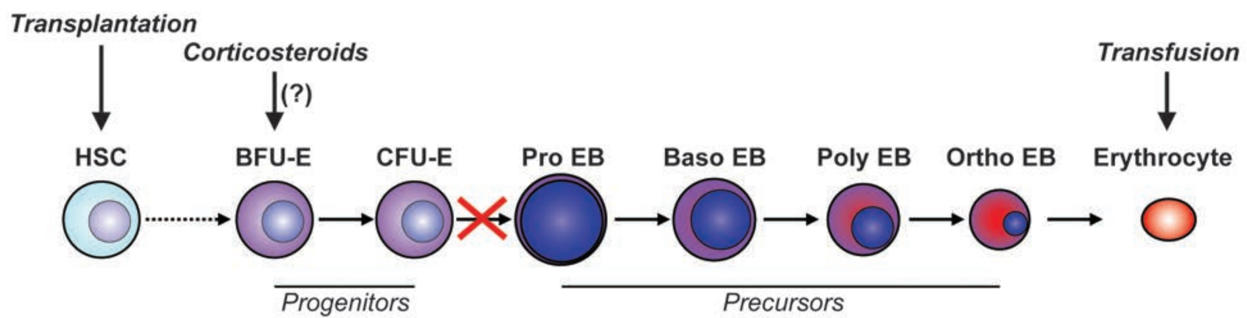

Figure 2. Erythroid development and differentiation. Cells of the erythroid lineage can be divided into erythroid progenitor and precursor cells. Erythroid progenitor cells are distinguished based on their differential growth factor requirements and colony-forming capacity *in vitro*. BFU-E progenitor cells are dependent on SCF and IL-3 signaling, while CFU-E progenitor cells are solely dependent on Epo. In contrast to the erythroid progenitor cells, erythroid precursor cells are recognized based on their morphology, which reflects the accumulation of erythroid-specific proteins, decrease in size and nuclear condensation. Scientific evidence suggests that although there is a proliferation deficiency throughout the hierarchy including at the level of hematopoietic stem cells and early progenitors, the main deficiency is at the level of the CFU-E - proerythroblast transition. The arrows indicate at which level of the hierarchy the different treatment modalities act. Blood and marrow transplantation rebuilds the hematopoietic system from the level of the stem cells and is curative if successful whereas chronic red cell transfusions just treat the anemia temporarily. It is known that corticosteroids increase the self-renewal of BFU-Es and thereby increase the total erythroid output but they may also have additional DBA-specific mechanisms of action. More detailed knowledge about the mechanism of action may allow a reduction in the corticosteroid dose by using other drugs that synergize with corticosteroids in combination.[31]

rapid G1 cell cycle phase, which results in a progressive decrease in the cell size.[33] Simultaneously, maturating precursor cells undergo alterations in morphology that reflect the accumulation of erythroid-specific proteins and nuclear condensation. Orthochromatic erythroblasts withdraw from the cell cycle and form reticulocytes by extruding their nuclei. Reticulocytes loose their mitochondria and ribosomes within a couple of days and mature into erythrocytes.[32] The main intrinsic regulator of erythropoiesis apart from the Epo receptor and c-Kit (SCF receptor) is the transcription factor GATA-1.[34,35]

The hematopoietic defect and cellular mechanisms in DBA

The success of bone marrow transplantation and studies using cultured cells from patients demonstrate the intrinsic cell nature of the hematopoietic defect in DBA. Erythroid progenitor cells are usually present, often in normal numbers, in the marrow of young patients suggesting that the main erythroid failure of DBA results from impaired terminal differentiation of erythroid progenitor cells rather than from their absence.[36,37] Consistent with these studies, Ohene-Abuakwa *et al.* used a liquid erythroid culture system in order to locate the erythroid defect at the onset of Epo-dependent terminal erythroid differentiation.[38] Furthermore, recent studies using mouse models for RPS19-deficient DBA located the most severe erythroid defect at the CFU-E-proerythroblast transition, corroborating the previous findings.[39] Some patients develop hypocellular bone marrow over time and this is often associated with neutropenia and thrombocytopenia.[7] Although the frequency of immature hematopoietic stem and progenitor cells in patients appears normal, their proliferative capacity is significantly lower compared to controls.[7,41] These findings suggest that the hematopoietic defect in DBA involves hematopoietic progenitors or even hematopoietic stem cells (HSCs) resulting in bone marrow failure. Supporting these conclusions are recent findings from an inducible Rps19-deficient mouse model.[39] In this study, transplantation of HSCs derived from mice that had been transiently exposed to Rps19 deficiency led to significantly reduced engraftment in the peripheral blood, demonstrating the irreversible exhaustion of HSCs.[39]

Disease severity and spontaneous remission

Despite recent advances in understanding the molecular basis of DBA, the natural course of the disease remains largely unpredictable. Approximately 20% of the patients enter spontaneous remission, often during the first decade of life, in which physiologically acceptable hemoglobin level is maintained without therapeutic intervention. Interestingly, there appears to be no clear correlation between the chance of remission and the type and duration of the therapy. The failure of the genotype to predict the hematopoietic phenotype is highlighted by the variable penetrance of genetic lesions in DBA pedigrees. However, there is a genotype-phenotype relationship when it comes to orofacial clefts since these are found in patients with *RPL5* and *RPL11* mutations and not in patients with mutated *RPS19*.[13,29]

It is of interest that the vast majority of patients in remission continue to exhibit elevated eADA and macrocytosis.[2,6] These findings suggest a continuous presence of the erythroid defect, which is compensated through extrinsic factors that stimulate the hematopoietic stem and progenitor cells, leading to increased influx of cells into the Epo-responsive stage. Indeed, Ohene-Abuakwa *et al*. demonstrated a consistent erythroid defect of patient cells *in vitro* regardless of the clinical severity.[38] Intriguingly, a similar defect was observed when culturing cells from asymptomatic first-degree relatives who shared the genetic lesion. Relapses tend to occur under conditions of hematopoietic stress, such as pregnancy, indicating the importance of the dynamics of the hematopoietic system in determining whether the patient is symptomatic or not.[41] Presentation of anemia in DBA normally coincides with the neonatal decline in HSC turnover.[42] Dynamics of the hematopoietic system could also directly influence the severity of the cellular defect of DBA. This is supported by the fact that the chance of relapse in remitted patients appears low, except during stress conditions.

5q minus syndrome

MDS comprise a heterogeneous group of clonal disorders characterized by dysplastic bone marrow and peripheral cytopenia. The 5q- syndrome is a distinct subtype of MDS, defined by an isolated interstitial deletion of chromosome 5q, and is characterized by macrocytic anemia, normal or elevated platelet counts, dysplastic megakaryocytes and elevated risk of AML.[43] Most patients respond to the treatment with lenalidomide, resulting in reduced transfusion requirement that is often combined with a complete cytogenetic response.[44] The 5q- common deleted region encompasses forty protein-coding genes.[45] By a systematic targeting of each gene using the short hairpin RNA (shRNA) technology, Ebert *et al*. identified *RPS14* as the critical gene for the erythroid phenotype.[46] Therefore, a similar mechanism underlies the erythroid phenotype in both 5q minus syndrome and DBA.

The molecular pathology in DBA

With the exception of a few DBA patients with *GATA1* mutations, all the identified mutations in DBA are found in ribosomal proteins. Therefore, defects in ribosome biogenesis are considered the key pathogenic mechanism in DBA. However, it is still not yet fully understood why the main phenotype, ineffective erythropoiesis, is relatively tissue-specific since ribosomal proteins have a generic function in all cell types. Below, we will discuss ribosomal stress, a possible role for p53, and the regulation of protein translation as possible molecular mechanisms causing the DBA phenotype.

Ribosome biogenesis and ribosomal stress

Ribosome biogenesis takes place in a specialized nuclear compartment, the nucleolus, which is formed around the actively transcribed rRNA genes. Transcription of rRNA genes by RNA polymerase I gives rise to a 47S precursor rRNA (pre-rRNA), which simultaneously asso-

ciates with trans-acting factors to form the 90S pre-ribosome. After a series of remodeling and pre-rRNA processing, 90S pre-ribosome splits into pre-40S and pre-60S subunits that are exported into the cytoplasm where the final maturation steps occur.[47,48] The modified pre-rRNA undergoes hierarchical endonucleolytic and exonucleolytic cleavages, eventually giving rise to 18S, 28S and 5.8S mature rRNAs.[47] Ribosomal proteins assemble with pre-rRNA in a hierarchical manner and facilitate its processing, nuclear export and cytoplasmic maturation, and deficiency of ribosomal proteins impairs the rRNA processing at distinct stages.[49-51] Perturbations to the dynamics and flow of this process have been associated with alterations in the regulation of cell size and cell cycle progression, leading to developmental defects and increased cancer susceptibility.[52] Pharmacological or genetic disruption of rRNA transcription and processing has shown to result in the activation of the tumor suppressor p53.[53-56] Similarly, numerous studies have demonstrated the activation of p53 in response to ribosomal protein deficiencies.[57,58] During normal growth conditions, the activity of p53 is kept low by the oncoprotein mouse double minute 2 (Mdm2). In the absence of stress, Mdm2 binds to p53 and functions as an ubiquitin ligase, targeting p53 for proteosomal degradation. Various cellular stresses disrupt the interaction between Mdm2 and p53, resulting in the stabilization and activation of p53. In case of ribosomal stress, impaired rRNA synthesis or processing leads to nuclear accumulation of free ribosomal proteins, which are able to bind to Mdm2 and inhibit its ubiquitin ligase function, resulting in the accumulation of p53 (Figure 3). Although multiple ribosomal proteins have been shown to interact with Mdm2, the recent evidence suggests that only RPL5 and RPL11, in a mutually dependent manner, are required for Mdm2 inhibition.[58]

Disease models suggest a role for p53

Several animal models with reduced expression of ribosomal proteins have been generated to define the role of ribosomal proteins in hematopoiesis and generate model systems for DBA (reviewed in McGowan and Mason[59]). rps19-deficient zebrafish models were generated using morpholino technology.[60,61] These models showed developmental and hematologic abnormalities. Furthermore, the loss of p53 rescued the phenotypic abnormalities observed upon rps19 haploinsufficiency.[60] In 2008,

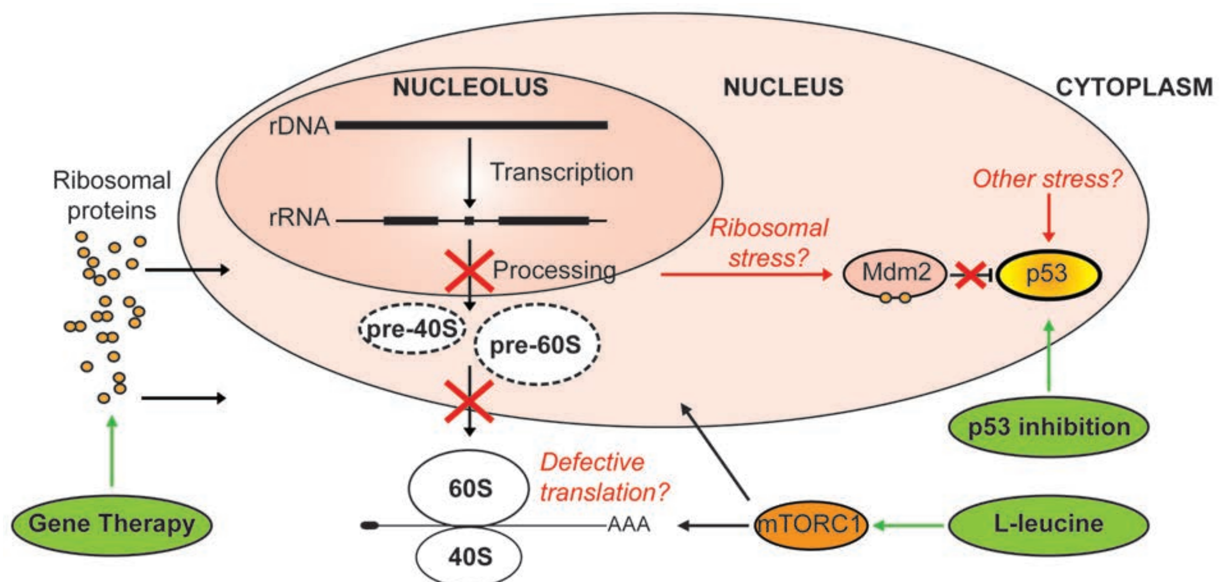

Figure 3. Ribosome biogenesis, ribosomal stress and development of possible mechanism-based therapies. Transcription of rDNA by RNA polymerase I gives rise to a 47S rRNA precursor, which associates with trans-acting factors that mediate a series of chemical modifications and nucleolytic cleavages. This results in the formation of pre-40S and pre-60S ribosomal subunits that are exported into the cytoplasm where the final maturation takes place. Ribosomal proteins associate with pre-rRNA in a hierarchical manner and facilitate its processing, nuclear export and maturation. Deficiency of ribosomal proteins impairs rRNA processing (indicated by the red X). Ribosomal stress is believed to be a key pathogenic mechanism in DBA. During steady state conditions, the levels of p53 are kept low through proteosomal degradation by Mdm2. Impaired rRNA synthesis or processing leads to nuclear accumulation of free ribosomal proteins, which are able to bind to Mdm2 and inhibit its ubiquitin ligase function, resulting in the accumulation of p53.58 The figure also shows three possible mechanistic approaches to treat DBA. Gene replacement therapy will cure the hematologic disease. Reduction in p53 activity will improve erythropoiesis in DBA although p53 reduction is not without risks. L-Leucine can activate the mTORC1 pathway. mTORC1 regulates ribosome biogenesis by promoting rRNA and ribosomal protein synthesis and enhancing translation initiation and elongation. Of these three possible approaches, L-Leucine therapy is least likely to cause severe side effects. Clinical trials using L-Leucine are under way.

McGowan *et al.* reported a novel mouse model for RPS19-deficient DBA that presents a missense mutation resulting in a single amino acid substitution in the Rps19 protein.[62] This mutation was embryonic lethal in a homozygous setting. However, the heterozygous mice exhibited dark skin, retarded growth and a mild macrocytic anemia with a reduction in erythrocyte number. Importantly, all of these features were rescued in a p53-deficient background. RPS19 can be down-regulated in hematopoietic cells using knockdown technology to generate a DBA-like phenotype *in vitro*.[63-65] This approach was taken to generate mouse models with inducible and graded downregulation of Rps19.[38] Depending on the level of Rps19 downregulation, mice with mild to lethal macrocytic anemia could be generated. Strikingly, crossing these mice into p53-deficient background almost fully corrected the lethal hematopoietic phenotype.[38]

As the studies using DBA animal models demonstrate an activation of p53 in response to ribosomal protein deficiencies, it is tempting to speculate that the erythroid failure in DBA patients is caused through p53-dependent mechanisms. Recently, downregulation of RPS19 or RPS14 in primary human bone marrow cells was shown to result in the erythroid-pronounced activation of p53.[66] Furthermore, the treatment of bone marrow cells with nutlin-3, a compound that activates p53 by preventing its interaction with Mdm2, led to an erythroid-biased activation of p53. Finally, inhibition of p53 with a small molecule pifithrin alpha rescued the erythroid defect in RPS19-deficient and RPS14-deficient human bone marrow cell cultures. Immunohistochemistry for p53 in the bone marrow biopsies from DBA patients demonstrated elevated levels of p53, although variation was observed in terms of the intensity and cell type-specificity of p53 staining.[66] However, a generic defect in ribosomal biogenesis may influence the translational apparatus in cells and influence other regulatory pathways than just p53.

Translational defects

Ribosomal protein haploinsufficiency has been shown to result in reduced rate of protein synthesis.[67] However, whether the global reduction in translation contributes to the severe anemia of DBA is not known. Studies in mice deficient for Flvcr, a heme exporter protein, have led to a hypothesis that defective globin synthesis contributes to the erythroid defect of DBA.[68] These findings suggest that the accumulation of free heme in proerythroblasts is toxic, raising a hypothesis that the dysregulation of heme synthesis and globin translation, resulting in a transient excess of free heme, could in part explain the erythroid defect of DBA.

Development of future therapies

Lenalidomide

Lenalidomide has proven to be highly effective in the treatment of patients with 5q- syndrome, causing both hematologic and cytogenetic responses.[44] Although the underlying mechanism remains elusive, lenalidomide has been reported to promote the erythroid differentiation of human CD34-positive bone marrow cells and the production of fetal hemoglobin.[69] This is due to its ability to stimulate CFU-E progenitor cells, possibly through the modulation the Epo receptor turnover.[70,71] As corticosteroids and lenalidomide promote erythropoiesis at distinct stages, use of these agents in combination could provide a more profound therapeutic effect in DBA.[70]

L-Leucine

Recently, based on the theory of inefficient translation as the underlying cause for the severe anemia in DBA, Pospisilova *et al*. reported one patient who became transfusion-independent in response to treatment with the amino acid L-leucine.[14] Similarly, L-leucine administration alleviated the developmental defects and in some cases also the anemia of rps19-deficient and rps14-deficient zebrafish models.[15] Furthermore, dietary L-leucine was shown to improve the anemia of Rps19-deficient mice.[16] L-leucine is an essential branched chain amino acid that plays an important role in the regulation of protein synthesis, and this response involves the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) pathway.[72] Thus the enhanced translation of ribosomal proteins could underlie the therapeutic effect of L-leucine. Irrespective of the mechanism, several large clinical trials are now ongoing or about to start. The future outcome of these trials could be exciting since the side effects of L-Leucine, if used in the correct dose, are expected to be relatively modest compared to the potential toxic effects of corticosteroids.

Targeting the p53 pathway

Based on the current experimental findings, it is tempting to speculate that the erythroid defect in DBA is largely caused through a p53-dependent mechanism. The identification of p53 could provide a novel therapeutic avenue for the treatment of DBA and related disorders. Inhibition of p53 with a small molecule pifithrin alpha rescues the erythroid defect of RPS19-deficient and RPS14-deficient human bone marrow cell cultures.[66] Indeed, a transient dampening of the p53 pathway could provide a therapeutic benefit in patients. However, direct interference with p53 raises concerns because of its role as a tumor suppressor. Strategies targeting disease-specific factors either upstream or downstream of p53 could provide a more promising alternative.

Gene therapy

Gene therapy is the only approach apart from allogeneic transplantation that can cure the hematopoietic defect in DBA. In a recent proof-of-principle experiment, the lethal bone marrow failure in Rps19-deficient mice could be cured by gene therapy.[73] However, as the current therapies, especially those with corticosteroids, have a relatively good outcome, moving gene therapy to the clinic will require a careful assessment of the risk-benefit ratio for this approach. We envisage that the first clinical trials could be applied to patients with a chronic transfusion-dependent DBA. Lentiviral vectors, in which the potent spleen focus-forming vector (SFFV) promoter drives the expression of codon-optimized human *RPS19* cDNA, were used to correct the DBA phenotype in mice.[73] However, for future clinical application, more moderate cellular promoters must be validated, as they are potentially safer with regards to the probability of insertional mutagenesis. Clinical trials for Fanconi anemia employing similar lentiviral vectors, in which the *PGK* promoter drives the expression of *FANCA* cDNA, are

being conducted.[74] However, the elongation factor 1α (EF1α) short promoter may prove to be an even more viable alternative.[75] Furthermore, a lentiviral vector utilizing the EF1α promoter combined with the locus control region of β-globin has been shown to allow a constitutive but erythroid-pronounced transgene expression.[76] The safety and efficacy of ongoing clinical trials using lentiviral vectors to treat disorders other than DBA will largely determine the future of DBA gene therapy. Although the follow-up time for these trials is still relatively short, no severe genotoxic side effects have been reported.[77] The development of a human gene therapy protocol for RPS19-deficient DBA is estimated to take approximately five years

References

1. Shimamura A, Alter BP. Pathophysiology and management of inherited bone marrow failure syndromes. Blood Rev. 2010;24:101-22.
2. Willig TN, Niemeyer CM, Leblanc T, Tiemann C, Robert A, Budde J, et al. Identification of new prognosis factors from the clinical and epidemiologic analysis of a registry of 229 Diamond-Blackfan anemia patients. DBA group of Société d'Hématologie et d'Immunologie Pédiatrique (SHIP), Gesellshaft für Pädiatrische Onkologie und Hämatologie (GPOH), and the European Society for Pediatric Hematology and Immunology (ESPHI). Pediatr Res. 1999;46:553-61.
3. Lipton JM, Atsidaftos E, Zyskind I, Vlachos A. Improving clinical care and elucidating the pathophysiology of Diamond Blackfan anemia: an update from the Diamond Blackfan Anemia Registry. Pediat Blood Cancer. 2006; 46:558-64.
4. Diamond LK, Wang WC, Alter BP. Congenital hypoplastic anemia. Adv Pediatr. 1976;22: 349-78.
5. Glader BE, Backer K, Diamond LK. Elevated erythrocyte adenosine deaminase activity in congenital hypoplastic anemia. N Engl J Med. 1983;309:1486-90.
6. Orfali KA, Ohene-Abuakwa Y, Ball SE. Diamond Blackfan anaemia in the UK: clinical and genetic heterogeneity. Br J Haematol. 2004;125:243-52.
7. Giri N, Kang EM, Tisdale JF, Follman D, Rivera M, Schwartz GN, et al. Clinical and laboratory evidence for a trilineage haematopoietic defect in patients with refractory Diamond-Blackfan anaemia. Br J Haematol. 2000;108:167-75.
8. Ball SE, McGuckin CP, Jenkins G, Gordon-Smith EC. Diamond-Blackfan anaemia in the U.K.: analysis of 80 cases from a 20-year birth cohort. Br J Haematol. 1996;94:645-53.
9. Ramenghi U, Garelli E, Valtolina S, Campagnoli MF, Timeus F, Crescenzio N, et al. Diamond-Blackfan anaemia in the Italian population. Br J Haematol. 1999;104:841-8.
10. Alter BP, Giri N, Savage SA, Peters JA, Loud JT, Leathwood L, et al. Malignancies and survival patterns in the National Cancer Institute inherited bone marrow failure syndromes cohort study. Br J Haematol. 2010;150:179-88.
11. Vlachos A, Rosenberg PS, Atsidaftos E, Alter BP, Lipton JM. Incidence of neoplasia in Diamond Blackfan anemia: a report from the Diamond Blackfan Anemia Registry. Blood. 2012;119:3815-9.
12. Vlachos A, Ball SE, Dahl N, Alter BP, Sheth S, Ramenghi U, et al. Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. Br J Haematol. 2008;142:859-76.
13. Vlachos A, Muir E. How I treat Diamond-Blackfan anemia. Blood. 2010;116:3715-23.
14. Pospisilova D, Cmejlova J, Hak J, Adam T, Cmejla R. Successful treatment of a Diamond-Blackfan anemia patient with amino acid leucine. Haematologica. 2007;92:66-7.
15. Payne E, Virgilio M, Narla A, Sun H, Levine M, Paw BH, et al. L-Leucine improves anemia and developmental defects associated with Diamond-Blackfan anemia and del(5q)MDS by activating the mTOR pathway. Blood. 2012;120:2214-24.
16. Jaako P, Debnath S, Olsson K, Bryder D, Flygare J, Karlsson S. Dietary L-leucine improves the anemia in a mouse model for Diamond-Blackfan anemia. Blood. 2012;120:2225-8.
17. Draptchinskaia N, Gustavsson P, Andersson B, Pettersson M, Willig TN, Dianzani I, et al. The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nat Genet. 1999;21:169-75.
18. Gazda HT, Grabowska A, Merida-Long LB, Latawiec E, Schneider HE, Lipton JM, et al. Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. Am J Hum Genet. 2006;79:1110-8.
19. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. Hum Mutat. 2007;28:1178-82.
20. Farrar JE, Nater M, Caywood E, McDevitt MA, Kowalski J, Takemoto CM, et al. Abnormalities of the large ribosomal subunit protein, Rpl35A, in diamond-blackfan anemia. Blood. 2008;112:1582-92.
21. Gazda HT, Sheen MR, Vlachos A, Choesmel V, O'Donohue MF, Schneider H, et al. Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. Am J Hum Genet. 2008;83:769-80.
22. Doherty L, Sheen MR, Vlachos A, Choesmel V, O'Donohue MF, Clinton C, et al. Ribosomal protein genes RPS10 and RPS26 are commonly mutated in Diamond-Blackfan anemia. Am J Hum Genet. 2010;86:222-8.
23. Farrar JE, Vlachos A, Atsidaftos E, Carlson-Donohoe H, Markello TC, Arceci RJ, et al. Ribosomal protein gene deletions in Diamond-Blackfan anemia. Blood. 2011;118:6943-51.
24. Gazda HT, Preti M, Sheen MR, O'Donohue MF, Vlachos A, Davies SM, et al. Frameshift mutation in p53 regulator RPL26 is associated with multiple physical abnormalities and a specific pre-ribosomal RNA processing defect in diamond-blackfan anemia. Hum Mutat. 2012;33:1037-44.
25. Kuramitsu M, Sato-Otsubo A, Morio T, Takagi M, Toki T, Terui K, et al. Extensive gene deletions in Japanese patients with Diamond-Blackfan anemia. Blood. 2012;119:2376-84.
26. Boria I, Quarello P, Avondo F, Garelli E, Aspesi A, Carando A, et al. A new database for ribosomal protein genes which are mutated in Diamond-Blackfan Anemia. Hum Mutat. 2008; 29:263-70.
27. Farrar JE, Dahl N. Untangling the phenotypic heterogeneity of Diamond Blackfan anemia. Semin Hematol. 2011;48:124-5.
28. Devlin EE, Dacosta L, Mohandas N, Elliott G, Bodine DM. A transgenic mouse model demonstrates a dominant negative effect of a point mutation in the RPS19 gene associated with Diamond-Blackfan anemia. Blood. 2010;116:2826-35.
29. Boria I, Garelli E, Gazda HT, Aspesi A, Quarello P, Pavesi E, et al. The ribosomal basis of Diamond-Blackfan anemia: mutation and database update. Hum Mutat. 2010;31:1269-79.
30. Sankaran VG, Ghazvinian R, Do R, Thiru P, Vergilio JA, Beggs AH, et al. Exome sequencing identifies GATA1 mutations resulting in Diamond-Blackfan anemia. J Clin Invest. 2012;122:2439-43.
31. Flygare J, Rayon Estrada V, Shin C, Gupta S, Lodish HF. HIF1alpha synergizes with glucocorticoids to promote BFU-E progenitor self-renewal. Blood. 2011;117:3435-44.
32. Testa U. Apoptotic mechanisms in the control of erythropoiesis. Leukemia. 2004;18:1176-99.
33. Von Lindern M. Cell-cycle control in erythropoiesis. Blood. 2006;108:781-2.
34. Cantor AB, Orkin SH. Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene. 2002;21:3368-76.
35. Hattangadi SM, Wong P, Zhang L, Flygare J, Lodish HF. From stem cell to red cell: regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. Blood. 2011;118:6258-68.
36. Lipton JM, Kudisch M, Gross R, Nathan DG. Defective erythroid progenitor differentiation system in congenital hypoplastic (Diamond-Blackfan) anemia. Blood. 1986;67: 962-8.
37. Casadevall N, Croisille L, Auffray I, Tchernia G, Coulombel L. Age-related alterations in erythroid and granulopoietic progenitors in Diamond-Blackfan anaemia. Br J Haematol. 1994;87:369-75.
38. Ohene-Abuakwa Y, Orfali KA, Marius C, Ball SE. Two-phase culture in Diamond Blackfan anemia: localization of erythroid defect. Blood. 2005;105:838-46.
39. Jaako P, Flygare J, Olsson K, Quere R, Ehinger M, Henson A, et al. Mice with ribosomal protein S19 deficiency develop bone marrow failure and symptoms like patients with Diamond-Blackfan anemia. Blood. 2011;118:6087-96.
40. Hamaguchi, I, Flygare J, Nishiura H, Brun AC, Ooka A, Kiefer T, et al. Proliferation deficiency of multipotent hematopoietic progenitors in ribosomal protein S19 (RPS19)-deficient diamond-Blackfan anemia improves following RPS19 gene transfer. Mol Ther. 2003;7:613-22.
41. Faivre L, Meerpohl J, Da Costa L, Marie I, Nouvel C, Gnekow A, et al. High-risk pregnancies in Diamond-Blackfan anemia:

a survey of 64 pregnancies from the French and German registries. Haematologica. 2006;91:530-3.
42. Rufer N, Brümmendorf TH, Kolvraa S, Bischoff C, Christensen K, Wadsworth L, et al. Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. J Exp Med. 1999;190:157-67.
43. Giagounidis AA, Germing U, Haase S, Hildebrandt B, Schlegelberger B, Schoch C, et al. Clinical, morphological, cytogenetic, and prognostic features of patients with myelodysplastic syndromes and del(5q) including band q31. Leukemia. 2004;18:113-9.
44. List A, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. N Engl J Med. 2006;355:1456-65.
45. Boultwood J, Fidler C, Strickson AJ, Watkins F, Gama S, Kearney L, et al. Narrowing and genomic annotation of the commonly deleted region of the 5q- syndrome. Blood. 2002;99:4638-41.
46. Ebert BL, Lee MM, Pretz JL, Subramanian A, Mak R, Golub TR, et al. An RNA interference model of RPS19 deficiency in Diamond-Blackfan anemia recapitulates defective hematopoiesis and rescue by dexamethasone: identification of dexamethasone-responsive genes by microarray. Blood. 2005;105:4620-6.
47. Hadjiolova KV, Nicoloso M, Mazan S, Hadjiolov AA, Bachellerie JP. Alternative pre-rRNA processing pathways in human cells and their alteration by cycloheximide inhibition of protein synthesis. Eur J Biochem. 1993;212:211-5.
48. Rouquette J, Choesmel V, Gleizes PE. Nuclear export and cytoplasmic processing of precursors to the 40S ribosomal subunits in mammalian cells. EMBO J. 2005;24:2862-72.
49. O'Donohue MF, Choesmel V, Faubladier M, Fichant G, Gleizes PE. Functional dichotomy of ribosomal proteins during the synthesis of mammalian 40S ribosomal subunits. J Cell Biol. 2010;190:853-6.
50. Flygare J, Aspesi A, Bailey JC, Miyake K, Caffrey J, Karlsson S, et al. Human RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. Blood. 2007;109:980-6.
51. Choesmel V, Bacqueville D, Rouquette J, Noaillac-Depeyre J, Fribourg S, Crètien A, et al. Impaired ribosome biogenesis in Diamond-Blackfan anemia. Blood. 2007;109:1275-83.
52. Ruggero D, Pandolfi PP. Does the ribosome translate cancer? Nat Rev Cancer. 2003;3:179-82.
53. Andera L, Wasylyk B. Transcription abnormalities potentiate apoptosis of normal human fibroblasts. Mol Med. 1997;3:852-63.
54. Pritchard DM, Watson AJ, Potten CS, Jackman AL, Hickman JA. Inhibition by uridine but not thymidine of p53-dependent intestinal apoptosis initiated by 5-fluorouracil: evidence for the involvement of RNA perturbation. Proc Natl Acad Sci USA. 1997;94:1795-9.
55. Yuan X, Zhou Y, Casanova E, Chai M, Kiss E, Gröne HJ, et al. Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis. Mol Cell. 2005;19:77-87.
56. Pestov DG, Strezoska Z, Lau LF, Evidence of p53-dependent cross-talk between ribosome biogenesis and the cell cycle: effects of nucleolar protein Bop1 on G(1)/S transition. Mol Cell Biol. 2001;21:4246-55.
57. Sulic S, Panic L, Barkic M, Mercep M, Uzelac M, Volarevic S. Inactivation of S6 ribosomal protein gene in T lymphocytes activates a p53-dependent checkpoint response. Genes Dev. 2005;19:3070-82.
58. Fumagalli S, Ivanenkov VV, Teng T, Thomas G. Suprainduction of p53 by disruption of 40S and 60S ribosome biogenesis leads to the activation of a novel G2/M checkpoint. Genes Dev. 2010;26:1028-40.
59. McGowan KA, Mason PJ. Animal models in Diamond Blackfan anemia. Semin Hematol. 2011M48:106-16.
60. Danilova N, Sakamoto K, Lin S. Ribosomal protein S19 deficiency in zebrafish leads to developmental abnormalities and defective erythropoiesis through activation of p53 protein family. Blood. 2008;112:5228-37.
61. Uechi T, Nakajima Y, Chakraborty A, Torihara H, Higa S, Kenmochi N. Deficiency of ribosomal protein S19 during early embryogenesis leads to reduction of erythrocytes in a zebrafish model of Diamond-Blackfan anemia. Hum Mol Genet. 2008;17:3204-11.
62. McGowan KA, Li JZ, Park CY, Beaudry V, Tabor HK, Sabnis AJ, et al. Ribosomal mutations cause p53-mediated dark skin and pleiotropic effects. Nat Genet. 2008;40:963-70.
63. Flygare J, Kiefer T, Miyake K, Utsugisawa T, Hamaguchi I, Da Costa L, et al. Deficiency of ribosomal protein S19 in CD34+ cells generated by siRNA blocks erythroid development and mimics defects seen in Diamond-Blackfan anemia. Blood. 2005;105:4627-34.
64. Ebert BL, Lee MM, Pretz JL, Subramanian A, Mak R, Golub TR, et al. An RNA interference model of RPS19 deficiency in Diamond-Blackfan anemia recapitulates defective hematopoiesis and rescue by dexamethasone: identification of dexamethasone-responsive genes by microarray. Blood. 2005;105:4620-6.
65. Miyake K, Flygare J, Kiefer T, Utsugisawa T, Richter J, Ma Z, et al. Development of cellular models for ribosomal protein S19 (RPS19)-deficient diamond-blackfan anemia using inducible expression of siRNA against RPS19. Mol Ther. 2005;11:627-37.
66. Dutt S, Narla A, Lin K, Mullally A, Abayasekara N, Megerdichian C, et al. Haploinsufficiency for ribosomal protein genes causes selective activation of p53 in human erythroid progenitor cells. Blood. 2011;117:2567-76.
67. Cmejlova J, Dolezalova L, Pospisilova D, Petrtylova K, Petrak J, Cmejla R. Translational efficiency in patients with Diamond-Blackfan anemia. Haematologica. 2006;91:1456-64.
68. Keel SB, Doty RT, Yang Z, Quigley JG, Chen J, Knoblaugh S, et al. A heme export protein is required for red blood cell differentiation and iron homeostasis. Science. 2008;319:825-8.
69. Moutouh-de Parseval LA, Verhelle D, Glezer E, Jensen-Pergakes K, Ferguson GD, Corral LG, et al. Pomalidomide and lenalidomide regulate erythropoiesis and fetal hemoglobin production in human CD34+ cells. J Clin Invest. 2008;118:248-8.
70. Narla A, Dutt S, McAuley JR, Al-Shahrour F, Hurst S, McConkey M, et al. Dexamethasone and lenalidomide have distinct functional effects on erythropoiesis. Blood. 2011;118:2296-304.
71. Basiorka AA, McGraw K, Clark J, Caceres G, Johnson J, Hall L, et al. Lenalidomide upregulates erythropoietin receptor expression in hematopoietic progenitors by modulating receptor turnover. Blood. 2011;118:2382.
72. Stipanuk MH. Leucine and protein synthesis: mTOR and beyond. Nutr Rev. 2007;65:122-9.
73. Jaako P, Debnath S, Olsson K, Schambach A, Baum C, Flygare J, Karlsson S. Gene therapy corrects the anemia and lethal bone marrow failure in mouse model for RPS19-deficient Diamond-Blackfan anemia. Blood. 2012;120:513.
74. Tolar J, Becker PS, Clapp DW, Hanenberg H, de Heredia CD, Kiem HP, et al. Gene therapy for fanconi anemia: one step closer to the clinic. Hum Gene Ther. 2012;23:141-4.
75. Zychlinski D, Schambach A, Modlich U, Maetzig T, Meyer J, Grassman E, et al. Physiological promoters reduce the genotoxic risk of integrating gene vectors. Mol Ther. 2008;16:718-25.
76. Montiel-Equihua CA, Zhang L, Knight S, Saadeh H, Scholz S, Carmo M, et al. The β-globin locus control region in combination with the EF1α short promoter allows enhanced lentiviral vector-mediated erythroid gene expression with conserved multilineage activity. Mol Ther. 2012;20:1400-9.
77. Biffi A, Bartolomae CC, Cesana D, Cartier N, Aubourg P, Ranzani M, et al. Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. Blood. 2011;117:5332-9.

Optimizing hematopoetic stem cell transplantation for bone marrow failure syndromes

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A B S T R A C T

Hematopoietic stem cell transplantation for marrow failure is overall a huge success story of modern medicine with 70%-90% long-term survival achieved in these patients. First-line treatment for acquired marrow failure includes immunosuppression with antithymocyte globulin and cyclosporine as well as marrow transplantation, and decision algorithms are useful to determine appropriate approaches. A series of prospective and observational studies have determined current standards for transplantation in patients with an HLA-identical sibling donor, and it is against these standards that all future progress has to be measured. In recent years, availability of well-matched unrelated donors has increased dramatically and results of unrelated donor transplantation are approaching those with sibling donor transplantation. This is in sharp contrast to results in the 1990s. In patients without a matched sibling or unrelated donor, alternative approaches, including cord blood transplants and transplants from haploidentical donors, are discussed.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe standard indications and procedures for allogeneic stem cell transplantation in aplastic anemia;
- determine the appropriate timing to start an unrelated donor search for patients with marrow failure;
- discuss choice of stem cell source for transplantation in patients with marrow failure.

Introduction

Marrow failures include aplastic anemia (AA), generally considered an autoimmune disease. This needs to be differentiated from hypoplastic MDS and PNH with aplasia. A number of congenital diseases are part of the marrow failure world. Most prominent are failures of DNA repair such as Fanconi anemia and much rarer failure of ribosomal apparatus e.g. Diamond Blackfan anemia and Schwachman-Diamond anemia, or of telomere elongation such as dyskeratosis congenita. In the latter congenital disorders, hematopoietic stem cell transplantation (HSCT) is often a consideration. HSCT will not correct the underlying congenital disease but can correct the marrow failure. Indications to transplant patients with congenital marrow failure and use of transplant technology should be reserved for specialized centers because of susceptibilities to toxicity and secondary cancers. This review will, therefore, focus on acquired marrow failure. Figure 1A and B shows the absolute numbers of allogeneic HSCT for marrow failure as reported in the activity survey of the European Group for Blood and Marrow Transplantation (EBMT). Separate lines are drawn for HSCT from sibling and unrelated donors, and separate graphs for AA and other marrow failure syndromes, the most important of these being Fanconi anemia. As is evident, in Europe, there has been a continuous increase in HSCT for these indications in the period 2004-2011.

Aplastic anemia (AA) is defined as pancytopenia with a hypocellular marrow. The incidence of acquired aplastic anemia in the Western hemisphere is around 1-2 per million of the population per year, and this is higher in East Asia. Age distribution shows peaks in children and young adults, and in patients over 60 years of age. Patients with AA commonly present with anemia and hemorrhage, or neutropenic infection. Diagnosis may not be very clear at the outset. Treatment decisions are complex, a watch and wait strategy is often used initially in cases of unexplained pancytopenia, but a prolonged interval from diagnosis to treatment is associated with worse outcome.[1] Prior to treatment, the patient should be stable in terms of controlling bleeding and treating infection. Once disease is confirmed, disease severity has been assessed, and family typing performed, it is time to initiate treatment.

First-line treatment

The decision for first-line treatment will depend on patient age, availability of an HLA-identical sibling donor, and, in part, on the severity of the disease.[2] Family HLA typing is, therefore, recommended at first suspicion of the disease. The standard first-line treatment for a newly diagnosed patient with AA is either allogeneic bone marrow transplantation (BMT) from an HLA-identical sibling donor or immunosuppressive therapy (IST) with a combination of ATG and cyclosporine A (ATG+CSA), with younger age and more severe disease favoring HSCT, and older age and less severe disease favoring IST as first-line treatment. Allogeneic BMT from an HLA-identical sibling donor is recommended as first-line treatment if the disease is severe or very severe, and if the patient is younger than 40-50 years of age.

HLA-identical sibling donor transplantation:

Transplantation for AA from an HLA-identical sibling donor has improved considerably over the years with a 75%-80% chance of long-term cure in more recent cohorts. Unresolved issues are: graft failure rates of 4%-14% including late graft failure, and graft-*versus*-host disease (GvHD), severe acute GvHD (grade III-IV) (which appears to occur less commonly now) and chronic GvHD, which still occurs in 3%-40% of patients.

There is controversy concerning the upper age limit for BMT as a first-line treatment as results vary in different case series. In more recent cohorts of patients reported to the EBMT, outcome of patients in the 20-30, 30-40 and 40-50 year age groups tend to be similar. The advantage of treating a patient with IST and transplanting only in case of IST failure is appealing, but outcome in patients undergoing transplantation after failing IST is worse than undergoing transplantation upfront.[3] In this study, the hazard ratio for mortality was 1.7 in patients receiving a transplant as part of a second-line treatment as compared to patients with up-front transplantation. For patients with an HLA-identical sibling donor in whom transplantation is not used as first-line treatment, BMT remains an option as second-line treatment in the case of IST failure.

Optimal transplantation strategies for HLA-identical sibling BMT are defined. It is recommended to use bone marrow stem cells rather than G-CSF mobilized peripheral blood stem cells. In retrospective studies, earlier engraftment occurred with peripheral blood but survival was worse with more chronic GVHD, using peripheral blood compared with bone marrow.[1,4] Figure 2 shows acute and chronic GvHD in recipients of peripheral blood and marrow transplants from sibling donors.[1] The effect of sex-mismatch between donor and recipient has been evaluated and shows better survival in patients with donors from the same gender. Male patients with female donors had risks of acute GvHD increased by 33% as compared to male-into-male transplant patients; female patients with male donors had increased risks of graft rejection.[5] The conditioning regimens and GVHD prophylaxis described below refer specifically to patients with acquired AA. In younger patients with AA, the standard conditioning proposed is cyclophosphamide 50 mg/kg x 4 + antithymocyte globulin

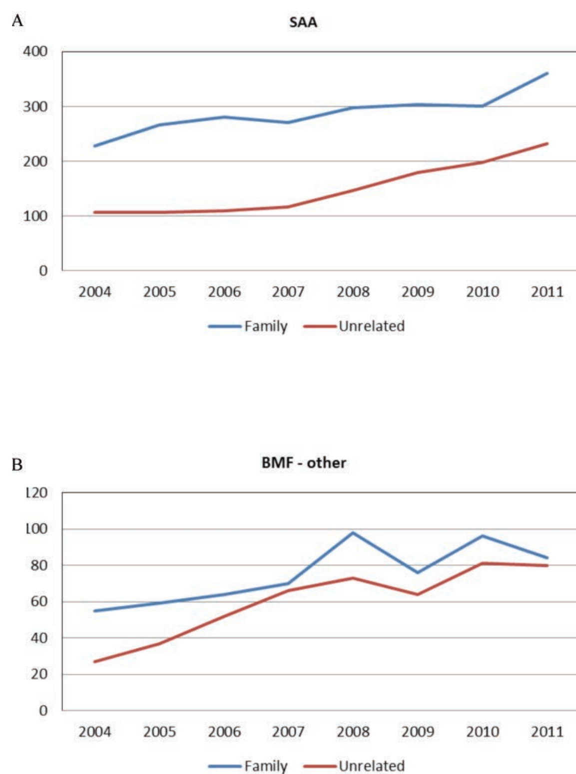


Figure 1. Data from the Activity Survey of the European Group of Blood and Marrow Transplantation. Frequencies of allogeneic Transplants for (A) severe aplastic anemia (SAA) and (B) other marrow failure syndromes (BMF-other) most of which are Fanconi Anemia.

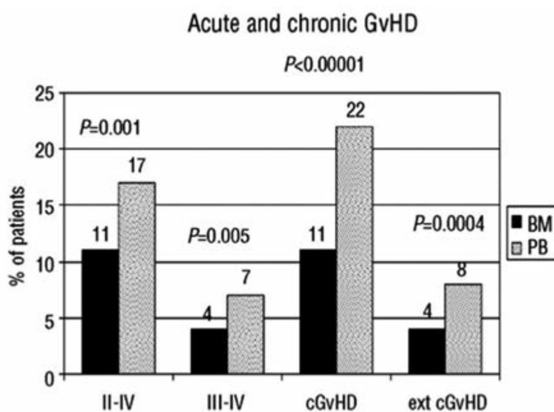
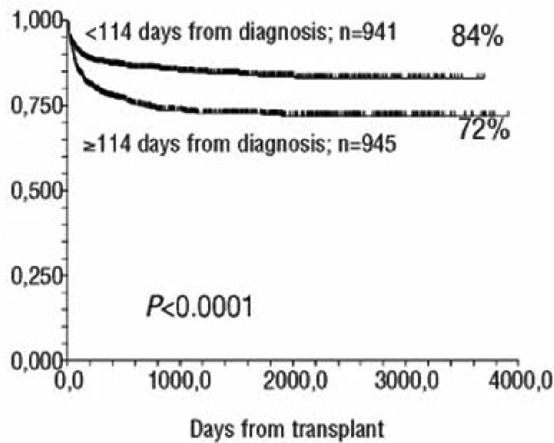
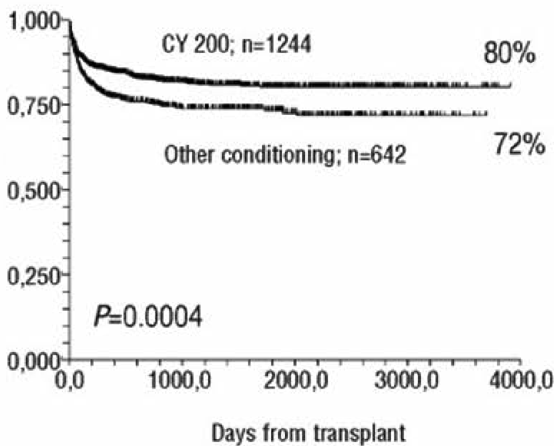


Figure 2. Sibling donor transplantation, differences in acute and chronic graft-versus-host (GvHD) disease by stem cell source comparing bone marrow versus peripheral blood.[1]





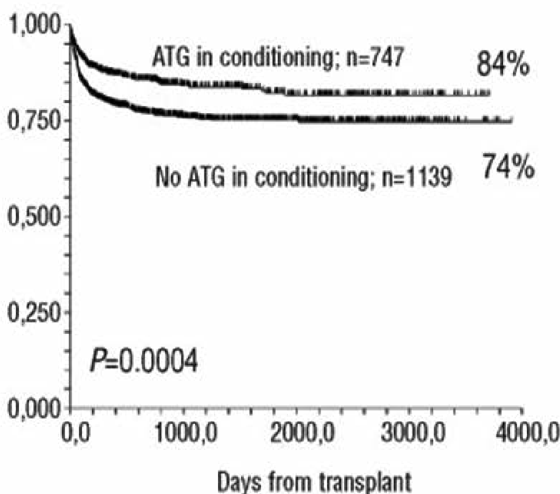


Figure 3. Survival of patients receiving sibling donor transplantation for aplastic anemia by disease duration prior to transplant, and use of conditioning regimens with cyclophosphamide and antithymocyte globulin.[1]

(ATG). This regimen is highly immunosuppressive to prevent graft rejection and GVHD. The benefit of adding ATG to cyclophosphamide is unclear, but a retrospective study showed better survival in recipients of ATG[1] (Figure 3). The recommended post-transplant immunosuppression is cyclosporine A (CSA) continued for at least 12 months with slow tapering and short course methotrexate, the superiority of the combination having been confirmed in a randomized controlled trial (RCT). Because of unsatisfactory results with older patients (e.g. >30 or >40 years of age) with sibling donor transplantation, and the fact that most of these patients received a transplant not as first- but as second-line treatment having a longer interval from diagnosis to transplantation and a higher transfusional load, several groups have tried to modify conditioning by adding, for example, fludarabine and by reducing the cyclophosphamide dose. Some interesting series have been published[7] but data are limited.

Unrelated donor transplantation

The outcome of unrelated donor transplants for patients with AA has improved in the last decade.[8,9] Improved selection of better HLA-matched donors most likely played a major role. Since this progress in high-resolution typing, with greater availability of HLA-A, B-, C-, DRB1-, DQB1-matched donors, the number of unrelated donor transplants for marrow failure has increased (Figure 1A and B). Outcomes after unrelated donor transplantation for AA continue to improve, as shown by the results from the EBMT database (Figure 4) with 72% 5-year survival in patients transplanted in the period 2006-2011. Slightly higher survival rates are reported in phase II studies (73%-80%[10,11]) as is often seen when comparing phase II single center data to data obtained from large observational registries.

Appropriate timing to start the search for an unrelated donor is an important issue. In patients who may become

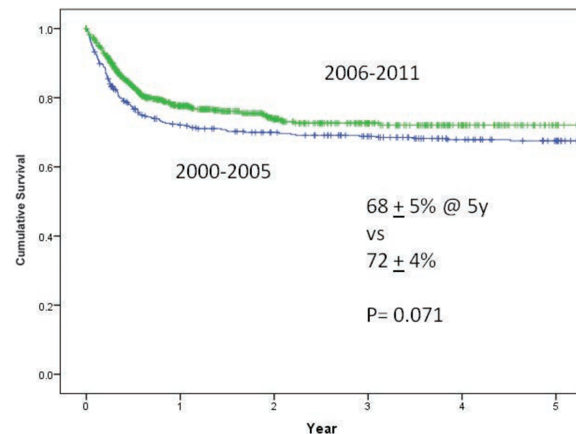


Figure 4. Survival after allogeneic Transplantation from unrelated donors in 2000-2205 and 2006-2011.

candidates for unrelated donor transplantation, donor search should start at diagnosis as response to immune suppressive treatment may require an appropriate time interval that will be used to identify an appropriate donor. Pediatric groups, in particular, discuss up-front unrelated donor transplantation[12] because with the increasing size of the donor pool, and the increasing proportion of unrelated donor typed at high-resolution level, the donor search may take much less time. Indeed, outcomes after unrelated donor transplantation in children are now similar to those after matched sibling donor transplantation.

Similar to sibling transplants, outcomes are improved if marrow is used rather than peripheral blood[13] as marrow failure patients derive little, if any benefit from the higher T-cell dose found in peripheral blood with the associated higher risks of chronic GvHD. Optimal conditioning regimens in unrelated donor transplantation are not known. Increasingly, regimens incorporating fludarabine, ATG or campath and small doses of total body irradiation (e.g. 2 Gy of TBI) are being used[10,14-16] with variable doses of cyclosphosphamide. An interesting study tested cyclophosphamide de-escalation[15] with toxicity at doses of over 100 mg/kg and graft failure with doses under 50 mg/kg when used in combination with fludarabine, ATG and TBI. Similar regimens are used successfully in patients with congenital marrow failure.[17]

Transplants from alternative donors

Umbilical cord blood as an alternative source of stem cells for transplantation has been used in a small number of patients with AA,[18] Umbilical cord blood transplantation (UCBT) has extended the availability of hematopoietic stem cell transplantation (HSCT) in the absence of a suitable donor. Outcome is excellent in the case of identical sibling cord (a situation that is rare, i.e. child with the disease of a mother who is pregnant) but less so with unrelated cord blood units. Double unrelated cord blood transplantation has been reported in only a few patients with marrow failure. In a study of 14 patients with congenital and acquired marrow failure who received double cord blood transplantation after a median follow up of 23 months, the estimated 2-year overall survival was 80±17% and 33±16% for patients with acquired and inherited marrow failure. Transplantation of two partially HLA-matched cord blood units thus enables salvage treatment of high-risk patients. In a series of 71 patients reported as an observational study,[19] with a median age of 13 years, the cumulative incidence of neutrophil recovery at Day 60 was 51±6% with a shorter time to engraftment with higher nucleated cell counts (>3.9×10^7/kg); the incidence of acute GVHD was 20±5% and chronic GVHD was 18±5%. Three year overall survival was 38±6%. Therefore, cord blood transplantation results are currently not equivalent to sibling or unrelated donor transplantation and further studies are needed. Interestingly, however, there is no direct comparison and it is likely that alternative donor transplant recipients are not comparable to patients receiving standard treatment.

In parallel to cord blood transplantation, haploidentical stem cell transplantation has undergone major modifications and progress. The advantage of haploidentical stem cell transplantation is the rapid availability of a one-haplotype mismatched donor for almost all patients. The most commonly used transplant technology is T-cell depleted grafts with high dose of $CD34^+$ cells.[20] More recently, unmanipulated haploidentical bone transplantation with post-transplant cyclophosphamide as GvHD prophylaxis has been reported,[21] although few patients with aplastic anemia have been treated. Problems of haploidentical stem cell transplantation include non-engraftment, poor immune reconstitution, and high rates of relapse in patients with active malignancy at the time of transplantation. Haploidentical transplants have been used only on an individual basis in AA and no large studies have been performed. In a series of 19 Chinese patients receiving a combination of G-CSF-primed marrow and G-CSF-mobilized peripheral blood stem cells from haploidentical family donors using a conditioning regimen with busulphan, cyclophosphamide and ATG, all patients engrafted. Survival was 64% with 56% reported with chronic GVHD.[22] Alternative donor transplantation will continue to be difficult to study in marrow failure as this is for patients with rare diseases, and of these, a minority fail strategies of conventional treatment or transplants using the more established techniques with matched sibling or unrelated donors.

Paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a disease with highly variable clinical manifestations and may resemble aplastic anemia. It is, however, more commonly a disease with the classical hemolytic or thromboembolic presentation. A recent comparative study[23] in which transplanted patients from the EBMT registry have been matched to patients without transplantation from the cohort of the French hematologic society, 5-year survival of transplanted patients was 68±3% in the transplanted group (54±7% in patients with thromboembolic presentation, 69±5% in patients with aplastic anemia presentation and 86±6% in patients with hemolytic presentation). Patients with thromboembolic presentation did not benefit from transplantation, whereas in patients presenting with aplastic anemia, a matched pair analysis could not be performed. The outcome of these patients is, however, similar to other patients reported to the registries. Interestingly, most of these patients were treated prior to the availability of complement inhibitors.

Congenital marrow failure

A considerable number of transplants are performed for marrow failure other than AA. In the 2011 European Group for Blood and Marrow Transplantation (EBMT) activity survey,[24] 499 allogeneic HSCT were for AA and 177 for other marrow failure syndromes. Most of these include the congenital marrow failure of Fanconi anemia, a DNA repair defect disease associated with increased cancer risks and other congenital defects. The EBMT database contains over a 1000 cases transplanted for Fanconi anemia. Other diseases are much rarer and include the Dyskeratosis congenita (a defect of telomere elongation),

Shwachman-Diamond syndrome, and pure red cell aplasia, mainly Diamond Blackfan anemia and few cases of congenital neutropenia, amegakaryocytic thrombocytopenia and others. It is beyond the scope of this paper to address details of transplantation of these diseases, as each of these entails particular considerations and transplants should be performed in specialized centers.

In a recent survey of patients with Fanconi anemia[25] analyzed 795 patients. Survival at 20 years was 49%; more recent year of transplant, younger age and marrow as a stem cell source was associated with better outcome. Chronic GvHD and secondary tumors were deleterious. In patients with Fanconi anemia, the choice of conditioning is of particular importance because of sensitivity to toxicity, chronic GvHD is poorly tolerated, and secondary tumors, in particular of the oral cavity problematic. Timing of HSCT for Fanconi anemia is crucial, particularly in patients with moderately severe marrow failure. Once transformation to MDS or leukemia has occurred outcome is impaired.[26] The choice of conditioning avoiding TBI, of preferable marrow stem cell source and of a well-matched donor have been discussed above. Cord blood transplantation from mismatched unrelated donors has been used with varying results[27,28] in patients with congenital marrow failure.

References

1. Bacigalupo A, Socié G, Schrezenmeier H, Tichelli A, Locasciulli A, Fuehrer M, et al; Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation (WPSAA-EBMT). Bone marrow versus peripheral blood as the stem cell source for sibling transplants in acquired aplastic anemia: survival advantage for bone marrow in all age groups. Haematologica. 2012;97(8):1142-8.
2. Bacigalupo A, Brand R, Oneto R, Bruno B, Socié G, Passweg J, et al. Treatment of acquired severe aplastic anemia: bone marrow transplantation compared with immunosuppressive therapy—The European Group for Blood and Marrow Transplantation experience. Semin Hematol. 2000;37(1):69-80.
3. Ades L, Mary JY, Robin M, Ferry C, Porcher R, Esperou H, et al. Long-term outcome after bone marrow transplantation for severe aplastic anemia. Blood. 2004;103(7):2490-7.
4. Schrezenmeier H, Passweg JR, Marsh JC, Bacigalupo A, Bredeson CN, Bullorsky E, et al. Worse outcome and more chronic GVHD with peripheral blood progenitor cells than bone marrow in HLA-matched sibling donor transplants for young patients with severe acquired aplastic anemia. Blood. 2007;110(4):1397-400.
5. Stern M, Passweg J, Locasciulli A, Socie G, Schrezenemeier H, Bekassy A, et al. Influence of donor/recipient sex matching on outcome of allogeneic hematopoietic stem cell transplantation. Transplantation. 2006;82(2):218-26.
6. Locatelli F, Bruno B, Zecca M, Van-Lint MT, McCann S, Arcese W, et al. Cyclosporin A and short-term methotrexate versus cyclosporin A as graft versus host disease prophylaxis in patients with severe aplastic anemia given allogeneic bone marrow transplantation from an HLA-identical sibling: results of a GITMO/EBMT randomized trial. Blood. 2000;96(5): 1690-7.
7. Maury S, Bacigalupo A, Anderlini P, Aljurf M, Marsh J, Socié G, et al.; Severe Aplastic Anemia Working Party, European Group for Blood and Marrow Transplantation (EBMT-SAAWP). Improved outcome of patients older than 30 years receiving HLA-identical sibling hematopoietic stem cell transplantation for severe acquired aplastic anemia using fludarabine-based conditioning: a comparison with conventional conditioning regimen. Haematologica. 2009;94(9):1312-5.
8. Maury S, Balere-Appert ML, Chir Z, et al. French Society of Bone Marrow Transplantation and Cellular Therapy (SFGM-TC). Unrelated stem cell transplantation for severe acquired aplastic anemia: improved outcome in the era of high-resolution HLA matching between donor and recipient. Haematologica. 2007;92:589-96.
9. Viollier R, Socié G, Tichelli A, Bacigalupo A, Korthof ET, Marsh J, et al. Recent improvement in outcome of unrelated donor transplantation for aplastic anemia. Bone Marrow Transplant. 2008;41(1):45-50
10. Bacigalupo A, Marsh JC. Unrelated donor search and unrelated donor transplantation in the adult aplastic anaemia patient aged 18-40 years without an HLA-identical sibling and failing immunosuppression. Bone Marrow Transplant. 2012 Nov 26. [Epub ahead of print]
11. Marsh JC, Gupta V, Lim Z, Ho AY, Ireland R, Hayden J, et al. Alemtuzumab with fludarabine and cyclophosphamide reduces chronic graft versus host disease after allogeneic stem cell transplantation for acquired aplastic anemia. Blood. 2011 April 25. [Epub ahead of print]
12. Samarasinghe S, Steward C, Hiwarkar P, Saif MA, Hough R, Webb D, et al. Excellent outcome of matched unrelated donor transplantation in paediatric aplastic anaemia following failure with immunosuppressive therapy: a United Kingdom multicentre retrospective experience. Br J Haematol. 2012;157 (3):339-46.
13. Eapen M, Rademacher JL, Antin JH, Champlin RE, Carreras J, Fay J, et al. Effect of stem cell source on outcomes after unrelated donor transplantation in severe aplastic anemia. Blood. 2011 June 15. [Epub ahead of print]
14. Deeg HJ, Amylon ID, Harris RE, et al. Marrow transplants from unrelated donors for patients with aplastic anemia: minimum effective dose of total body irradiation. Biol Blood Marrow Transplant. 2001;7:208-15.
15. Tolar J, Deeg HJ, Arai S, Horwitz M, Antin JH, McCarty JM, et al. Fludarabine-based conditioning for marrow transplantation from unrelated donors in severe aplastic anemia: early results of a cyclophosphamide dose deescalation study show life-threatening adverse events at predefined cyclophosphamide dose levels. Biol Blood Marrow Transplant. 2012;18(7):1007-11.
16. Kojima S, Matsuyama T, Kato S, Kigasawa H, Kobayashi R, Kikuta A, et al. Outcome of 154 patients with severe aplastic anemia who received transplants from unrelated donors: the Japan Marrow Donor Program. Blood. 2002;100:799-803.
17. Shimada A, Takahashi Y, Muramatsu H, Hama A, Ismael O, Narita A, et al. Excellent outcome of allogeneic bone marrow transplantation for Fanconi anemia using fludarabine-based reduced-intensity conditioning regimen. Int J Hematol. 2012;95 (6):675-9.
18. Peffault de Latour R, Rocha V, Socié G. Cord blood transplantation in aplastic anemia. Bone Marrow Transplant. 2013 Jan 7. [Epub ahead of print]
19. Peffault de Latour R, Purtill D, Ruggeri et al. Influence of Nucleated Cell Dose on Overall Survival of Unrelated Cord Blood Transplantation for Patients with Severe Acquired Aplastic Anemia: A Study by Eurocord and the Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation. BBMT 2010;(1):78-85.
20. Yair Reisner, David Hagin, and Massimo F. Martelli. Haploidentical hematopoietic transplantation: current status and future perspectives. Blood. 2011;118(23):6006-17.
21. Luznik L, O'Donnell PV, Fuchs EJ. Post-transplantation cyclophosphamide for tolerance induction in HLA-haploidentical bone marrow transplantation. Semin Oncol. 2012;39(6):683-93.
22. Xu LP, Liu KY, Liu DH, Han W, Chen H, Chen YH. A novel protocol for haploidentical hematopoietic SCT without in vitro T-cell depletion in the treatment of severe acquired aplastic anemia. Bone Marrow Transplant. 2012;47(12):1507-12.
23. Peffault de Latour R, Schrezenmeier H, Bacigalupo A, Blaise D, de Souza CA, Vigouroux S, et al. Allogeneic stem cell transplantation in paroxysmal nocturnal hemoglobinuria. Haematologica. 2012;97(11):1666-73.
24. Passweg JR, Baldomero H, Bregni M, Cesaro S, Dreger P, Duarte RF, et al. Hematopoietic SCT in Europe: data and trends in 2011. Bone Marrow Transplant. 2013 Apr 15. [Epub ahead of print]
25. Peffault de Latour R Allogeneic HSCT in Fanconi anemia, the EBMT experience. Bone Marrow Transplant. 2013; 48(2 Suppl):114.
26. Ayas M, Saber W, Davies SM, Harris RE, Hale GA, Socie G, et al. Allogeneic Hematopoietic Cell Transplantation for Fanconi Anemia in Patients With Pretransplantation Cytogenetic Abnormalities, Myelodysplastic Syndrome, or Acute Leukemia. J Clin Oncol. 2013 Apr 1. [Epub ahead of print]
27. Bizzetto R, Bonfim C, Rocha V, Socié G, Locatelli F, Chan K, Ramirez O, et al; Eurocord and SAA-WP from EBMT. Outcomes after related and unrelated umbilical cord blood transplantation for hereditary bone marrow failure syndromes other than Fanconi anemia. Haematologica. 2011;96(1):134-41.
28. Gluckman E, Rocha V, Ionescu I, Bierings M, Harris RE, Wagner J, et al; Eurocord-Netcord and EBMT. Results of unrelated cord blood transplant in fanconi anemia patients: risk factor analysis for engraftment and survival. Biol Blood Marrow Transplant. 2007;13(9):1073-82.

Immune signaling in chronic lymphocytic leukemia

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A B S T R A C T

Surface Ig (sIg), the key receptor for normal B cells, is retained by the majority of B-cell malignancies. In CLL, sIgM appears to influence tumor cell behavior via transient engagement with antigen in lymphoid tissue. Importantly, inhibitors of sIgM-activated intracellular pathways are showing clinical promise. However, CLL includes two major prognostic subsets that differ in mutational status of the Ig V-genes. The sIgM of unmutated (U) CLL is less down-regulated by antigen, with cells remaining more responsive to stimulation *in vitro*, and possibly *in vivo*. Downstream effects of sIgM signaling include upregulation of MYC proto-oncoprotein expression and induction of MYC-regulated targets, including cyclin D2, with both proteins detected in proliferation centers. Cell survival is also promoted, with inactivation of the pro-apoptotic activity of BIM(EL) via enhanced phosphorylation. The ability to phosphorylate BIM(EL) was highly correlated with mutational status and with requirement for treatment. U-CLL also preferentially expresses CXCR4 and CD49d, both important in migration to tissue. Intraclonal analysis of individual CLL cases reveals small subgroups with high sIgM/CXCR4, apparently dangerously primed for tissue-based proliferative stimulation. Unlike normal B cells, this is an iterative process exposing proliferating CLL cells, especially U-CLL but some M-CLL cases, to further genetic change.

Learning goals

At the conclusion of this activity, participants should be able to:
- understand the role of the B-cell receptor in CLL and to gain insight into the signals mediated via engagement of surface Ig;
- use that knowledge to interpret the clinical outcome of BCR pathway inhibitors.

Introduction

CLL is a relatively indolent B-cell tumor with a variable clinical course. It may be preceded by B-cell expansions, termed monoclonal B-cell lymphocytosis (MBL), a finding difficult to define, since it includes patients with early disease and also those who do not develop overt tumors.[1] Even clear cases of CLL can occasionally regress.[2,3] This range of features. and the fact that many patients remain untreated, at least for some time, allows investigation of the pathogenetic steps. Chromosomal changes are quite limited in early disease, the most common being deletion at 13q14, a region which encodes two microRNAs, *miR-15a/miR-16-1*.[4] This deletion can be detected at the stage of MBL and, since the miRNAs are apparently negative regulators of BCL-2, the consequence is to up-regulate BCL2 expression.[5] There may be an analogy here with follicular lymphoma, where the first step toward tumor development, insufficient by itself, is the t(14;18) translocation which also up-regulates BCL-2 expression.[6-8]

A further advantage of CLL for the investigator is that, although cases look similar by routine hematologic investigation, the disease can be divided into two major subsets. These are distinguished by the level of somatic hypermutation (SHM) in the Ig variable (V) region genes, into so-called unmutated CLL (U-CLL) (approx. 40% of cases) and mutated CLL (M-CLL) (approx. 60%).[9,10] This feature indicates that the B cells of origin of the two subsets had reached distinct points of differentiation. U-CLL is likely to derive from a pre-germinal center (GC) B cell, whereas M-CLL appears to have undergone the normal process of SHM and antigen selection in the GC prior to transformation. This is not just a biological curiosity but has profound effects on clinical behavior, with U-CLL being more aggressive. Although this is proving clinically useful, there are some exceptions, such as the relatively rarely used IGHV3-21 gene. Usage of this gene seems to mark an aggressive tumor even when classified as M-CLL; however, the mutational frequency does tend to be quite low, falling at the border between the two subsets.[11]

The two subsets are not interconvertible and use different IGV genes, indicating separate development. There is differential asymmetry of IGV (H and L) gene usage, with the most dramatic being the increased level (20-30%) of IGHV1-69[12] in U-CLL.[9,10] Conservation of HCDR3 sequences is also evident, especially in U-CLL, and the various conserved sequences, suggestive of common antigen

recognition, have been defined as 'stereotypes'.[13,14] We used the conserved sequences of the IGHV1-69 gene to probe for the potential B cells of origin of U-CLL, and detected them in the normal naïve B-cell population in blood.[15] This has been confirmed by recent gene expression profiling data, which also found that the cells are $CD5^+CD27^-$.[16] The origin of M-CLL is more difficult to track by V-gene analysis, but GEP suggests that this subset may be derived from a circulating $CD5^+CD27^+$ B-cell population.[16] Further differences between the two subsets have emerged, with ZAP-70 expression detected mainly in U-CLL.[17] The function of this protein in CLL cells remains unclear but it has potential effects on signaling,[18] sIgM endocytosis[19] and migration.[20] CD38 is also more often expressed in U-CLL and tends to be up-regulated in tissue sites, appearing as a marker of cell division and growth *in vivo*.[21] While these two proteins are useful prognostic indicators, their association with U-CLL underlines the differential biology of the two subsets. Understanding this biology, and the changes that occur during circulation through tissue sites, should reveal new ways of specific drug targeting of CLL cells.

CLL in tissue sites

CLL cells of both subsets proliferate in tissue sites, mainly lymph nodes, and migration from blood to tissue is clearly required. For normal B cells, this involves first, extravasation from blood vessels via interaction with L-selectin, chemokines and adhesion molecules, and second, following chemokine gradients along the fibroblastic reticular cell network to the follicle (reviewed in [22]). If antigen and $CD4^+$ T cells are engaged, a GC will be formed and B cells will undergo antigen selection and differentiation. The picture for CLL cells indicates exclusion from follicles, a likely outcome in the absence of cognate T-cell help. Exposure to antigen can still occur in the extrafollicular site, and proliferating CLL cells efface follicular structures, forming loose aggregates containing $Ki\text{-}67^+$ cells together with stromal cells and T cells, termed proliferation centers.[23]

The antigens recognized by CLL cells are probably not a single entity but, from the pattern of follicular exclusion of tumor cells and from the apparently persistent stimulatory effects, they are most likely to be autoantigens, with several candidates already identified.[24,25] One possibility is that they are not the antigens which stimulated the B cells of origin, but are cross-reactive substitutes of lower affinity. However, specificity for the initiating antigen may be retained, as illustrated by the finding that a small number of cases of U-CLL encoded by the IGHV1-69 gene react with a phosphoprotein of cytomegalovirus.[26] For M-CLL, a recent analysis found that a proportion of CLL IgM molecules encoded by the IGHV3-7/IGKV2-24 genes recognized fungal β-(1,6)-glucan.[27] Following antigen engagement, CLL cells may proliferate, die or be anergized. Exit from the lymph node requires desensitization of chemokine receptors and upregulation of the sphingosine-1-phosphate receptor. There is some evidence that the latter may be less efficient in U-CLL.[28] It is the cells which enter the blood and circulate which are usually available for study and these retain a temporary imprint of events in the tissue.

Signaing via the B-cell receptor

The signaling pathways activated in CLL cells by engagement of sIgM *in vitro* have been described,[29] and the links between positive signals, downstream events and biological outcome are summarized in Figure 1. Signaling may be modulated either positively or negatively by co-receptors, and can be curtailed either by endocytosis of the receptor or by intracellular phosphatases. BCR-induced membrane-proximal events include LYN-mediated phosphorylation of Igα/β followed by recruitment of the tyrosine kinase Syk. Signal propagation then involves various effectors, including BTK, PLCγ2 and BLNK. LYN-dependent phosphorylation of CD19 also triggers the recruitment and activation of PI3K that plays a central role in promoting cell survival in CLL. Inhibitors aimed at the BCR-associated tyrosine kinases LYN (dasatinib), Syk (fostamatinib) and BTK (ibrutinib, CC-292), or at phosphatidylinositol 3-kinase (GS-1101), are all in clinical trials for CLL. The interesting outcome of treatment with the latter three inhibitors is to reduce lymph node size and cause a temporary lymphocytosis which generally clears over time.[30] This strongly suggests that chemokine-induced migration and BCR-mediated events are critical for maintenance of CLL cells.

BCR-signaling in CLL subsets

The consequence of antigen encounter in tissue sites is low level proliferation, but also the induction of anergy. One of the features of anergy in normal human B cells is

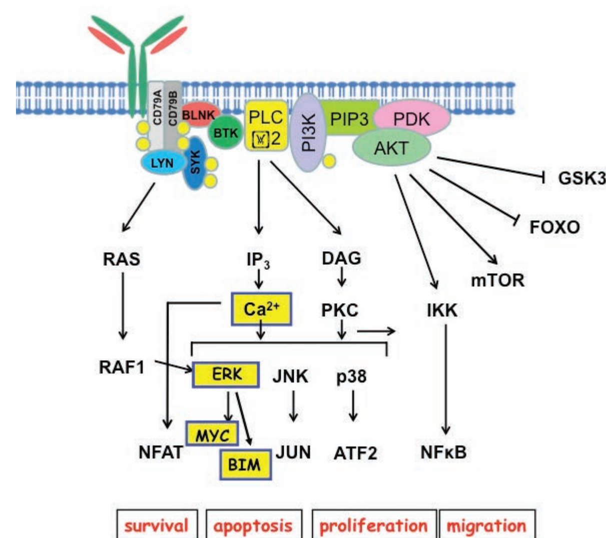

Figure 1. Downstream effects of positive B-cell receptor signaling in CLL. Activation via the signalosome comprising LYN, SYK, BLNK, BTK, PLC-γ2 and PI3K, leads to multiple downstream effects, including Ca^{2+} mobilization, ERK1/2 phosphorylation, upregulation of MYC, and phosphorylation of BIM (EL), with effects on a range of cell functions.

downregulation of expression of sIgM, but not of sIgD.[31] This 'endocytosis *in vivo*' is evident in blood cells of both U-CLL and M-CLL, and the fact that it is antigen driven is clear from the finding that sIgM expression can recover *in vitro.*[32] Recovery argues against the concept that CLL cells are responding to neighboring IgM-derived peptides either in *cis* or in *trans,*[33] unless that interaction is distinct from the downregulation of sIgM. Reversal of antigen-induced changes affecting the N-glycosylation status of the sIgM is also apparently occurring to variable extents *in vivo.*[34] However, there is a difference between U-CLL and M-CLL in that signaling ability as measured *in vitro* by intracellular Ca^{+2} mobilization or by ERK1/2 phosphorylation is higher in the former, due either to a weaker anergizing signal in the tissue, or to a more rapid recovery from the signal. The outcome is retention of signal capacity by U-CLL cases[32] and this has clinical significance.[35] It may be that this subtle difference accounts for the differential clinical behavior of the two subsets.

The functional significance of positive BCR signaling can be assessed by analyzing downstream events (Figure 1). We have detected BCR-mediated upregulation of MYC proto-oncoprotein expression and induction of MYC-regulated target genes including cyclin D2,[36] with both proteins detected in proliferation centers.[37] This induction is relevant since MYC is pivotal for controlling cell proliferation, apoptosis and metabolism.[38] Pathways to increased cell survival are BCR-mediated increase of the anti-apoptotic MCL1 protein and inactivation of the pro-apoptotic activity of BIM(EL) via enhanced phosphorylation.[39] The ability to phosphorylate BIM(EL) was highly correlated with mutational status and with requirement for treatment.[39] Interestingly, this also appeared to identify cases of M-CLL which progressed. These findings indicate that BCR engagement can lead to expression of functionally important molecules, preferentially in U-CLL, and that this is occurring in tissues.

Expression of CXCR4 in CLL

Trafficking of CLL cells through tissue sites is of obvious importance and the complex array of interacting molecules involved has been elegantly reviewed.[40] Chemokine receptors are involved and CLL cells express a range of these including CXCR4, CXCR5 and CCR7, which bind to CXCL12 (SDF-1), CXCL13 (BCA-1) and CCL19/21, respectively, all secreted by stromal cells. CXCR4 expression in CLL is labile and rapidly down-regulated by its ligand CXCL12, as expected for a receptor which has to respond to a chemokine gradient. Once in tissue sites, CXCL12, held on stromal cell surfaces by binding to heparan sulphate, stabilizes the gradient, so that cells can migrate in an orientated manner.[41] Consistent with this interaction, expression of CXCR4 is lower in tissue sites than in blood.[42] Our reciprocal finding is that CLL cells in blood rapidly increase expression of CXCR4 when incubated *in vitro* in the absence of ligand (*data not shown*). Even though expression of CXCR4 is labile, a higher expression on circulating cells has been found to be associated with stage IV disease[42] and correlates negatively with survival.[43] In the overall analysis, although most cases of U-CLL expressed higher levels, there was no significant correlation with mutational status. In common with some other features, this might be due to heterogeneity within M-CLL, since analysis of this subset alone revealed a negative correlation with survival (CS Pepper and FK Stevenson, unpublished observations, 2013).

Intraclonal subgroups within CLL cases

Expression of sIgM clearly varies within CLL cases and we used non-endocytosable Dynabeads covalently linked to $F(ab')_2$ anti-IgM to probe this heterogeneity. When we exposed the CLL cells to a 2:1 ratio of beads:cells, we were able to separate the clones into 4 major intraclonal subgroups (SG 1-4) of increasing sIg expression (Figure 2). As expected, signaling mediated by the bead-bound anti-IgM, detected by phosphorylation of ERK1/2 or PLCγ was highest in the small high sIgM subgroup, SG4. It was inhibitable (75-100%) by the BTK inhibitor ibrutinib. SG4 also had a higher expression of CXCR4. Interestingly, the lowest subgroup (SG1) included the Ki-67^+ population, leading to the suggestion that these are cells that have divided, presumably in tissue sites, and then entered the circulation as an ex-proliferative population. The fate of cells in SG1 could be apoptosis, or survival with re-expression of sIgM and CXCR4. Recovered cells could potentially repopulate the small CSG4 population of potentially dangerous cells ready for migration to tissue and for BCR stimulation. SG1 appears to relate to that identified by $^{(2)}$H-labelled DNA, which was also CXCR4 (dim).[44] Although sIgM expression was not analyzed in that study, there was a CXCR4 (bright) population which was CD5 low. In a preliminary analysis, expression of CD5 on SG4 was, in fact, higher than in SG1; this suggests that our clonal dissection based on sIgM is revealing different subpopulations.

Functional linkage between sIgM and CXCR4

Functional connection between sIgM and CXCR4 is evident from the co-downregulation of expression of the two molecules on BCR engagement.[45] The loss of CXCR4 expression also occurred using solid phase anti-IgM[46] which is unlikely to endocytose and provides a persistent signal. In this case, there was a concomitant decrease in migration to CXCL12. However, although the weaker signal mediated by soluble anti-IgM also decreased expression of CXCR4, migration was increased rather than decreased.[47] While functional outcome remains uncertain, both groups show that BCR-mediated signals can affect a remote receptor. CXCR4 is not the only receptor affected by BCR signaling since expression and function of the integrin VLA-4 α4β1 (CD49d/CD29) is also down-regulated.[48]

Receptor crosstalk in anergic B cells and in CLL cells

The influence of BCR engagement on CXCR4 in CLL cells is reminiscent of crosstalk described in normal B cells anergized by chronic engagement of the surface Ig by self antigen. The anergic state in normal B cells involves direct effects on the BCR with downregulation of sIgM,

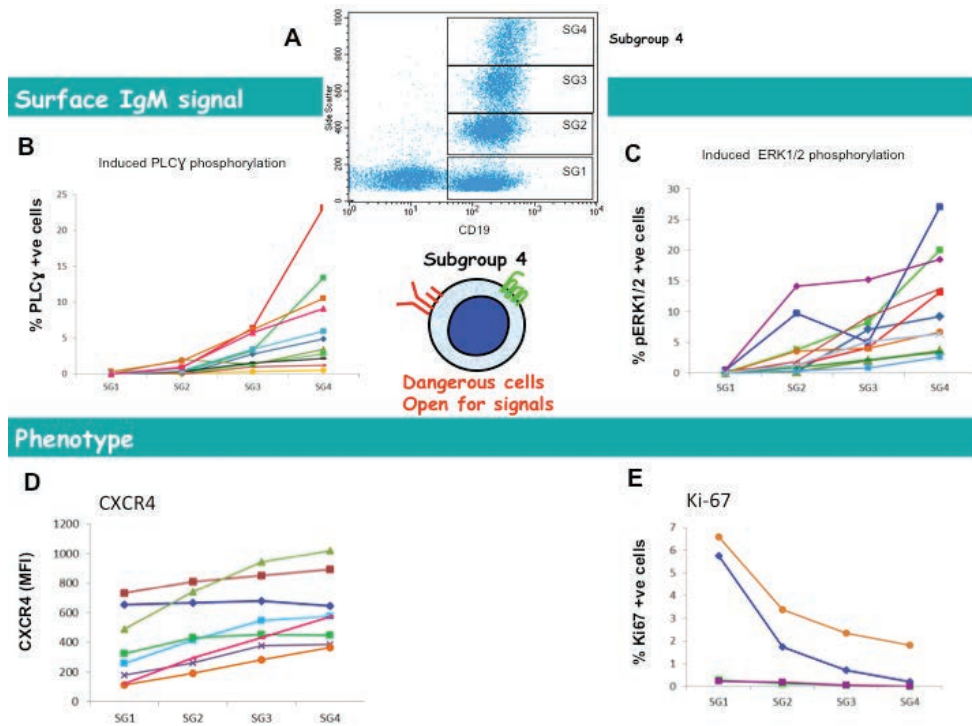

Figure 2. Functional analysis of intraclonal subgroups. (A) Separation of CLL cells from a representative case into subgroups (SG), based on the ability of CD19+ cells to bind beads coated with (Fab')$_2$ anti-IgM. (B and C) Phosphorylation of PLCγ (B) or ERK1/2 (C) induced by binding of beads for 30 min at 37°C, with levels at 0°C subtracted. (D) Expression of CXCR4 (MFI) detected by FACS, and (E) percentage of Ki-67-positive cells, in SGs from individual cases.

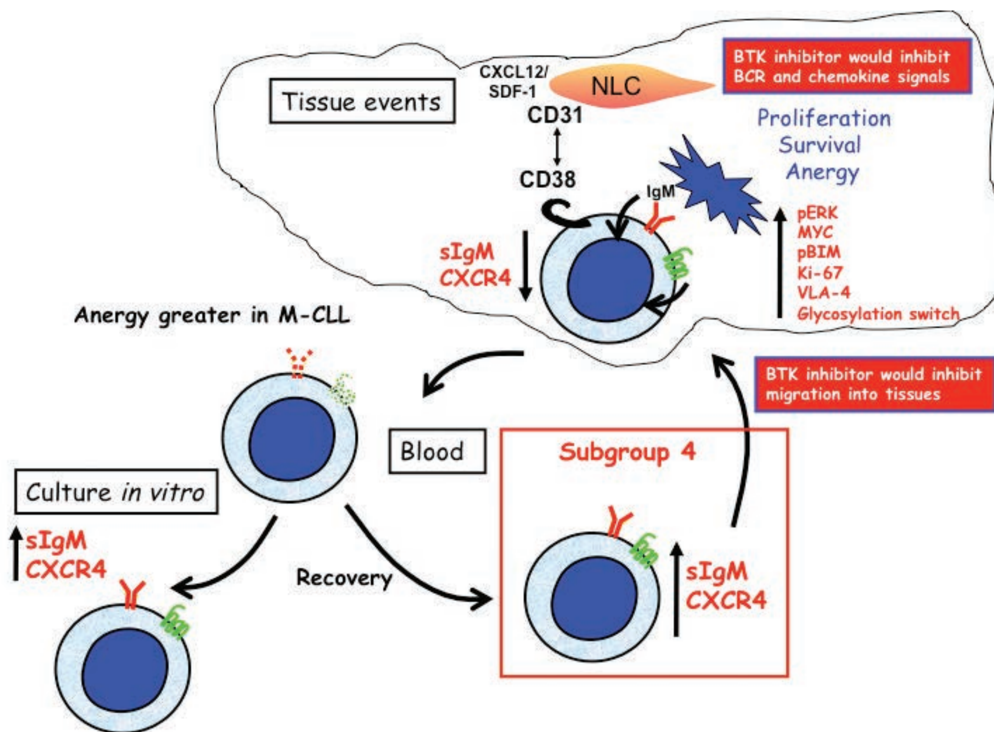

Figure 3. A working model of the dynamics of the B-cell receptor in CLL. Interaction with antigen and with microenvironmental elements occurs in tissue sites and activates proliferative and survival events, but also can lead to anergy. Downregulation of sIgM and CXCR4, apparently greater in M-CLL, is followed by release into the blood and recovery of expression. Subgroup 4 is a small population within each CLL clone which appears most recovered and primed for re-entry to tissue. BTK inhibitors will affect both BCR signaling and chemokine-induced migration.

but not sIgD,[31] exactly as seen in CLL cells.[32] However, anergy also involves a more global indirect inhibitory influence on other remote receptors. This trans-inhibition is known to affect chemokine receptors such as CXCR4 and can occur by downregulation of receptor expression or by reduction in function. Evidence from mouse models points to the inositol phosphatase SHIP-1 as a major mediator of these effects.[49] In a preliminary study of CLL, SHIP-1 levels, together with activating phosphorylation, were found to be higher in ZAP-70-negative (presumably mainly M-CLL) cases.[50]

Conclusions

The unfolding story of CLL is an example of how biological understanding can inform clinical strategies. The B-cell receptor is now center stage, both as a driver of tumor cell responses and as a target for inhibitory drugs. Perhaps because of this, there is a multitude of papers describing components of the intracellular pathways, and the ability of drugs to inhibit these. Since tissue-based events are of obvious importance,[51] and appear to be affected by the drugs, there is interest in the cell surface molecules involved in migration and tissue location as well as in the interactions operating in the microenvironment.[52] Genomic studies and deep sequencing are also revealing details of chromosomal lesions, and locating significant mutations which could affect disease development and progression.[53] It is clearly impossible to review this vast and expanding literature. Instead, this Educational Review provides a synthesis of how the BCR might be influencing tumor behavior. An analogy with normal anergic B cells can be drawn, recognizing that these would be susceptible to death due to high levels of pro-apoptotic BIM.[54] The key to survival of anergized CLL cells is likely to be the increase in BCL-2 which opposes the action of BIM by sequestration.[55] Targeting of BCL-2 is, therefore, another attractive therapeutic approach. However, clinical effectiveness of an inhibitor, navitoclax (ABT-263), in CLL was limited by its effects on BCL-X(L) which led to on-target thrombocytopenia.[56] A modified version (ABT-199), more specific for BCL-2, is now being tested with encouraging preliminary results.[57]

Biologically, U-CLL and M-CLL are different and reflect the properties of their cell of origin. Clearly, antigen-induced anergy is more complete in M-CLL, but this subset is particularly heterogeneous both in sIgM expression and in clinical behavior; therefore, more insight into the reasons for this is required. It is the tumor cells that escape from anergy and are open to positive BCR signaling which represent the challenge. However, CLL is not a static disease, since cells engage antigen in tissue sites, proliferate and then exit to blood where they recover their potential to respond (Figure 3). These processes are accompanied by many reversible phenotypic and functional changes making investigation of circulating populations difficult to quantify. This iterative process appears to depend on interaction with an autoantigen and it is possible that CLL cells are selected for these specificities and for their ability to respond in this way. It will be interesting to look for any flaring effect on CLL of re-exposure to the initiating antigen, such as the fungal glycan described for M-CLL.[27] Paradoxically, it might be the success of the new drugs in the clinic which will close down the investigations of the natural pathogenesis of CLL. Hopefully, it will reveal the major pathways activated via the BCR that are successfully subverted by malignant B cells.

References

1. Ghia P, Caligaris-Cappio F. Monoclonal B-cell lymphocytosis: right track or red herring? Blood. 2012;119(19):4358-62.
2. Del Giudice I, Chiaretti S, Tavolaro S, De Propris MS, Maggio R, Mancini F, et al. Spontaneous regression of chronic lymphocytic leukemia: clinical and biologic features of 9 cases. Blood. 2009;114(3):638-46.
3. Rossi D, Sozzi E, Puma A, De Paoli L, Rasi S, Spina V, et al. The prognosis of clinical monoclonal B cell lymphocytosis differs from prognosis of Rai 0 chronic lymphocytic leukaemia and is recapitulated by biological risk factors. Br J Haematol. 2009;146(1):64-75.
4. Calin GA, Pekarsky Y, Croce CM. The role of microRNA and other non-coding RNA in the pathogenesis of chronic lymphocytic leukemia. Best Pract Res Clin Haematol. 2007;20(3):425-37.
5. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA. 2005;102(39):13944-9.
6. Tsujimoto Y, Yunis J, Onorato-Showe L, Erikson J, Nowell PC, Croce CM. Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t(11;14) chromosome translocation. Science.1984;224(4656):1403-6.
7. Roulland S, Faroudi M, Mamessier E, Sungalee S, Salles G, Nadel B. Early steps of follicular lymphoma pathogenesis. Adv Immunol. 2011;111:1-46.
8. Stevenson FK, Stevenson GT. Follicular lymphoma and the immune system: from pathogenesis to antibody therapy. Blood. 2012;119(16):3659-67.
9. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood. 1999;94(6):1840-7.
10. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94(6):1848-54.
11. Tobin G, Thunberg U, Johnson A, Thorn I, Soderberg O, Hultdin M, et al. Somatically mutated Ig V(H)3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood. 2002;99(6):2262-4.
12. Kipps TJ, Tomhave E, Pratt LF, Duffy S, Chen PP, Carson DA. Developmentally restricted immunoglobulin heavy chain variable region gene expressed at high frequency in chronic lymphocytic leukemia. Proc Natl Acad Sci USA. 1989;86(15):5913-7.
13. Messmer BT, Albesiano E, Efremov DG, Ghiotto F, Allen SL, Kolitz J, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med. 2004;200(4):519-25.
14. Stamatopoulos K, Belessi C, Moreno C, Boudjograh M, Guida G, Smilevska T, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. Blood. 2007;109(1):259-70.
15. Forconi F, Potter KN, Wheatley I, Darzentas N, Sozzi E, Stamatopoulos K, et al. The normal IGHV1-69-derived B-cell repertoire contains stereotypic patterns characteristic of unmutated CLL. Blood. 2010;115(1):71-7.
16. Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, Durig J, et al. Cellular origin and pathophysiology of chronic lymphocytic leukemia. J Exp Med. 2012;209(12):2183-98.
17. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood. 2003;101(12):4944-51.
18. Chen L, Huynh L, Apgar J, Tang L, Rassenti L, Weiss A, et al. ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. Blood. 2008;111(5):2685-92.
19. Gobessi S, Laurenti L, Longo PG, Sica S, Leone G, Efremov DG. ZAP-70 enhances B-cell-receptor signaling despite

absent or inefficient tyrosine kinase activation in chronic lymphocytic leukemia and lymphoma B cells. Blood. 2007;109(5):2032-9.
20. Calpe E, Codony C, Baptista MJ, Abrisqueta P, Carpio C, Purroy N, et al. ZAP-70 enhances migration of malignant B lymphocytes toward CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation. Blood. 2011;118(16):4401-10.
21. Chiorazzi N. Implications of new prognostic markers in chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program. 2012;2012:76-87.
22. Girard JP, Moussion C, Forster R. HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. Nat Rev Immunol. 2012;12(11):762-73.
23. Ravetch JV, Clynes RA. Divergent roles for Fc receptors and complement in vivo. Annu Rev Immunol. 1998;16:421-32.
24. Herve M, Xu K, Ng YS, Wardemann H, Albesiano E, Messmer BT, et al. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. J Clin Invest. 2005;115(6):1636-43.
25. Lanemo Myhrinder A, Hellqvist E, Sidorova E, Soderberg A, Baxendale H, Dahle C, et al. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood. 2008;111(7):3838-48.
26. Steininger C, Widhopf GF 2nd, Ghia EM, Morello CS, Vanura K, Sanders R, et al. Recombinant antibodies encoded by IGHV1-69 react with pUL32, a phosphoprotein of cytomegalovirus and B-cell superantigen. Blood. 2012;119 (10):2293-301.
27. Hoogeboom R, van Kessel KP, Hochstenbach F, Wormhoudt TA, Reinten RJ, Wagner K, et al. A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. J Exp Med. 2013;210(1):59-70.
28. Capitani N, Patrussi L, Trentin L, Lucherini OM, Cannizzaro E, Migliaccio E, et al. S1P1 expression is controlled by the pro-oxidant activity of p66Shc and is impaired in B-CLL patients with unfavorable prognosis. Blood. 2012;120 (22):4391-9.
29. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. Blood. 2011;118(16):4313-20.
30. Davids MS, Brown JR. Targeting the B cell receptor pathway in chronic lymphocytic leukemia. Leuk Lymphoma. 2012;53 (12):2362-70.
31. Duty JA, Szodoray P, Zheng NY, Koelsch KA, Zhang Q, Swiatkowski M, et al. Functional anergy in a subpopulation of naive B cells from healthy humans that express autoreactive immunoglobulin receptors. J Exp Med. 2009;206(1):139-51.
32. Mockridge CI, Potter KN, Wheatley I, Neville LA, Packham G, Stevenson FK. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. Blood. 2007;109(10):4424-31.
33. Duhren-von Minden M, Ubelhart R, Schneider D, Wossning T, Bach MP, Buchner M, et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. Nature. 2012;489(7415):309-12.
34. Krysov S, Potter KN, Mockridge CI, Coelho V, Wheatley I, Packham G, et al. Surface IgM of CLL cells displays unusual glycans indicative of engagement of antigen in vivo. Blood. 2010;115(21):4198-205.
35. Cesano A, Perbellini O, Evensen E, Chu CC, Cioffi F, Ptacek J, et al. Association between B-cell receptor responsiveness and disease progression in B-cell chronic lymphocytic leukemia: results from single cell network profiling studies. Haematologica. 2012. [Epub ahead of print].
36. Krysov S, Dias S, Paterson A, Mockridge CI, Potter KN, Smith KA, et al. Surface IgM stimulation induces MEK1/2-dependent MYC expression in chronic lymphocytic leukemia cells. Blood. 2012;119(1):170-9.
37. Igawa T, Sato Y, Takata K, Fushimi S, Tamura M, Nakamura N, et al. Cyclin D2 is overexpressed in proliferation centers of chronic lymphocytic leukemia/small lymphocytic lymphoma. Cancer Sci. 2011;102(11):2103-7.
38. Dang CV. MYC on the path to cancer. Cell. 2012;149(1):22-35.
39. Paterson A, Mockridge CI, Adams JE, Krysov S, Potter KN, Duncombe AS, et al. Mechanisms and clinical significance of BIM phosphorylation in chronic lymphocytic leukemia. Blood. 2012;119(7):1726-36.
40. Davids MS, Burger JA. Cell Trafficking in Chronic Lymphocytic Leukemia. Open Journal of Hematology. 2012; 3(S1).
41. Lortat-Jacob H. The molecular basis and functional implications of chemokine interactions with heparan sulphate. Curr Opin Struct Biol. 2009;19(5):543-8.
42. Ghobrial IM, Bone ND, Stenson MJ, Novak A, Hedin KE, Kay NE, et al. Expression of the chemokine receptors CXCR4 and CCR7 and disease progression in B-cell chronic lymphocytic leukemia/ small lymphocytic lymphoma. Mayo Clinic Proceedings. 2004;79(3):318-25.
43. Majid A, Lin TT, Best G, Fishlock K, Hewamana S, Pratt G, et al. CD49d is an independent prognostic marker that is associated with CXCR4 expression in CLL. Leuk Res. 2011;35(6):750-6.
44. Calissano C, Damle RN, Marsilio S, Yan XJ, Yancopoulos S, Hayes G, et al. Intraclonal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. Mol Med. 2011;17(11-12):1374-82.
45. Quiroga MP, Balakrishnan K, Kurtova AV, Sivina M, Keating MJ, Wierda WG, et al. B-cell antigen receptor signaling enhances chronic lymphocytic leukemia cell migration and survival: specific targeting with a novel spleen tyrosine kinase inhibitor, R406. Blood. 2009;114(5):1029-37.
46. Vlad A, Deglesne PA, Letestu R, Saint-Georges S, Chevallier N, Baran-Marszak F, et al. Down-regulation of CXCR4 and CD62L in chronic lymphocytic leukemia cells is triggered by B-cell receptor ligation and associated with progressive disease. Cancer Res. 2009;69(16):6387-95.
47. Quiroga MP, Burger JA. BCR-mediated decrease of CXCR4 and CD62L in CLL. Cancer Res. 2010;70(12):5194.
48. de Rooij MF, Kuil A, Geest CR, Eldering E, Chang BY, Buggy JJ, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. Blood. 2012;119(11):2590-4.
49. Yarkoni Y, Getahun A, Cambier JC. Molecular underpinning of B-cell anergy. Immunol Rev. 2010;237(1):249-63.
50. Gabelloni ML, Borge M, Galletti J, Canones C, Calotti PF, Bezares RF, et al. SHIP-1 protein level and phosphorylation status differs between CLL cells segregated by ZAP-70 expression. Br J Haematol. 2008;140(1):117-9.
51. Fecteau JF, Kipps TJ. Structure and function of the hematopoietic cancer niche: focus on chronic lymphocytic leukemia. Frontiers in Bioscience. 2012;4:61-73.
52. Scielzo C, Bertilaccio MT, Simonetti G, Dagklis A, ten Hacken E, Fazi C, et al. HS1 has a central role in the trafficking and homing of leukemic B cells. Blood. 2010;116 (18):3537-46.
53. Zenz T, Mertens D, Dohner H, Stilgenbauer S. Importance of genetics in chronic lymphocytic leukemia. Blood Rev. 2011;25(3):131-7.
54. Marechal Y, Pesesse X, Jia Y, Pouillon V, Perez-Morga D, Daniel J, et al. Inositol 1,3,4,5-tetrakisphosphate controls proapoptotic Bim gene expression and survival in B cells. Proc Natl Acad Sci USA. 2007;104(35):13978-83.
55. Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. J Clin Invest. 2007;117(1):112-21.
56. Roberts AW, Seymour JF, Brown JR, Wierda WG, Kipps TJ, Khaw SL, et al. Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. J Clin Oncol. 2012;30(5):488-96.
57. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med. 2013. [Epub ahead of print.]

Do we need novel prognostic markers?

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A B S T R A C T

Factors able to predict the rate of disease progression and/or response to treatment are particularly valuable in heterogeneous diseases such as chronic lymphocytic leukemia (CLL). Although many such factors have been identified in CLL, current management is predominantly based on clinical factors, and loss or mutation of the *TP53* gene is the only biomarker recommended for routine use. Although many other potentially valuable biomarkers have been reported, evidence for clinical utility is frequently lacking, often due to lack of precision. Novel markers, especially acquired genomic abnormalities and detection of minimal residual disease, offer the prospect of individualized patient management but their eventual role will require evaluation in large validated studies and will also depend on the effectiveness of emerging novel therapies.

Learning goals

At the conclusion of this activity, participants should know that:
- to be clinically useful, prognostic factors must predict the natural history and/or response to treatment of individual patients with a high degree of accuracy;
- currently, patients should be screened for a *TP53* abnormality prior to treatment with standard chemotherapy or chemo-immunotherapy regimens;
- a panel of biomarkers including IGHV sequencing, CD38 expression and a screen for 11q loss and *TP53* abnormalities can provide additional prognostic information in patients with early stage disease;
- in the future, screening patients for driver mutations pre-therapy and at relapse, and testing for minimal residual disease post therapy offers the potential for personalized treatment.

Why do we need prognostic factors?

The introduction to the majority of papers on chronic lymphocytic leukemia (CLL) includes a statement on the heterogeneity of the disease both in terms of its natural history and the variable outcome of patients receiving identical treatments. This heterogeneity has a number of causes. It partly reflects the arbitrary time of diagnosis, as more than 80% of patients are identified following a routine blood count performed for an incidental reason. Heterogeneity between patients, including genetic factors, age at diagnosis and the presence of co-morbidities, is a further potent factor accounting for variable outcomes. However, even among fit, younger patients with a similar tumor burden, differences in the rate of disease progression and response to therapy persist. This has provided the stimulus for identifying both prognostic factors (defined as those which distinguish the clinical outcome of patients in the absence of any future therapies) and predictive factors (those which identify patients who will or will not derive substantial benefit from treatment). The potential benefits of these factors are well recognized. For patients with no immediate indication for treatment, they can provide information about the likelihood of their condition subsequently affecting their quality of life and/or requiring treatment. They can inform the need for and frequency of follow up and can identify patients at high risk of disease progression who may be suitable for trials of early treatment, especially with novel agents that have a low risk of both short- and long-term toxicity. In contrast, predictive factors can be used to influence the nature, dose and duration of treatment and avoid the use of either ineffective or excessively toxic therapy. They also enable patient stratification within clinical trials to ensure compatibility between patient groups.

Why are so few prognostic/ predictive biomarkers used in routine practice?

Despite the enthusiasm for research into prognostic factors and the number of papers published, it is salutary to reflect that less than 1% of published cancer biological factors (biomarkers) enter clinical practice. This is also evident in CLL in which *TP53* loss or mutation is currently the only biomarker rec-

ommended for routine clinical use.[1,2] The journey between an initial exploratory study indicating a possible association between a biological factor and a clinically important outcome and its routine clinical application is both long and arduous. Figure 1 describes the various steps involved in evaluating novel biomarkers. There are many reasons why initially promising biomarkers are either not adopted into routine practice or subsequently lose their value.[3] Many studies suffer from incomplete or flawed evaluation due, for example, to problems with study design, statistical methods, data analysis or reporting biases although, paradoxically, the value of the most powerful prognostic markers may be evident even from imperfect studies. A further problem is failure to demonstrate clinical utility despite evidence for biological, analytical and clinical validity. This in turn has a number of possible causes. 1) Many prognostic factors lack precision such that a single or group of prognostic markers may enable the subdivision of patient populations into risk groups but do not predict with sufficient accuracy the outcome of an individual patient. This is especially important for predictive markers. 2) There may be a surfeit of markers providing comparable information such that the new marker has no added value. 3) The introduction of a new therapy may obviate the need for a predictive biomarker for a previous therapy. 4) Despite predicting a poor outcome, there may be no good alternative treatment. 5) Finally, the inability to demonstrate both validity and utility may also reflect difficulties in obtaining funding for prognostic marker studies and in overcoming regulatory hurdles.

These problems are well documented and considerable effort has been and continues to be expended on measures to overcome them and avoid the expenditure of time, expertise and money on prognostic markers that have a very small chance of being clinically useful.[4-11]

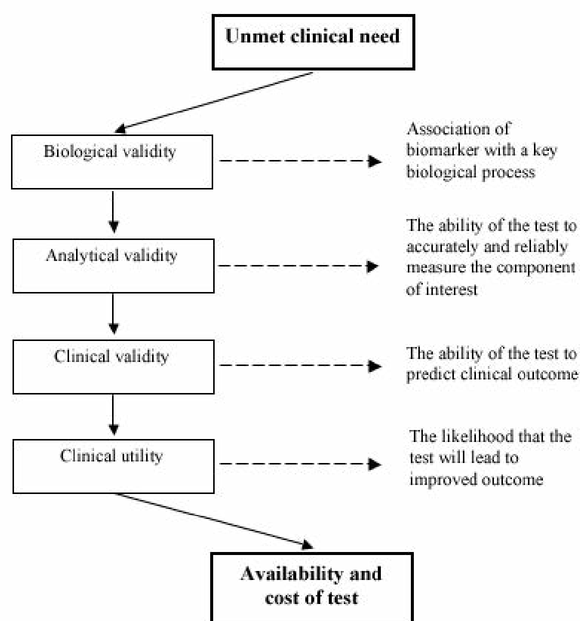

Figure 1. Evaluation of biomarkers.

These initiatives include:

- the publication of standards for the reporting of biomarker studies (Remark) and sample collection and storage (BRISq) with shared responsibility between researchers, editors and funders to ensure these recommendations are implemented;
- the use of comparative effectiveness research utilizing ‘real-world data’ and ‘patient-reported outcomes’ to supplement conventional clinical trial data. Randomized clinical trials (RCT) are indispensable for evaluating novel agents and have been improved by the introduction of adaptive designs. However, too many new biomarkers are being identified for them all to be evaluated in RCTs and the latter frequently have restricted entry criteria such that patients who may benefit from novel therapies are excluded from them;
- the creation of a regulatory and funding environment that would promote collaboration among national and international research groups, facilitate sample exchange between cell banks, and enable large well conducted and, ideally, prospective studies with external validation to be undertaken;
- a recommendation to establish an International registry of biomarker studies analogous to the clinical trials registry to avoid duplication of studies.

An additional and sometimes neglected issue is to ensure that prognostic information given to patients is done so in a comprehensible and compassionate manner.[12]

Current use of prognostic and predictive factors in CLL

The current management of CLL routinely utilizes a series of prognostic and predictive factors that are commonly classified as patient, disease or treatment-related. Patient-related factors include age, performance status and co-morbidities, and can either directly affect overall survival or limit the use of effective therapies. Disease-related factors include tumor burden, marrow failure, the rate of disease progression, immunodeficiency, lymphomatous transformation, and loss/mutation of the *TP53* gene, while treatment-related factors include the type of treatment, and the degree and duration of response.

Although useful, the factors listed above have important limitations. Measurement of tumor burden and marrow failure encapsulated in the Binet and Rai staging systems have been the main tools for predicting outcome in CLL and remain key elements in the current International Workshop on Chronic Lymphocytic Leukemia (IWCLL) guidelines on indications for treatment. However, both staging systems are insensitive to the heterogeneity within cases presenting with a low tumor burden and are poor predictors of response to treatment. *TP53* loss /mutation identifies a group of patients who fail to benefit from treatment with alkylating agents, purine analogs with or without anti-CD20 antibodies and for whom alternative treatments are recommended. However, only approximately 50% of patients refractory to a purine analog-containing regimen have a *TP53* abnormality.[13] In addition, there is heterogeneity among patients with a *TP53* abnormality in that some are unresponsive to treatment while others respond but have a short progression-free survival (PFS)

suggesting that other factors such as clone size, the site and/or functional consequence of the *TP53* mutation, or co-existing genomic abnormalities may be important. These observations highlight the potential value of better prognostic and predictive factors.

The potential role for new prognostic and predictive markers in CLL

Advances in the understanding of the biology of CLL have highlighted the importance of the cell(s) of origin, the ability of leukemic cells to respond to antigen, the interaction of leukemic cells with their microenvironment, and the acquisition of genomic abnormalities as key factors determining the rate of disease progression and response to therapy. A list of biomarkers frequently measured in patients entered into clinical trials (B2M, IGHV mutational status and IGHV3-21 use, CD38 and ZAP70 expression, fluorescence *in situ* hybridization (FISH) for chromosome 11q, 13q, 17p loss and 12 gain using the Dohner hierarchical model), together with some of the more recently described markers and methods for their detection (serum free light chains,[14-16] gene mutations,[17-20] genomic complexity,[21] epigenetic abnormalities,[22-26] gene expression,[27-31] telomere abnormalities,[32-35] BcR stereotypy[36,37] and functional assays[38-41]) is given in Table 1. Recently, many excellent reviews have covered both the range and potential use of biomarkers in CLL.[42-46] I will briefly review recent data on novel genomic abnormalities, summarize the results of studies using both established and newer prognostic and predictive markers, and then consider the role of minimal residual disease (MRD) detection.

Acquired genomic abnormalities

The application of single nucleotide polymorphism (SNP) and comparative genomic hybridization (CGH) arrays and next generation sequencing (NGS) technologies to CLL has enabled high-resolution genome-wide screening for genomic loss, gains, rearrangements, loss of heterozygosity, gene mutations, abnormal DNA methylation and aberrant transcription. Although the number of genomic abnormalities per case detected in CLL is low compared to some other hematologic malignancies and solid tumors, novel prognostic indicators are being identified. Recent SNP array studies indicate that gains of 8q24 or 3q26.3 involving the PIK3CA gene, genomic complexity and chromothripsis may all have prognostic or predictive significance in univariate analysis.[47,48] NGS studies on small discovery cohorts has revealed an increasing number of novel mutations in CLL clustered within well-defined pathways such as NOTCH, WNT, NFKB, TLR and BcR signaling, DNA repair and RNA splicing. Targeted resequencing of the commoner mutations, involving the *NOTCH1*, *SF3B1*and *BIRC3* genes in larger cohorts consistently shows associations with poor outcome in univariate analyses.[49-52] These studies also enable the prognostic value of established genomic abnormalities, such as deletions of 13q and 11q, to be refined and show that clone size, deletion size and mutations of genes on the remaining allele may all have prognostic significance.

Clonal evolution

Sequential cytogenetic and/or FISH studies demonstrated clonal evolution in up to 30% of patients, indicating that heterogeneity exists not only between patients but also between leukemic cells from the same individual. A linear pattern of evolution was usually observed in which new subclones show additional abnormalities while retaining those present in the original clone. Clinically, both clonal evolution and genomic complexity have been associated with poor outcome independent of a *TP53* or 11q abnormality. Newer technologies have provided a more

Table 1. Biological prognostic markers in CLL.

Serum markers		B2M, STK, sCD23, sFLC
Genomic abnormalities	Copy number variation	del 13q, del 11q, p53 loss, gain8q24, +12
	Genomic complexity	
	Chromothripsis	
	Genetic mutations	TP53, ATM, NOTCH1, SF3B1, BIRC3
	Gene SNPs	
DNA methylation	Global arrays	
	Specific genes	ZAP 70
Gene expression	mRNAs	CLLU1, LPL, AID
	miRs	21, 29c,34a, 181b,223
	Protein	CD38, CD49d, CD69, ZAP70, TCL1
	Global assays	Gene expression profiles, proteomics
IGVH genes		Mutational load, VH gene usage, stereotypye
Telomere abnormalities	Telomere length	
	Telomerase activity	
Functional assays	BCR, CD40 signaling	
	P53 function	

accurate picture of the incidence and patterns of clonal evolution in CLL.[53-55] Comparable to other tumor types, a branching pattern of evolution is frequently observed in which small subclones present early in the disease may emerge as dominant clones or subclones following therapy or at transformation to diffuse large B-cell lymphoma (Figure 2).

Schuh *et al.*[56] performed whole genomic sequencing on peripheral blood leukemic cells and buccal cells from 3 patients at five separate time points for a period of up to seven years. All patients received several courses of therapy, and based on standard biomarkers all would have been predicted to respond to standard chemo-immunotherapy. In each case, driver mutations were found in all cells (representing founder events) and also in emerging subclones. Interestingly, the relative frequency of various subclones prior to therapy and following relapse varied among cases. In one case, the predominant pre-treatment clone was almost completely replaced, in relapse, by a subclone that was present as a small subpopulation pre-treatment, while in another patient, all five subclones present pre-treatment re-emerged following therapy.

Landau *et al.*[57] performed whole exomic sequencing and SNP arrays in 149 patients with CLL. Twenty putative cancer driver genes were identified of which trisomy 12, del 13q and mutations of *MYD88* were present in the majority of cells indicating that these were early events in leukemogenesis while mutations of *ATM*, *TP53* and *SF3B1* were present only in subclones consistent with their acquisition later in the course of the disease. Subclonal mutations were associated with a shorter time from sample collection to first therapy and shorter PFS indedendent of IGHV mutational status, del 11q or TP53 loss. Among the 18 cases tested sequentially clonal evolution was detected in 10 of 12 who received intervening therapy but in only one of 6 who were not treated. Subclones present pre- treatment tended to become clonal on re-testing post therapy.

Studies performed in CLL and other malignancies have also found subclones emerging post therapy that could not be detected earlier in the disease, indicating that treatment itself may be mutagenic. These types of study require replication in larger cohorts, both at diagnosis and within clinical trials, and have important implications for the use of genomic screening as a prognostic and predictive marker. Although clonal evolution is most often detected in patients who relapse following treatment, it also occurs in untreated patients and can limit the ability of genomic screening at a single time point to predict the long-term natural history of the disease. Whilst clonal evolution is more common in patients with unmutated IGHV genes, a *TP53* abnormality or deletion of 11q, the mechanism(s) underlying genomic instability in an individual patient is frequently unclear. Of the many factors that have been associated with genomic instability, expression of AID[58] and short telomere length[34,35] are promising candidates.

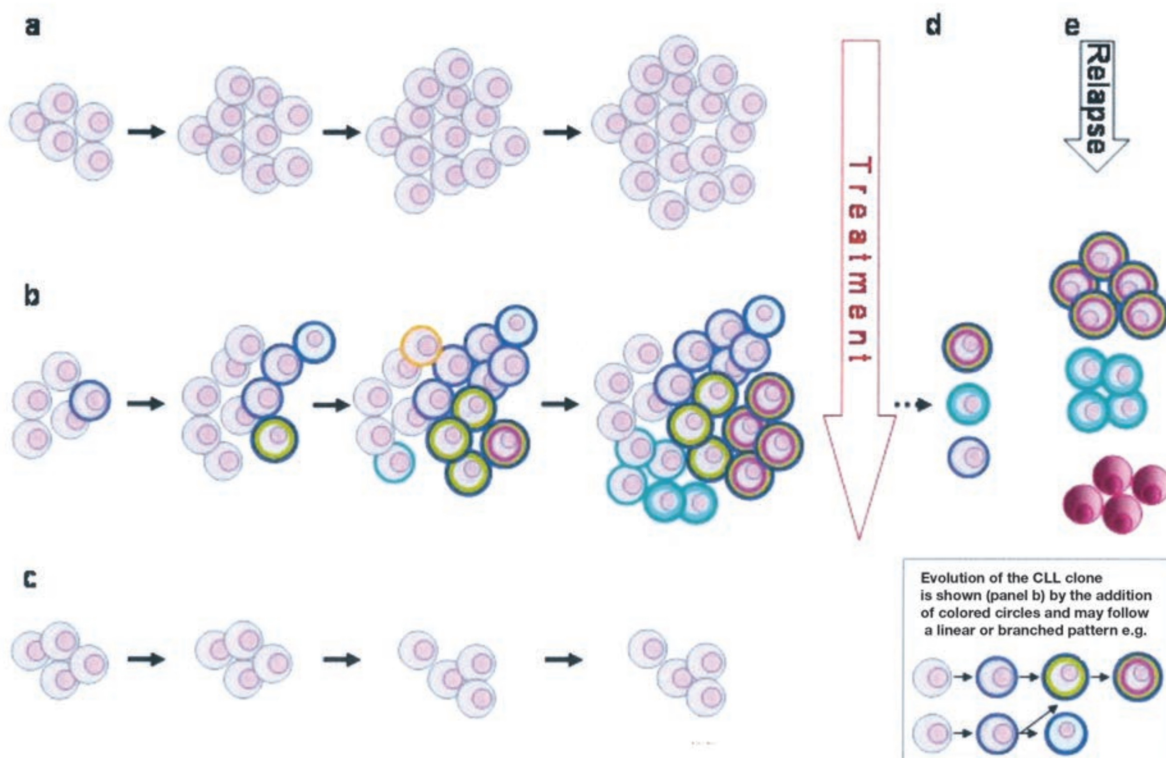

Figure 2. (a) Tumor slowly increasing in size. (b) Tumor increasing in size and acquiring genomic abnormalities sequentially (shown by colored circles), given rise to multipe sub-clones. (c) Tumor unchanging over time. (d) Post therapy , MRD small resisant sub-clones remain (e) At relapse resitant clones expand and new sub-clones may emerge.

A recent study suggests that very short telomeres detectable using the STELA assay may be a more powerful predictor of TTFT than established biomarkers (D Baird, personal communication, 2013). For patients requiring initial or subsequent treatment, screening for recurring genomic abnormalities and detection of subclones pre-treatment, at relapse and conceivably in MRD positive disease post-treatment, may have implications for the choice and duration of treatment, particularly if therapies targeted to specific genomic abnormalities become more widely available.[59,60]

Role of predictive markers

Clinical and biomarkers have been evaluated both retrospectively and in phase III trials for their ability to predict treatment response, progression-free (PFS) and overall survival (OS). In the UK LRF CLL4 trial[61,62] which randomized 777 patients to fludarabine alone, in combination with cyclophosphamide or to chlorambucil, 3 risk groups were identified: poor risk (6%) with *TP53* loss >10%; intermediate risk (72%) without *TP53* loss and with at least one of: unmutated IGHV genes and/or IGHV*3-21* gene usage, 11q deletion, β2M >4 mg/L; good risk (22%) with mutated IGHV genes and none of the above factors. Neither CD38 nor ZAP70 expression retained prognostic significance in multivariate analysis, consistent with other studies suggesting that markers reflecting cell activation and proliferation are more predictive of TTFT than outcome following treatment (see below). The phase III GSGCLL8 trial[63] randomized 817 previously untreated, predominantly Binet stage B/C patients to receive FC with or without rituximab (FCR). In a multivariate analysis, age, gender, FCR treatment, sTK, β2M, unmutated IGHV genes and del(17p) were all independent factors predicting PFS or OS. Of particular interest was the observation that del(11q) was not associated with shorter PFS or OS in the FCR arm. The predictive value of *TP53* mutations has been confirmed in both the UK CLL4 and GCLLSG CLL4 studies[64,65] and in the UK CLL4 trial, patients with *ATM* loss and mutation had a shorter PFS and OS than cases with monoallelic *ATM* abnormalities.[66] In the UK CLL4 trial, *NOTCH1* and *SF3B1* mutations were detected in 10% and 17% of patients, respectively. Both were independent factors for PFS and OS, but TP53 abnormalities remained the strongest adverse prognostic factor.[67] Similarly, *NOTCH1* and *SF3B1* mutations were found in 10% and 18.4% of patients in the GCLLSG CLL8 trial and both were independent markers for PFS. *NOTCH1* mutations appeared to identify a subgroup of patients who did not benefit from the addition of rituximab to FC.[68]

Neither *TP53*, *NOTCH1* nor *SF3B1* mutations affected OS in patients with fludarabine-refractory CLL treated with alemtuzumab in the GCLLSG 2H trial,[69] nor those receiving allogeneic transplantation in the GCLLSG CLL3X trial,[70] highlighting the importance of evaluating predictive markers within a clearly defined clinical context.

Role of prognostic markers

Many studies have sought to identify factors that predict time to first treatment (TTFT) and/or overall survival from diagnosis, especially for patients with no immediate indication for treatment. Table 2 lists the ‘established’ prognostic factors that have remained independent markers for TTFT in recent publications.[72-76] These studies differ in a number of important respects, such as whether they include all or only early stage patients and whether they are derived from a local community or referred to a specialist center. But a number of interesting points emerge. 1) Only a single factor (IGHV mutational status) is consistently represented among all 6 studies. 2) TP53 status appears to be less important as a prognostic than a predictive marker, partly reflecting a subgroup of early stage patients with TP53 abnormalities and mutated IGHV genes who have stable disease.[77,78] 3) The Rai staging system provides prognostic information in patients with Binet stage A disease. 4) Easily measured parameters such as B-cell count and lymphocyte doubling time retain prognostic significance in multivariate analyses that include large panels of biomarkers (Table 2).

Although the panel of markers used in the above studies can identify the majority of patients at high risk of early progression, they are less useful for distinguishing between patients (most of whom have mutated IGHV genes) destined to have either stable or slowly progressive disease. Preliminary studies of newer biomarkers indicate that they may be clinically useful predictors of progressive disease. Expression of CD38, and the more recently described markers, CD49d[30] and CD69,[31] reflect cellular activation and both recent cell proliferation and a higher incidence of genomic abnormalities are associated with cells expressing CD38.[79,80] It is, therefore, unsurprising that their expression correlates with disease progression. However, there is still uncertainty as to the level of expression that best predicts outcome and expression of both CD38 and CD49d are higher in patients with trisomy 12,

Table 2. Independent risk factors for TTFT in multivariate analyses.

Study	N. of patients	Stage	Rai stage	ALC	B2M	IGVH	CD38	ZAP-70	del 11q	del 17p	Other
Shanafelt[71] 2010	585	all	√	√	N/A	√	√	X	X	X	
Wierda[72] 2011	687	all	X	X	X	√	X	X	√	√	
Pepper[73] 2012	1154	A	N/A	N/A	N/A	√	√	X	X	X	≥3 node sites, LDH
Bulian[74] 2012	291	A< 70 yrs	N/A	X	√	√	√	X	√	X	Age, LDT
Molica[75] 2012	328	A	√	√	√	√	X	X	X	X	
Scarfo[76] 2012	614	Rai 0 cMBL	N/A	√	N/A	√	X	N/A	X	X	B-cell count >10.37x10^{9}

including some with stable disease.[81] Approximately 30% of cases have closely related (stereotyped) BcR sequences and several subsets show characteristic antigen reactivity, gene expression and epigenetic profiles and clinical behaviour.[36,37] Larger studies are in progress to determine whether stereotypes are independent markers of disease progression. Recently, the prognostic significance of *NOTCH1* and *SF3B1* mutations has also been evaluated in retrospective studies of patients at diagnosis.[82,83] Although the incidence of mutations is lower than in patients with advanced disease, they are independent factors for TTFT and OS, and enhance the prognostic value of the Dohner hierarchical model. As an example, Rossi *et al*.[84] were able to identify a subgroup of patients with del13q as their only abnormality whose 10-year survival was similar to that of the matched general population.

Evidence that biomarkers can identify patients who benefit from early treatment awaits the results of randomized studies, such as the CLL7 trial of the German and French CLL study groups, in which Binet stage A patients with a high risk of disease progression (defined as having at least 2 of the following 4 parameters: sTK >10U/l, unmutated IGHV genes, del11q, del17p, trisomy12 or a lymphocyte doubling time of <12 months) were randomized to observation or treatment with FCR.[84] It is important to recognize that markers predicting TTFT based on IWCLL criteria may be insensitive to other CLL-related problems that can arise in patients with no indication for immediate treatment; these include infections secondary to hypogammaglobulinemia, an increased risk of secondary malignancies and transformation to high-grade lymphoma (see below).

Prediction of CLL transformation to Richter's syndrome

Rossi *et al*. studied 185 consecutive CLL cases in whom the actuarial incidence of RS (all DLBCL) was 16.2% at ten years.[85] Univariate analysis of both clinical and biological parameters showed that unmutated IGHV genes, IGHV*4-39* usage, absence of del(13)(q14), CD38>30% ZAP70>20%, size and number of lymph nodes, advanced Binet stage and LDH were predictive of transformation to RS. In a multivariate model, only lymph node size over 3 cm and absence of del(13)(q14) remained significant. Subsequent studies incorporating newer biomarkers have shown that short telomere length, single nucleotide polymorphisms within the *CD38* and low density lipoprotein receptor 4 (*LRP4*) genes, stereotyped BCRs (especially subset 8 which utilizes the IGHV*4-39/IGHD6-13/IGHJ5* genes) and *NOTCH1* mutations are additional risk factors for transformation RS.[17,86,87] Interestingly, these risk factors differ from those predicting progression of CLL.

Detection of minimal residual disease

One of the best predictors of overall survival is response to treatment. The introduction of sensitive clone-specific PCR and 4-color flow cytometric assays has enabled the reproducible detection of one leukemic cell in a background of 10^4 normal B cells.[88,89] Cases in which residual disease post therapy is either undetectable or present in less than 1 in 10^4 cells are currently considered to be minimal residual disease negative (MRD negative). Many studies have shown that MRD negativity following either initial treatment with chemotherapy, alemtuzumab for relapsed/refractory disease, autologous or allogeneic transplantation is associated with a longer PFS and OS.[90-92]

MRD levels were measured in the GCLLSG CLL8 Trial, comparing FC with FCR, and patients categorized into low ($<10^{-4}$), intermediate ($>10^{-4}$ to $<10^{-2}$) and high level ($>10^{-2}$) groups.[93] Low MRD levels were predictive of longer PFS and OS independent of TP53 abnormalities, IGHV mutation status and treatment arm, although a higher percentage of patients treated with FCR achieved a low MRD level. These results have important implications for patients receiving intensive therapies and raise the possibility of using MRD levels and kinetics to influence the duration of therapy, the need for maintenance treatment and the early treatment of relapse.[94] These issues are starting to be addressed in randomized clinical trials. The GCLLSG CLLM1 trial randomizes patients with high MRD levels or those with intermediate levels and either a TP53 abnormality or unmutated IGHV genes to receive lenalidomide or placebo following first-line therapy. In the UK, the CLARET study will randomize patients with a good response to previous treatment and who remain MRD positive to either obinutuzumab or placebo.

Conclusions

Prognostic factors are generated from 3 main sources: from data collected as part of routine management, from focused research into the mechanisms underlying treatment resistance and, most frequently, as a by-product of advances into the biology of CLL. Unless treatments become available that are non-toxic, universally effective, affordable and to which resistance does not occur, there will continue to be a need for predictive markers.[95,96] Similarly, for the majority of patients presenting with early disease, there is a continuing need for the more precise identification of those at high risk of disease progression and those destined to have stable asymptomatic disease in whom over-diagnosis, over-investigation and over-treatment must be avoided. Prognostic and predictive factors with the potential to achieve these goals are becoming available and will continue to be discovered, enabling a shift from the allocation of patients to risk groups to individualized risk assessment. However, it will remain important to use markers in well-defined clinical contexts, and to base clinical decisions on all available clinical and laboratory data, using a panel rather than a single biomarker. The greater challenge is in performing the studies demonstrating that these factors have clinical utility.

References

1. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Dohner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008;111:5446-56.
2. Oscier D, Dearden C, Erem E, Fegan C, Follows G, Hillmen P, et al. Writing group: On behalf of the British Committee for Standards in Haematology. Guidelines on the diagnosis, investigation and management of Chronic Lymphocytic Leukaemia. Br J Haematol. 2012;159:541-64.
3. Kern SE. Why your new cancer biomarker may never work: recurrent patterns and remarkable diversity in biomarker failures. Cancer Res. 2012;72:6097-101.
4. Riley RD, Abrams KR, Sutton AJ, Lambert PC, Jones DR, Heney D, et al. Reporting of prognostic markers: current prob-

lems and development of guidelines for evidence-based practice in the future. Br J Cancer. 2003;88:1191-8.
5. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer. 2005:93:387-91.
6. Teutsch SM, Bradley LA, Palomaki GE, Haddow JE, Piper M, Calonge N, et al. The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Initiative: methods of the EGAPP Working Group. Genet Med. 2009;11:3-14.
7. Moore HM, Kelly AB, Jewell SD, McShane LM, Clark DP, Greenspan R, et al. Biospecimen reporting for improved study quality (BRISQ). J Proteome Res. 2011; 10:3429-38.
8. McShane LM, Hayes DF. Publication of tumor marker research results: The necessity for complete and transparent reporting. J Clin Oncol. 2012;30:4223-32.
9. Ginsburg GS, Kuderer NM. Comparative effectiveness research, genomics-enabled personalised medicine, and rapid learning health care: A common bond. J Clin Oncol. 2012;30:4233-42.
10. Armstrong K. Methods in comparative effectiveness research. J Clin Oncol. 2012;30:4208-14.
11. Novik A, Salek S, Ionova T. Guidelines Patient-Reported Outcomes in Hematology. The European Hematology Association, The Hague, The Netherlands, 2012.
12. Gigerenzer G, Gaissmaier W, Kurz-Milcke E, Schwartz LM, Woloshin S. Helping doctors and patients to make sense of health statistics. Psychol Sci Public Interest. 2007;8:53-96.
13. Zenz T, Gribben JG, Hallek M, Döhner H, Keating MJ, Stilgenbauer S. Risk categories and refractory CLL in the era of chemoimmunotherapy. Blood. 2012;119:4101-7.
14. Pratt G, Harding S, Holder R, Fegan C, Pepper C, Oscier D, et al. Abnormal serum free light chain ratios are associated with poor survival and may reflect biological subgroups in patients with chronic lymphocytic leukaemia. Br J Haematol. 2009; 144:217-22.
15. Maurer MJ, Cerhan JR, Katzmann JA, Link BK, Allmer C, Zent CS, et al. Monoclonal and polyclonal serum free light chains and clinical outcome in chronic lymphocytic leukemia. Blood. 2011;118 :2821-6.
16. Morabito F, De Filippi R, Laurenti L, Zirlik K, Recchia AG, Gentile M, et al. The cumulative amount of serum-free light chain is a strong prognosticator in chronic lymphocytic leukemia. Blood. 2011;118:6353-61.
17. Fabbri G, Rasi S, Rossi D, Trifonov V, Khiabanian H, Ma J, et al. Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. J Exp Med. 2011;208:1389-401.
18. Puente XS, Pinyol M, Quesada V, Conde L, Ordonez GR, Villamor N, et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature. 2011;475:101-5.
19. Wang L, Lawrence MS, Wan Y, Stojanov P, Sougnez C, Stevenson K, et al. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. N Engl J Med. 2011;365:2497-506.
20. Quesada V, Conde L, Villamor N, Ordonez G, Jares P, Bassaganyas L, et al. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. Nat Genet. 2012;44:47-52.
21. Kujawski L, Ouillette P, Erba H, Saddler C, Jakubowiak A, Kaminski M, et al. Genomic complexity identifies patients with aggressive chronic lymphocytic leukemia. Blood. 2008;112:1993-2003.
22. Corcoran M, Parker A, Orchard J, Davis Z, Wirtz M, Schmitz OJ, et al. ZAP-70 methylation status is associated with ZAP-70 expression status in chronic lymphocytic leukemia. Haematologica. 2005;90:1078-88.
23. Claus R, Lucas DM, Stilgenbauer S, Ruppert AS, Yu LB, Zucknick M, et al. Quantitative DNA Methylation Analysis Identifies a Single CpG Dinucleotide Important for ZAP-70 Expression and Predictive of Prognosis in Chronic Lymphocytic Leukemia. J Clin Oncol. 2012;30:2483-91.
24. Irving L, Mainou-Fowler T, Parker A, Ibbotson RE, Oscier DG, Strathdee G. Methylation markers identify high risk patients in IGHV mutated chronic lymphocytic leukemia. Epigenetics. 2011;6:300-6.
25. Cahill N, Bergh AC, Kanduri M, Goransson-Kultima H, Mansouri L, Isaksson A, et al. 450K-array analysis of chronic lymphocytic leukemia cells reveals global DNA methylation to be relatively stable over time and similar in resting and proliferative compartments. Leukemia. 2012;27:150-8.
26. Kulis M, Heath S, Bibikova M, Queiros AC, Navarro A, Clot G, et al. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. Nat Genet. 2012;44:1236-42.
27. Sevov M, Rosenquist R, Mansouri L. RNA-based markers as prognostic factors in chronic lymphocytic leukemia. Expert Rev Hematol. 2012;5:69-79.
28. Gonzalez D, Else M, Wren D, Usai M, Buhl AM, Parker A, et al. CLLU1 expression has prognostic value in CLL after first-line therapy in younger patients and those with mutated IGHV genes. Haematologica. 2013;98:274-8.
29. Rossi S, Shimizu M, Barbarotto E, Nicoloso MS, Dimitri F, Sampath D, et al. microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. Blood. 2010;116:945-52.
30. Gattei V, Bulian P, Del Principe MI, Zucchetto A, Maurillo L, Buccisano F, et al. Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. Blood. 2008;111(2):865-73.
31. Del Poeta G, Del Principe MI, Zucchetto A, Luciano F, Buccisano F, Rossi FM, et al. CD69 is independently prognostic in chronic lymphocytic leukemia: a comprehensive clinical and biological profiling study. Haematologica. 2012;97:279-87.
32. Rossi D, Lobetti Bodoni C, Genuardi E, Monitillo L, Drandi D, et al. Telomere length is an independent predictor of survival, treatment requirement and Richter's syndrome transformation in chronic lymphocytic leukemia. Leukemia. 2009;23:1062-72.
33. Brugat T, Nguyen-Khac F, Grelier A, Merle-Béral H, Delic J. Telomere dysfunction-induced foci arise with the onset of telomeric deletions and complex chromosomal aberrations in resistant chronic lymphocytic leukemia cells. Blood. 2010;116:239-49.
34. Roos G, Kröber A, Grabowski P, Kienle D, Bühler A, Döhner H, et al. Short telomeres are associated with genetic complexity, high-risk genomic aberrations, and short survival in chronic lymphocytic leukemia. Blood. 2008;111:2246-52.
35. Lin TT, Letsolo BT, Jones RE, Rowson J, Pratt G, Hewamana S, et al. Telomere dysfunction and fusion during the progression of chronic lymphocytic leukemia: evidence for a telomere crisis. Blood. 2010;116:1899-907.
36. Stamatopoulos K, Belessi C, Moreno C, Boudjograh M, Guida G, Smilevska T, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. Blood. 2007;109:259-70.
37. Maura F, Cutrona G, Fabris S, Colombo M, Tuana G, Agnelli L, et al. Relevance of stereotyped B-cell receptors in the context of the molecular, cytogenetic and clinical features of chronic lymphocytic leukemia. PLoS One. 2011;6:e24313.
38. Mohr J, Helfrich H, Fuge M, Eldering E, Buhler A, Winkler D, et al. DNA damage induced transcriptional program in CLL: biological and diagnostic implications for functional p53 testing. Blood. 2011;117:1622-32.
39. de Viron E, Michaux L, Put N, Bontemps F, Van Den Neste E. Present status and perspectives in functional analysis of p53 in chronic lymphocytic leukemia. Leuk Lymphoma. 2012;53: 1445-51.
40. Scielzo C, Apollonio B, Scarfò L, Janus A, Muzio M, Ten Hacken E, et al. The functional in vitro response to CD40 ligation reflects a different clinical outcome in patients with chronic lymphocytic leukemia. Leukemia. 2011;25:1760-7.
41. Le Roy C, Deglesne PA, Chevallier N, Beitar T, Eclache V, Quettier M, et al. The degree of BCR and NFAT activation predicts clinical outcomes in chronic lymphocytic leukemia. Blood. 2012;120:356-65.
42. Van Bockstaele F, Verhasselt B, Philippe J. Prognostic markers in chronic lymphocytic leukemia: a comprehensive review. Blood Rev. 2009;23:25-47.
43. Stamatopoulos K, Ghia P, Rosenquist R, eds. New biological prognostic markers in chronic lymphocytic leukemia. Wolter Kluwer: Lippincott Williams and Wilkins; 2010.
44. Bazargan A, Tam CS, Keating MJ. Predicting survival in chronic lymphocytic leukemia. Expert Rev Anticancer Ther. 2012;12:393-403.
43. Sellner L, Dietrich S, Dreger P, Glimm H, Zenz T. Can prognostic factors be used to direct therapy in chronic lymphocytic leukemia? Curr Hematol Malig Rep. 2012;7:3-12.
46. Chiorazzi N. Implications of new prognostic markers in chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program. 2012;2012:76-87.
47. Brown JR, Hanna M, Tesar B, Werner L, Pochet N, Asara JM, et al. Integrative genomic analysis implicates gain of PIK3CA at 3q26 and MYC at 8q24 in chronic lymphocytic leukemia. Clin Cancer Res. 2012;18:3791-802.
48. Edelmann J, Holzmann K, Miller F, Winkler D, Bühler A,

Zenz T, et al. High-resolution genomic profiling of chronic lymphocytic leukemia reveals new recurrent genomic alterations. Blood. 2012;120:4783-94.
49. Rossi D, Rasi S, Fabbri G, Spina V, Fangazio M, Forconi F, et al. Mutations of NOTCH1 are an independent predictor of survival in chronic lymphocytic leukemia. Blood. 2012;119:521-9.
50. Villamor N, Conde L, Martínez-Trillos A, Cazorla M, Navarro A, Beà S, et al. NOTCH1 mutations identify a genetic subgroup of chronic lymphocytic leukemia patients with high risk of transformation and poor outcome Leukemia. 2012 Dec 6. [Epub ahead of print].
51. Rossi D, Bruscaggin A, Spina V, Rasi S, Khiabanian H, Messina M, et al. Mutations of the SF3B1 splicing factor in chronic lymphocytic leukemia: association with progression and fludarabine-refractoriness. Blood. 2011;118:6904-8.
52. Rossi D, Fangazio M, Rasi S, Vaisitti T, Monti S, Cresta S, et al. Disruption of BIRC3 associates with fludarabine chemorefractoriness in TP53 wild-type chronic lymphocytic leukemia. Blood. 2012;119:2854-62.
53. Gunnarsson R, Mansouri L, Isaksson A, Goransson H, Cahill N, Jansson M, et al. Array-based genomic screening at diagnosis and during follow-up in chronic lymphocytic leukemia. Haematologica. 2011;96:1161-9.
54. Knight SJL, Yau C, Clifford R, Timbs AT, Akha ES, Dreau HM, et al. Quantification of subclonal distributions of recurrent genomic aberrations in paired pre-treatment and relapse samples from patients with B-cell chronic lymphocytic leukemia. Leukemia. 2012;26:1564-75.
55. Braggio E, Kay NE, Vanwier S, Tschumper RC, Smoley S, Eckel-Passow JE, et al. Longitudinal genome-wide analysis of patients with chronic lymphocytic leukemia reveals complex evolution of clonal architecture at disease progression and at the time of relapse. Leukemia. 2012;26:1698-701.
56. Schuh A, Becq J, Humphray S, Alexa A, Burns A, Clifford R, et al. Monitoring chronic lymphocytic leukemia progression by whole genome sequencing reveals heterogeneous clonal evolution patterns. Blood. 2012;120:4191-6.
57. Landau D, Carter S, Stojanov P, Mckenna A, Stevenson K, Lawrence M, et al. The Evolution and Impact of Subclonal Mutations in Chronic Lymphocytic Leukemia. Cell. 2013; 152:714-26.
58. Patten PEM, Chu CC, Albesiano E, Damle RN, Yan X-J, Kim D, et al. IGHV-unmutated and IGHV-mutated chronic lymphocytic leukemia cells produce activation-induced deaminase protein with a full range of biologic functions. Blood. 2012;120:4802-11.
59. Wu CJ. CLL clonal heterogeneity: an ecology of competing subpopulations. Blood. 2012;120:4117-8.
60. Aparicio S, Caldas C. The Implications of Clonal Genome Evolution for Cancer Medicine. N Engl J Med. 2013;368:842-51.
61. Catovsky D, Richards S, Matutes E, Oscier D, Dyer MJS, Bezares RF, et al. Fludarabine plus cyclophosphamide improves response and progression-free survival in chronic lymphocytic leukemia. A report from the LRF CLL4 trial. Lancet. 2007;370:230-9.
62. Oscier D, Wade R, Davis Z, Morilla A, Best G, Richards S, et al. Chronic Lymphocytic Leukaemia Working Group, UK National Cancer Research Institute. Prognostic factors identified three risk groups in the LRF CLL4 trial, independent of treatment allocation. Haematologica. 2010;95:1705-12.
63. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. Lancet. 2010;376:1164-74
64. Zenz T, Eichhorst B, Busch R, Denzel T, Häbe S, Winkler D, et al. TP53 mutation and survival in chronic lymphocytic leukemia. J Clin Oncol. 2010;28:4473-9.
65. Gonzalez D, Martinez P, Wade R, Hockley S, Oscier D, Matutes E, et al. Mutational status of the TP53 gene as a predictor of response and survival in patients with chronic lymphocytic leukemia: results from the LRF CLL4 Trial p53 mutation in CLL4. J Clin Oncol. 2011;29:2223-9.
66. Skowronska A, Parker A, Ahmed G, Oldreive C, Davis Z, Richards S, et al. Biallelic ATM Inactivation Significantly Reduces Survival in Patients Treated on the United Kingdom Leukemia Research Fund Chronic Lymphocytic Leukemia 4 Trial. J Clin Oncol. 2012;30:4524-32.
67. Oscier DG, Rose-Zerilli MJ, Winkelmann N, Gonzalez de Castro D, Gomez B, Forster J, et al. The clinical significance of NOTCH1 and SF3B1 mutations in the UK LRF CLL4 trial. Blood. 2013;121:468-75.
68. Stilgenbauer S, Busch R, Schnaiter A, Paschka P, Rossi M, Döhner K, et al. Gene Mutations and Treatment Outcome in Chronic Lymphocytic Leukemia: Results From the CLL8 Trial Blood. ASH Annual Meeting Abstracts. 2012;120:433.
69. Schnaiter A, Rossi M, Paschka P, Cazzola M, Döhner K, Edelmann J, et al. NOTCH1, SF3B1 and TP53 Mutations in Fludarabine-Refractory CLL Patients Treated with Alemtuzumab: Results From the CLL2H Trial of the Gcllsg Blood. ASH Annual Meeting Abstracts. 2012;120:710.
70. Dreger P, Schnaiter A, Zenz T, Böttcher S, Rossi M, Paschka P, et al. TP53, SF3B1, and NOTCH1 mutations and outcome of allotransplantation for chronic lymphocytic leukemia: six-year follow-up of the GCLLSG CLL3X trial. Blood. 2013 Feb 22. [Epub ahead of print]
71. Shanafelt TD, Rabe KG, Kay NE, Zent CS, Jelinek DF, Reinalda MS, et al. Age at diagnosis and the utility of prognostic testing in patients with chronic lymphocytic leukemia. Cancer. 2010;116:4777-87.
72. Wierda WG, O'Brien S, Wang X, Faderl S, Ferrajoli A, Do KA, et al. Multivariable model for time to first treatment in patients with chronic lymphocytic leukemia. J Clin Oncol. 2011;29:4088-95.
73. Pepper C, Majid A, Lin T, Hewamana S, Pratt G, Walewska R, et al. Defining the prognosis of stage A chronic lymphocytic leukaemia patients Br J Haematol. 2012;156;499-507.
74. Bulian P, Rossi D, Forconi F, Del Poeta G, Bertoni F, Zucca E, et al. IGHV gene mutational status and 17p deletion are independent molecular predictors in a comprehensive clinical-biological prognostic model for overall survival prediction in chronic lymphocytic leukemia. J Transl Med. 2012;10:18.
75. Molica S, Giannarelli D, Gentile M, Cutrona G, Di Renzo N, Di Raimondo F, et al. External validation on a prospective basis of a nomogram for predicting the time to first treatment in patients with chronic lymphocytic leukemia. Cancer. 2012 Dec 7 [Epub ahead of print].
76. Scarfò L, Zibellini S, Tedeschi A, Maura F, Neri A, Bertazzoni P, et al. Impact of B-cell count and imaging screening in cMBL: any need to revise the current guidelines? Leukemia. 2012;26:1703-7.
77. Best OG, Gardiner AC, Davis ZA, Tracy I, Ibbotson RE, Majid A, et al. A subset of Binet stage A CLL patients with TP53 abnormalities and mutated IGHV genes have stable disease. Leukemia. 2009;23:212-4.
78. Tam CS, Shanafelt TD, Wierda WG, Abruzzo LV, Van Dyke DL, O'Brien S, et al. De novo deletion 17p13.1 chronic lymphocytic leukemia shows significant clinical heterogeneity: the M. D. Anderson and Mayo Clinic experience. Blood. 2009;114:957-64.
79. Calissano C, Damle RN, Hayes G, et al. In vivo intraclonal and interclonal kinetic heterogeneity in B-cell chronic lymphocytic leukemia. Blood. 2009;114:4832-42.
80. Grubor V, Krasnitz A, Troge JE, Meth JL, Lakshmi B, Kendall JT, et al. Novel genomic alterations and clonal evolution in chronic lymphocytic leukemia revealed by representational oligonucleotide microarray analysis (ROMA). Blood. 2009;113:1294-303.
81. Ibbotson R, Athanasiadou A, Sutton LA, Davis Z, Gardiner A, Baliakas P, et al. Co-existence of trisomies of chromosomes 12 and 19 in chronic lymphocytic leukemia occurs exclusively in the rare IgG positive variant. Leukemia. 2012;26;170-2.
82. Rossi D, Rasi S, Spina V, Bruscaggin A, Monti S, Ciardullo C, et al. Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. Blood. 2012 Dec 14. [Epub ahead of print].
83. Mansouri L, Cahill N, Gunnarsson R, Smedby KE, Tjönnfjord E, Hjalgrim H, et al. NOTCH1 and SF3B1 mutations can be added to the hierarchical prognostic classification in chronic lymphocytic leukemia. Leukemia. 2012 Nov 6. [Epub ahead of print].
84. Bergmann MA, Eichhorst BF, Busch R, Adorf D, Stilgenbauer S, Eckart M, et al. Prospective evaluation of prognostic parameters in early stage chronic lymphocytic leukemia (CLL): Results of the CLL1-protocol of the German CLL Study Group (GCLLSG). Blood. 2007;110:625a.
85. Rossi D, Cerri M, Capello D, Deambrogi C, Rossi FM, Zucchetto A, et al. Biological and clinical risk factors of chronic lymphocytic leukaemia transformation to Richter syndrome. Br J Haematol. 2008;142:202-15.
86. Rossi D, Spina V, Cerri M, Rasi S, Deambrogi C, De Paoli L, et al. Stereotyped B-cell receptor is an independent risk factor of chronic lymphocytic leukemia transformation to Richter syndrome. Clin Cancer Res. 2009;15:4415-22.
87. Rossi D, Spina V, Deambrogi C, Rasi S, Laurenti L, Stamatopoulos K, et al. The genetics of Richter syndrome

reveals disease heterogeneity and predicts survival after transformation. Blood. 2011;117(12):3391-401.
88. Ritgen M, Bottcher S, Stilgenbauer S, Bunjes D, Schubert J, Cohen S, et al. Quantitative MRD monitoring identifies distinct GVL response patterns after allogeneic stem cell transplantationfor chronic lymphocytic leukemia: results from the GCLLSG CLL3X trial. Leukemia. 2008;22:1377-86.
89. Rawstron AC, Böttcher S, Letestu R, Villamor N, Fazi C, Kartsios H, et al. Improving efficiency and sensitivity: European Research Initiative in CLL (ERIC) update on the international harmonised approach for flow cytometric residual disease monitoring in CLL. Leukemia. 2013;27:142-9.
90. Moreton P, Kennedy B, Lucas G, Leach M, Rassam S, Haynes A, et al. Eradication of minimal residual disease in B-cell chronic lymphocytic leukemia after alemtuzumab therapy is associated with prolonged survival. J Clin Oncol. 2005;23: 2971-9.
91. Bosch F, Ferrer A, Villamor N, González M, Briones J, González-Barca E, et al. Fludarabine, cyclophosphamide, and mitoxantrone as initial therapy of chronic lymphocytic leukemia: high response rate and disease eradication. Clin Cancer Res. 2008;14:155-61.
92. Dreger P, Dohner H, Ritgen M, Bottcher S, Busch R, Dietrich S, et al. Allogeneic stem cell transplantation provides durable disease control in poor-risk chronic lymphocytic leukemia:long-term clinical and MRD results of the German CLL Study Group CLL3X trial. Blood. 2010;116:2438-47.
93. Bottcher S, Ritgen M, Fischer K, Stilgenbauer S, Busch M, Fingerle-Rowson G, et al. Minimal residual disease quantification is an independent predictor of progression-free and overall survival in chronic lymphocytic leukemia: a multivariate analysis from the randomized GCLLSG CLL8 trial. J Clin Oncol. 2012;30:980-8.
94. Ghia P. A look into the future: can minimal residual disease guide therapy and predict prognosis in chronic lymphocytic leukemia? Hematology Am Soc Hematol Educ Program. 2012;2012:97-104.
95. Roberts AW, Seymour JF, Brown JR, Wierda WG, Kipps TJ, Khaw SL, et al. Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. J Clin Oncol. 2012;30:488-96.
96. Garraway LA, Jänne PA. Circumventing cancer drug resistance in the era of personalized medicine. Cancer Discov. 2012;2:214-26.

Old and new treatments for relapsed chronic lymphocytic leukemia

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A B S T R A C T

Fludarabine, cyclophosphamide and rituximab (FCR) is the standard of care for most patients with chronic lymphocytic leukemia (CLL) requiring treatment. This regimen achieves high overall response rates (ORR) and complete response (CR) in 50-70% of patients with durable remission durations. Treatment of relapsed CLL is less standardized; repeat therapy with FCR is possible and bendamustine combined with rituximab is also a popular regimen. Once chemo-immunotherapy is no longer a viable consideration, the only other approved drug that is readily available is ofatumumab, the humanized monoclonal antibody to CD20. However, this drug has a very limited label, being approved for patients previously refractory to fludarabine and alemtuzumab. Several new agents with completely different mechanisms of action are in clinical trials and appear promising. Lenalidomide is an approved agent for the treatment of multiple myeloma and myelodysplastic syndrome. It has good efficacy in patients with CLL; phase II trials suggest enhanced efficacy when lenalidomide is combined with an anti-CD20 antibody. The B-cell receptor inhibitors include ibrutinib, which targets Bruton's tyrosine kinase, and idelalisib, targeting P13K delta. Both are oral agents that are not myelosuppressive, have excellent efficacy and good tolerability; they are both currently in randomized trials. The excitement generated by these new agents also leads to the consideration of how effective non-toxic drugs might be easily incorporated into front-line regimens. Several publications have stressed the importance of minimal residual disease (MRD) in CLL and its excellent correlation with progression-free survival (PFS) and overall survival (OS). Such end points should be incorporated into clinical trials and discussed with the American and European authorities as viable surrogates to move forward with registration trials in previously untreated patients with CLL. Chimeric antigen receptors (CARs) redirect T cells in an effort to eradicate CLL cells. This approach has great potential but further development is needed to abrogate toxicities and ramp up for large-scale usage.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe current and emerging therapies for patients with CLL;
- discuss oral agents currently in registration trials for the treatment of CLL;
- discuss side effects and pattern of activity with the oral agents, and differentiate those patterns from early progressive disease signs;
- describe the use of chimeric antigen receptor (CAR) T cells in CLL, side effects, and early efficacy data.

Front-line therapy

In the past ten years, the combination of fludarabine, cyclophosphamide and rituximab (FCR) has become the standard front-line treatment for most patients with chronic lymphocytic leukemia (CLL). This regimen was developed at the MD Anderson Cancer Center where a large phase II trial was conducted and produced the highest complete response (CR) rates seen for any regimen used in CLL.[1] The German CLL Group subsequently performed a randomized trial, CLL8, comparing FCR to FC chemotherapy.[2] FCR produced an overall response (OR) rate of 95%; the CR rate seen with FCR was double that seen with FC (44% *vs.* 22%). A recent update showed that the median progression-free survival (PFS) with FCR is five years,[3] thus, remissions are quite durable. In addition, this was the first trial to show a survival advantage for a front-line regimen in the treatment of CLL.

In Europe, this regimen is the standard of care for most patients with CLL; the exception would be in elderly or infirm patients where chlorambucil remains the standard. Although FCR is the most commonly used regimen in the Unites States, the off-label use of drugs allows utilization of other regimens such as fludarabine and rituximab (FR)[4] and bendamustine and rituximab (BR).[5] The Intergroup is conducting a randomized trial comparing FCR to FR for front-line treatment of CLL.[6] However, given the importance of the alkylating agent in patients with 11q deletion, these patients receive FCR followed by lenalidomide maintenance. Although this trial reached accrual in 2012, data has not yet been presented. The current front-line German CLL trial is randomizing patients to FCR *versus*

BR.[7] Thus, 2 other commonly used chemoimmunotherapy regimens are being compared to the standard of care and have the potential for changing this standard.

Therapy for relapse

Whereas there is a relative standard of care in the front-line treatment of CLL, there is arguably no standard relapse regimen. This is partly due to the fact that the choice of regimen is dictated by several factors, including length of the first remission, the patient's ability to tolerate chemoimmunotherapy, and fluorescence *in situ* hybridization (FISH) results. Use of FCR as a salvage regimen was recently described by Badoux *et al*. in 234 previously treated patients with CLL.[8] Seventy-eight patients had received prior multi-agent chemotherapy, predominantly fludarabine-based, including prior FCR. The overall response rate was 74% with a median progression-free survival (PFS) of 21 months. Only 42% of patients were able to complete 6 cycles and a substantial number needed dose reduction. As expected, patients older than 70 years were less likely to complete 3 or more courses of therapy compared with younger patients. In patients who had previously received fludarabine-based therapy, the OR rate was 73% and median PFS was 19 months, so this is still a reasonable option for patients who received FCR upfront.

FCR has also been compared to FC in a randomized trial for relapsed patients with CLL.[9] In the REACH trial the response rate with FCR and FC were 70% and 58% respectively (P=0.003); CR rates were 24% and 13% (P=0.0007). The primary end point of PFS was longer in the FCR arm at 30.6 months *versus* 20.6 months with FC. However, the REACH trial excluded patients who had received more than one prior treatment and those previously treated with FC or rituximab.

BR is a commonly used salvage regimen, particularly in patients receiving fludarabine-based therapy initially. The frequent use of BR (as opposed to repeating FCR) is likely related to the fact that this is a significantly less myelosuppressive regimen, a factor that becomes more relevant in a relapsed patient population. The use of BR in the treatment of relapsed CLL was investigated in a retrospective analysis conducted in 24 Italian centers.[10] Eighty-seven patients received the BR combination and 22 patients received bendamustine alone (this was not a randomized trial). The overall response rate was 70%; CRs were significantly higher with BR (34%) compared to bendamustine (14%). The median PFS for the whole group was 16 months and the median duration of response was 13 months; patients in CR had a significantly longer duration of response than those in PR. Of note, this population was fairly heavily pre-treated with a median of 3 prior regimens; more than one-third of the cases had received fludarabine (38%) in combination with alkylating agents and/or rituximab (39%).

Fischer treated 78 patients with BR in a prospective clinical trial.[11] The OR rate was 59% and the CR rate was 9%. Patients who were fludarabine sensitive were more likely to respond (60.5%) than those who were fludarabine resistant (46%). The median event-free survival was 14.7 months. Eighty-one percent of the patients had previously received fludarabine alone and/or fludarabine containing combination therapies although only 7 patients (9%) had received ritixumab-containing therapies.

Once patients have failed chemoimmunotherapy, options are limited. Alemtuzumab, a monoclonal antibody targeting CD52, was previously approved for the treatment of CLL but is no longer commercially available; it is only available by compassionate investigational new drug (IND) applications.[12] Ofatumumab, a humanized monoclonal antibody targeting CD20, has been approved in both the United States and Europe.[13] This drug is given weekly for eight weeks and then monthly for four weeks. In a patient population that was both fludarabine and alemtuzumab refractory, this agent produced a 50% response rate. It was well tolerated with infusion reactions being the predominant side effect. Importantly, even though this was a heavily pre-treated population, the drug was not myelosuppressive. Although the response rate was impressive given such a refractory patient group, remissions were partial and the duration of response was six months. In other words, once the monthly treatments were stopped, most patients began to relapse. One of the limitations of using this drug is that both in the United States and in Europe the label is restricted to the patient population treated in the pivotal trial, i.e. those refractory to both alemtuzumab and fludarabine. Interestingly, given the lack of availability of alemtuzumab, one wonders how this will or could impact the use of the drug, since it would be almost impossible to fulfill the requirements of the label.

However, ofatumumab is currently being compared to Physicians' Choice in a randomized trial in patients with CLL and bulky fludarabine refractory disease being conducted in Europe.[14] The Committee for Medicinal Products for Human Use (CHMP) required that such a trial be conducted (a randomized trial) as a condition of approval for ofatumumab in the European Union (EU). Patients progressing on the control arm will have the option of receiving ofatumumab.

Ofatumumab is in a pivotal trial that could potentially lead to approval in a front-line setting (Table 1). In a randomized trial, previously untreated patients with CLL receive chlorambucil or chlorambucil plus ofatumumab.[15] Patients eligible for the trial are those who are considered inappropriate for fludarabine-based therapy, so this trial will likely be enriched for an older population where FCR would not be considered standard of care.

Ofatumumab is also being evaluated as maintenance therapy in patients with CLL in second or third remission.[16] The randomization is to observation as there is no standard maintenance strategy in CLL, and this trial, if successful, could provide another approved use for the agent in CLL. Of interest are a number of promising oral agents in clinical trials for the treatment of CLL. This review will focus on several that are oral and further along in development.

Oral agents in clinical trials

Lenalidomide, a second-generation imid, is an approved drug for the treatment of multiple myeloma and myelodysplastic syndrome with 5q- abnormality.[17,18] It is an oral agent that is given either at 25 mg daily for three out of four weeks (myeloma schedule) or 10 mg daily continuously (MDS schedule). The most common side effect of this drug is neutropenia and this is the most frequent rea-

son for dose reduction. Other side effects include gastrointestinal complaints, rash, and fatigue. Several trials have documented the efficacy of this drug in the treatment of CLL in the relapsed setting where doses ranged from 10-25 mg daily;[19,20] increased efficacy of lenalidomide appeared to be associated with a higher dose. Although response rates are greater with higher doses of lenalidomide, most patients are unable to tolerate more than 5-10 mg a day. The neutropenia seen with lenalidomide is easily abrogated by the use of colony stimulating factors and the use of such agents is an alternative to dose reduction. Some toxicities appear particular to patients with CLL; one of these is tumor lysis.[21] This is actually uncommon when beginning the treatment with doses at 5 or 10 mg daily but frequent with initiation of therapy at higher doses. Tumor lysis can also occur during dose escalation. Another interesting toxicity is tumor flare.[22] This syndrome is associated with lymph node swelling, which is sometimes painful, rash, and low-grade temperature. It tends to occur early in treatment or at a time when the dose is being increased. It is important to recognize this phenomenon and not diagnose this as tumor progression with subsequent cessation of the treatment. Some data suggest that the occurrence of the flare may be associated with a higher likelihood of CR. The exact mechanism of the flare is not known.

Lenalidomide is currently in a number of pivotal trials (Table 1). These include a randomized trial of lenalidomide *versus* placebo as maintenance therapy for patients with CLL in second remission.[23] One of the attractions of using this drug in the maintenance setting is that the issues of tumor lysis and tumor flare become almost irrelevant since patients are already 'debulked' by their prior therapy. Similarly, the German CLL Study Group is conducting a randomized trial of lenalidomide *versus* placebo in high-risk patients in first remission.[24] Lenalidomide is also being compared to chlorambucil in a trial for previously untreated older patients with CLL (>65 years) with the hypothesis that this would be better tolerated as compared to chemoimmunotherapy, and more effective than chlorambucil in that group of patients.[25]

Lenalidomide has also been combined with anti-CD20 B-cell monoclonal antibodies in relapsed patients with CLL. Ferrajoli *et al*. compared their results using rituximab and lenalidomide to the results from their prior trial using single agent lenalidomide in a similar relapsed patient population.[26] The ORR with the combination was 64%; this is double the response rate that they had seen with single agent lenalidomide. In addition, the schedule that was developed, which gave 2 doses of rituximab prior to the lenalidomide, reduced the severity of tumor flare. The CLL Consortium also presented data combining rituximab with lenalidomide using a different schedule wherein lenalidomide was initiated prior to rituximab; the incidence of flare was greater than seen when the rituximab preceded lenalidomide.[27] Lenalidomide has also been combined with ofatumumab in relapsed patients with CLL.[28] Response rates looked identical to those seen with lenalidomide and rituximab.

Another exciting class of agents that are being developed in CLL, as well as in lymphoma, are the B-cell receptor inhibitors. Ligation of the B-cell receptor provides a strong proliferation and survival signal to both normal and malignant B cells. Thus interfering with such signaling could have a positive effect on B-cell diseases. Ibrutinib is an oral agent which irreversibly binds to Bruton's tyrosine kinase (BTK), a kinase in the B-cell receptor signaling pathway.[29] A phase I trial with this agent showed that the receptor site on BTK was completely occupied by Ibrutinib at doses of over 2.5 mg/kg/day.[30] No dose-limiting toxicity was seen and the most common side effect was mild diarrhea which was often self-limited. The phase Ib trial was conducted in patients with relapsed and refractory CLL or in a treatment naïve cohort of patients over the age of 65 years. Data from this trial were recently presented at ASH 2012.[31] In 85 relapsed/refractory patients, the ORR was 71% with 2% CR. These values were 68% and 10% in the treatment naïve group. The pattern of activity of the drug is interesting; initially there is rapid and significant shrinkage of lymph nodes while simultaneously the absolute lymphocyte count increases. To some extent this represents a compartment shift, although not

Table 1. Agents in pivotal randomized trials in CLL.

Agents in pivotal randomized trials in CLL			
	Population	**Trial**	**NCT#**
Ofatumumab – anti-CD20 monoclonal antibody	Fludarabine refractory	Ofatumumab vs. Physician's Choice	01313689
	Untreated; inappropriate for fludarabine-based therapy	Chlorambucil alone vs. chlorambucil + ofatumumab	00748189
	2nd or 3rd remission	*vs.* observation as maintenance	01039376
Lenalidomide – IMID	2nd remission	*vs.* placebo as maintenance therapy	00774345
	1st remission in high risk	*vs.* placebo as maintenance	01556776
	Untreated >65 years old	*vs.* chlorambucil	00910910
Ibrutinib – BTK inhibitor	Relapsed/refractory	*vs.* ofatumumab	01578707
	Relapsed/refractory	BR +/- ibrutinib	01611090
	Untreated	*vs.* chlorambucil	01722487
Idelalisib – P13K kinase delta isoform	Relapsed/refractory; not fit for chemotherapy	Rituximab +/- idelalisib	01539512
	Relapsed/refractory	Ofatumumab +/- idelalisib	01659021
	Relapsed/refractory	BR +/- idelalisib	01569295

fully, since the increase in the lymphocyte count is not proportional to the amount of shrinkage in the lymph nodes. Over time, this lymphocytosis gradually resolves. Early on in the assessment period, patients may have 70-80% reduction in lymph nodes but may not qualify as a PR using the IWCLL criteria because their baseline lymphocytosis has not decreased by more than 50%. This group of patients are sometimes referred to as nodal responders or those with PR with lymphocytosis. If one adds this group to the patients achieving PR, then the ORR for relapsed/refractory patients becomes 89% and for the treatment naïve it is 81%. Importantly, known poor prognostic factors for either response or remission duration after chemoimmunotherapy are not associated with a reduced response rate to ibrutinib. This is particularly striking in a very poor prognostic group, namely those with a 17p deletion. Twenty-eight of the 85 patients with relapsed/refractory disease had a 17p deletion; the overall response rate in this group was 68%. At 26 months, the progression-free survival of the treatment naïve group was 96%; it was 75% in the relapsed/refractory patients. These are very durable remission durations for a heavily pre-treated group of patients (median number of prior regimens 4.) Although the PFS curve for patients with relapsed 17p deletion disease is somewhat lower, 57% were progression-free at 26 months, which is still better data than those seen for any published survival curves for patients with relapsed 17p deletion disease.

Ibrutinib has been combined with BR chemotherapy in a phase II trial in patients with relapsed CLL.[32] The results indicated that using chemotherapy with ibrutinib resulted in much more rapid reduction in lymphocytosis than was seen with single agent ibrutinib. No additional toxicities were noted over those expected with chemotherapy; the response rate of 83% appeared higher than the published response rate for a previous trial with BR alone. Ibrutinib has also been combined with rituximab and, as expected, addition of the antibody also abrogates the lymphocytosis such that remissions occur more quickly; a response assessment at 3-6 months in 40 relapsed patients with CLL showed an ORR of 91% lymphocytosis, with 3% CR, 80% PR and 8% PR.[33]

There are currently four clinical trials with this agent for potential registration in the United States and Europe (Table 1).[34-37] These include two trials in relapsed/refractory patients; one trial randomizes relapsed patients who are not good candidates for chemotherapy to ofatumumab or ibrutinib. The second trial is a randomized trial of BR +/- ibrutinib. The front-line trial is a randomized trial of ibrutinib *versus* chlorambucil and, finally, there is a single arm trial of ibrutinib in patients with relapsed 17p deletion CLL. In this group, there is considered to be no standard of care to which these patients could be randomized because all therapies are inadequate and the expected outcome is poor.

Another oral B-cell receptor inhibitor currently in FDA pivotal trials is idelalisib (GS-1101, CAL-101) (Table 1). This agent targets PI3K kinase, another enzyme in the B-cell receptor signaling pathway. This inhibitor specifically binds to the delta isoform which is a prevalent isoform in hematologic malignancies.[38,39] Other drugs including pan PI3 kinase inhibitors are in clinical trials for the treatment of solid tumors; some data suggest that these pan inhibitors may cause hyperglycemia, probably related to the fact that the alpha isoform is involved in insulin signaling. Idelalisib is given twice daily and in a phase I trial marked activity was seen in patients with lymphomas as well as CLL.[40,41] There was no maximum tolerated dose (MTD), but one of the more common toxicities was elevation of transaminases. When this occurred, the drug was held and the transaminitis promptly resolved; the drug then was resumed at the same or lower dose. In the phase Ib trial in CLL, 55 highly refractory patients were treated (medium number of prior regimens, 5) with idelalisib.[42] Similarly to ibrutinib, the initial response is manifested by increasing lymphocytosis and rapid and dramatic shrinkage in lymphadenopathy. Eighty-four patients had a nodal response (at least 50% reduction of lymphoid mass) and 24% had a PR using IWCLL criteria. Thus, the majority of responders had some degree of lymphocytosis. Medium progression-free survival appeared to be about 18 months. Idelalisib has been combined with ofatumumab; the latter was given after an initial 300 mg dose at 1,000 mg weekly for eight weeks and then monthly for 4 more doses.[43] Patients continued on idelalisib after completion of ofatumumab. As expected, with the addition of the antibody the lymphocytosis was rapidly resolved, and the overall response rate was 94% (n=15). Thus, in contrast to the use of the single agent, the use of the combination results in more responses by IWCLL criteria because of the eradication of lymphocytosis. Idelalisib has also been combined with rituximab, bendamustine, and bendamustine plus rituximab.[44] These were small pilot trials evaluating the toxicity of the combinations. Treatment with idelalisib continued after completion of chemotherapy/antibody. No new toxicities were noted with any of the combinations and, as expected, the most common side effects with the chemotherapy regimens were myelosuppression and infection. The ORR to idelalisib plus rituximab was 79% (n=19), with bendamustine 78% (n=18), and with bendamustine and rituximab 87% (n=15). Median PFS was not reached and 1-year PFS was 68.7%. Grade 3-4 transaminase elevation was seen in 10% of patients.

Idelalisib is also in pivotal registration trials in Europe and the United States. Both trials are in the relapsed/refractory population of patients with CLL. For patients who are not good candidates for chemotherapy, there are two trials. One trial is rituximab +/- idelalisib.[45] The other trial is ofatumumab *versus* ofatumumab and idelalisib.[46] For patients who are good candidates for chemotherapy, the trial design is BR +/- idelalisib.[47]

Clinical trial end points

As exciting as it is to have these relatively non-toxic and highly effective oral drugs in clinical trials, one of the issues of bringing them forward into the front-line setting is the fact that the medium PFS with front-line FCR is 5-6 years. Thus, any attempt to conduct a randomized trial to compare FCR to either another regimen, or FCR in combination with an investigational agent, would necessitate very large trials with very prolonged time to completion and, consequently, would be very expensive. Alternative options for pharmaceutical companies seeking to have earlier employment of their agent would be to pick a population where FCR would not be considered the standard of therapy, such as elderly/infirm patients or patients with

17p deletion, to give just two examples. However, 17p deletion is an uncommon abnormality in patients who were previously untreated, representing only around 5-10% of front-line patients. Thus, the applicability of the results to other front-line patients would be very restricted. In addition, although FCR produces high response rates and durable remission durations, PFS curves continue to decline. Although it may take more extended follow up to ascertain whether there is a cure fraction, it is clear that such a cure fraction will encompass a minority of patients and most likely those with mutated IGVH genes. In addition, FCR has significant short-term toxicities as well as late toxicities. There is a lot of interest, even for patients who might successfully be treated with FCR, in developing novel regimens that avoid the use of chemotherapy. This may be especially important in patients with CLL who have a higher incidence of second malignancies just by virtue of having the disease, and DNA damaging agents are inherently unattractive in this setting. Thus, in order to be able to conduct front-line trials in patients with CLL that are not limited to a small patient population, the CLL community will need a surrogate end point that clearly is associated with PFS.

Emerging data indicates that minimal residual disease (MRD) is likely to be that end point. Several trials have been published with various treatments including alemtuzumab, FC, and FCR, clearly indicating a strong correlation between PFS, OS and MRD. MRD can be measured by both PCR and/or flow cytometry but most published data have used flow cytometry; there are also commercial assays for immunophenotypic detection of MRD. Recently, the MRD data from the CLL8 trial, the randomized trial of FCR to FC, were published.[48] MRD levels were prospectively quantified in over 1,700 blood and bone marrow samples from 493 patients, randomized to either FC or FCR. Patients were categorized into MRD low (less than 10^{-4}), intermediate ($\geq 10^{-4}$ to $<10^{-2}$) and high level ($\geq 10^{-2}$). Median PFS was estimated to be 68.7, 40.5 and 15.4 months for low, intermediate and high MRD levels, respectively, when assessed two months after completion of therapy. Median OS was 48.4 months in patients with high MRD and not reached for the lower MRD levels. Importantly, MRD remained predictive for OS and PFS in multivariate analyses that included the most important pre-treatment risk factors. Another crucial point is that PFS and OS did not differ between the treatment arms within each MRD category. In other words, although patients randomized to FC were less likely to achieve the best MRD status, patients who did achieve such status behaved exactly as those who had received FCR (in the same MRD category.) This is important because it clearly suggests that this is not a regimen-related marker but rather truly an assessment of residual disease. Other trials have also found MRD to be a significant predictor of outcome after treatment with alemtuzumab, the humanized monoclonal antibody to CD52.[49]

Immunological therapy

The fact that allogeneic hematopoietic stem cell transplant (HCST) can cure some patients with CLL indicates the powerful therapeutic effect of T cells.[50,51] The potency of this therapy has also been shown in the form of donor lymphocyte infusions (DLI) which effectively treat patients with relapsed CLL post HCST.[52] However, graft-*versus*-host disease (GVH) following HCST and DLI illustrates one of the most significant limitations of non-directed cellular therapy. Single-chain chimeric antigen receptors (CARs) can re-direct T-cell specificity to a tumor-derived antigen expressed on the cell surface, which is independent of HLA.[53,54] The CAR fuses a mouse monoclonal antibody that binds to a tumor antigen, triggering activation and effector functions. The specificity of the CAR is achieved by the antigen-binding motif, usually a monoclonal antibody that links V_H and V_L sequences resulting in a single chain fragment variable (scFv) region. The most common antigen targeted on CLL thus far has been CD19. CD19 is B-cell surface antigen that is expressed on CLL cells as well as the cells from most B-cell malignant disorders. It is not expressed on cells other than those of B lineage, is not shed into the circulation, and pre-clinical studies have shown that CD19 positive tumor cells can be lysed by T cells expressing CD19-specific CAR. The exodomain of the CAR is completed by a hinge and is expressed on the T-cell surface via a trans-membrane domain.

After binding to the targeted antigen the CAR activates T cells via an endodomain that typically includes cytoplasmic domains from CD3 or high affinity receptor FcεRI. There are now 1st, 2nd and 3rd generation CARs designed with 1, 2 or 3 signaling motifs within the endodomain. Most trials in CLL thus far have administered T cells that are 2nd generation CAR designs. Savoldo *et al.* administered a mixture of CARs containing either T cells with both CD3 zeta endodomain and CD28, or only the CD3 zeta endodomain, to 6 patients with relapsed lymphoma. They showed that CARs containing the CD28 endodomain had enhanced expansion and persistence, confirming the superiority of CARs with dual signal domains.[55]

There have been several clinical trials targeting CD19 on CLL cells by CAR positive T cells. Kochenderfer *et al.* used an anti-CD19 CAR joined to part of the CD28 molecule and the signaling domains of the CD3 zeta molecule to treat a patient with B-cell lymphoma after a lymphocyte-depleting regimen of cyclophosphamide and fludarabine.[56] They also administered IL-2 after the T-cell infusion. Although an impressive PR was seen, this remission was short lived at 32 weeks.

Brentjens *et al.* reported on 10 patients with chemo-refractory CLL or ALL treated with an anti-CD19 CAR that also included CD28 in the signaling domain.[57] The first cohort of 3 patients who were treated without cyclophosphamide conditioning had no evidence of disease response. Subsequent patients received cyclophosphamide chemotherapy prior to the CAR infusion. In these patients, T cells were more readily detected over time in the blood and bone marrow consistent with previously published reports indicating enhanced persistence of adoptively transferred tumor-specific T cells after chemotherapy. Porter *et al.* administered CARs targeting CD19 that included the co-stimulatory receptor 4-1BB as well as CD3 zeta.[58] They reported on an initial patient with refractory CLL who had expansion of T cells *in vivo* to more than 1,000 times as high as the initial engraftment level. This was associated with the delayed tumor lysis as well as CR. A specific toxicity associated with this eradication

of B cells was loss of normal B cells and hypogammaglobulin that was treated with IVIG. The remission was ongoing ten months after treatment. Genetically modified cells were present in the bone marrow at high levels for at least six months after the infusion.

One difference in this CAR compared to the others previously described is that this is a 2nd generation CAR based on the incorporation of CD137 (4-1BB). The authors hypothesize that it is the presence of this 4-1BB signaling domain that is responsible for the prolonged persistence of the CARs. Additionally, they also hypothesize that CAR mediated elimination of normal B cells facilitated the development of tolerance to the CAR, since the CAR cells that expressed a single chain Fv antibody fragment and contained murine sequences were not rejected. An update of this trial was recently presented at ASH in 2012.[59] Ten patients received CAR T19 cells including 9 adults with refractory CLL and one child with relapsed refractory ALL. All the patients with CLL received chemotherapy 4-6 days before the infusion. Median follow up was 5.6 months. There were no deaths and no infusional toxicities greater than Grade 2. Four of the 9 patients achieved CR (3 CLL, 1 ALL). The patients who achieved remission appeared to have a higher level of expansion of the CARs in the blood than those achieving a PR or not responding. No patient with CR has yet relapsed. All patients developed cytokine release syndrome manifested by fever with variable amounts of nausea, anorexia, hypotension, and hypoxia. This cytokine release syndrome was temporally associated with significant elevations in serum IL-6. Four patients were treated with the IL-6 receptor antagonist tocilizumab on Days 3-10 with prompt resolution of symptoms. Persistence of the anti-CD19 CAR was seen for up to two years.

Conclusion

Fludarabine, cyclophosphamide and rituximab remains the standard of care for most patients with CLL, and does produce high response rates and durable remission duration. But it is unlikely to result in cure in the majority of patients. Several new, non-chemotherapy agents are in clinical trials. Only 3 of them have been mentioned in this review due to space limitations and because these are the ones in pivotal registration trials that are likely to make them available within the next 2-3 years. In order to move such agents, as well as other exciting new agents in clinical trials, forward into the front-line treatment of CLL, we will need surrogate end points to avoid conducting very large, very long randomized trials. Enough data are now available to suggest that MRD should be exactly that end point and an important component of future trial design. Chimeric antigen receptors (CARs) harness the power of T cells to eradicate CLL cells by targeting a B-cell related antigen. Excitement is high regarding this approach, although more needs to be done to standardize this technique and make it more amenable to large-scale therapy.

References

1. Keating MJ, O'Brien S, Albitar M, Lerner S, Plunkett W, Giles F, et al. Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. J Clin Oncol. 2005;23(18):4079-88.
2. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. Lancet. 2010;376(9747):1164-74.
3. Fischer K, Bahlo J, Fink AM, Busch R, Böttcher S, Mayer J, et al. Extended Follow up of the CLL8 Protocol, a Randomized Phase-III Trial of the German CLL Study Group (GCLLSG) Comparing Fludarabine and Cyclophosphamide (FC) to FC Plus Rituximab (FCR) for Previously Untreated Patients with Chronic Lymphocytic Leukemia (CLL): Results On Survival, Progression-Free Survival, Delayed Neutropenias and Secondary Malignancies Confirm Superiority of the FCR Regimen. ASH Annual Meeting 2012. Abstract 435.
4. Woyach JA, Ruppert AS, Heerema NA, Peterson BL, Gribben JG, Morrison VA, et al. Chemoimmunotherapy with fludarabine and rituximab produces extended overall survival and progression-free survival in chronic lymphocytic leukemia: long-term follow-up of CALGB study 9712. J Clin Oncol. 2011;29(10):1349-55.
5. Fischer K, Cramer P, Busch R, Bottcher S, Bahlo J, Schubert J, et al. Bendamustine in combination with rituximab for previously untreated patients with chronic lymphocytic leukemia: a multicenter phase II trial of the German Chronic Lymphocytic Leukemia Study Group. J Clin Oncol. 2012;30(26):3209-16.
6. US National Institutes of Health. Fludarabine and Rituximab With or Without Lenalidomide or Cyclophosphamide in Treating Patients With Symptomatic Chronic Lymphocytic Leukemia. [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT00602459
7. US National Institutes of Health. FCR or BR in Patients With Previously Untreated B-Cell Chronic Lymphocytic Leukemia (CLL10). [Internet] Available from: http://www. clinicaltrials.gov/ct2/show/NCT00769522
8. Badoux XC, Keating MJ, Wang X, O'Brien SM, Ferrajoli A, Faderl S, et al. Cyclophosphamide, fludarabine, alemtuzumab, and rituximab as salvage therapy for heavily pretreated patients with chronic lymphocytic leukemia. Blood. 2011;118(8):2085-93.
9. Robak T, Dmoszynska A, Solal-Celigny P, Warzocha K, Loscertales J, Catalano J, et al. Rituximab plus fludarabine and cyclophosphamide prolongs progression-free survival compared with fludarabine and cyclophosphamide alone in previously treated chronic lymphocytic leukemia. J Clin Oncol. 2010;28(10):1756-65.
10. Iannitto E, Morabito F, Mancuso S, Gentile M, Montanini A, Augello A, et al. Bendamustine with or without rituximab in the treatment of relapsed chronic lymphocytic leukaemia: an Italian retrospective study. Br J Haematol. 2011;153(3):351-7.
11. Fischer K, Cramer P, Busch R, Stilgenbauer S, Bahlo J, Schweighofer CD, et al. Bendamustine combined with rituximab in patients with relapsed and/or refractory chronic lymphocytic leukemia: a multicenter phase II trial of the German Chronic Lymphocytic Leukemia Study Group. J Clin Oncol. 2011;29(26):3559-66.
12. Hillmen P, Skotnicki AB, Robak T, Jaksic B, Dmoszynska A, Wu J, et al. Alemtuzumab compared with chlorambucil as first-line therapy for chronic lymphocytic leukemia. J Clin Oncol. 2007;25(35):5616-23.
13. Wierda WG, Kipps TJ, Mayer J, Stilgenbauer S, Williams CD, Hellmann A, et al. Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. J Clin Oncol. 2010;28(10):1749-55.
14. US National Institutes of Health. Ofatumumab vs Physician's Choice in Subjects With Bulky Fludarabine-Refractory Chronic Lymphocytic Leukemia. [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01313689
15. US National Institutes of Health. Ofatumumab + Chlorambucil vs Chlorambucil Monotherapy in Previously Untreated Patients With Chronic Lymphocytic Leukemia (COMPLEMENT 1). [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT00748189
16. US National Institutes of Health. Ofatumumab Maintenance Treatment vs No Further Treatment in Relapsed CLL Responding to Induction Therapy (PROLONG). [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01039376
17. List A, Kurtin S, Roe DJ, Buresh A, Mahadevan D, Fuchs D, et al. Efficacy of lenalidomide in myelodysplastic syndromes.

N Engl J Med. 2005;352(6):549-57.
18. Richardson P. Management of the relapsed/refractory myeloma patient: strategies incorporating lenalidomide. Semin Hematol. 2005;42(Suppl 4):S9-15.
19. Chanan-Khan A, Miller KC, Musial L, Lawrence D, Padmanabhan S, Takeshita K, et al. Clinical efficacy of lenalidomide in patients with relapsed or refractory chronic lymphocytic leukemia: results of a phase II study. J Clin Oncol. 2006;24(34):5343-9.
20. Ferrajoli A, Lee BN, Schlette EJ, O'Brien SM, Gao H, Wen S, et al. Lenalidomide induces complete and partial remissions in patients with relapsed and refractory chronic lymphocytic leukemia. Blood. 2008;111(11):5291-7.
21. Chen CI, Bergsagel PL, Paul H, Xu W, Lau A, Dave N, et al. Single-agent lenalidomide in the treatment of previously untreated chronic lymphocytic leukemia. J Clin Oncol. 2011;29(9):1175-81.
22. Chanan-Khan A, Miller KC, Lawrence D, Padmanabhan S, Miller A, Hernandez-Illatazurri F, et al. Tumor flare reaction associated with lenalidomide treatment in patients with chronic lymphocytic leukemia predicts clinical response. Cancer. 2011;117(10):2127-35.
23. US National Institutes of Health. A Study to Evaluate the Efficacy and Safety of Lenalidomide as Maintenance Therapy for Patients With B-Cell Chronic Lymphocytic Leukemia (CLL) Following Second Line Therapy. [Internet] Available from: http://www.clinicaltrials.gov/ct2/results?term=nct00774345
24. US National Institutes of Health. A Phase 3 Study of the Efficacy and Safety of Lenalidomide Maintenance for High-risk Patients With CLL Following First-line Therapy (CLLM1) [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01556776
25. US National Institutes of Health. Study Of The Effectiveness & Safety Of Lenalidomide Versus Chlorambucil As First Line Therapy For Elderly Patients With B-Cell CLL (The ORIGIN Trial) [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT00910910
26. Badoux XC, Keating MJ, Wen S, Wierda WG, O'Brien SM, Faderl S, et al. Phase II Study of Lenalidomide and Rituximab As Salvage Therapy for Patients With Relapsed or Refractory Chronic Lymphocytic Leukemia. J Clin Oncol. 2012. [Epub ahead of print]
27. James DF, Brown JR, Werner W, Wierda WG, Barrientos JC, Castro J, et al. Lenalidomide and Rituximab for the Initial Treatment of Patients with Chronic Lymphocytic Leukemia (CLL) A Multicenter Study of the CLL Research Consortium. ASH Annual Meeting 2011. Abstract 291.
28. Ferrajoli A, Falchi L, O'Brien SM, Wierda W, Faderl S, Smith SC, et al. Combination of Ofatumumab and Lenalidomide in Patients with Relapsed Chronic Lymphocytic Leukemia (CLL): Results of a Phase II Trial. ASH Annual Meeting 2012. Abstract 720.
29. Honigberg LA, Smith AM, Sirisawad M, Verner E, Loury D, Chang B, et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. Proc Natl Acad Sci USA 2010;107(29):13075-80.
30. Advani RH, Buggy JJ, Sharman JP, Smith SM, Boyd TE, Grant B, et al. Bruton Tyrosine Kinase Inhibitor Ibrutinib (PCI-32765) Has Significant Activity in Patients With Relapsed/Refractory B-Cell Malignancies. J Clin Oncol. 2013;31(1):88-94.
31. Byrd JC, Furman RR, Coutre S, Flinn IW, Burger JA, Blum KA, et al. The Bruton's Tyrosine Kinase (BTK) Inhibitor Ibrutinib (PCI-32765) Promotes High Response Rate, Durable Remissions, and Is Tolerable in Treatment Naïve (TN) and Relapsed or Refractory (RR) Chronic Lymphocytic Leukemia (CLL) or Small Lymphocytic Lymphoma (SLL) Patients Including Patients with High-Risk (HR) Disease: New and Updated Results of 116 Patients in a Phase Ib/II Study. ASH Annual Meeting 2012, Abstract 189.
32. O'Brien SM, Barrientos JC, Flinn IW, Barr PM, Burger JA, Navarro T, et al. Combination of the Bruton's tyrosine kinase (BTK) inhibitor PCI-32765 with bendamustine (B)/rituximab (R) (BR) in patients (pts) with relapsed/refractory (R/R) chronic lymphocytic leukemia (CLL): Interim results of a phase Ib/II study. ASH Annual Meeting 2012, Abstract 6515.
33. Burger JA, Keating MJ, Wierda WG, Hoellenriegel J, Ferrajoli A, Faderl S, et al. The Btk Inhibitor Ibrutinib (PCI-32765) in Combination with Rituximab Is Well Tolerated and Displays Profound Activity in High-Risk Chronic Lymphocytic Leukemia (CLL) Patients. ASH Annual Meeting 2012. Abstract 187.
34. US National Institutes of Health. A Phase 3 Study of Ibrutinib (PCI-32765) Versus Ofatumumab in Patients With Relapsed or Refractory Chronic Lymphocytic Leukemia (RESONATE™). [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01578707
35. US National Institutes of Health. A Study of Ibrutinib in Combination With Bendamustine and Rituximab in Patients With Relapsed or Refractory Chronic Lymphocytic Leukemia or Small Lymphocytic Lymphoma. [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01611090
36. US National Institutes of Health. A Multicenter, Open-label, Phase 3 Study of the Bruton's Tyrosine Kinase Inhibitor PCI-32765 Versus Chlorambucil in Patients 65 Years or Older With Treatment-naive Chronic Lymphocytic Leukemia or Small Lymphocytic Lymphoma (RESONATE™-2). [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01722487
37. US National Institutes of Health. A Multicenter Phase 2 Study of PCI-32765 (Ibrutinib) in Patients With Relapsed or Refractory Chronic Lymphocytic Leukemia (CLL) or Small Lymphocytic Lymphoma (SLL) With 17p Deletion. [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01744691
38. Lannutti BJ, Meadows SA, Herman SE, Kashishian A, Steiner B, Johnson AJ, et al. CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. Blood. 2011;117(2):591-4.
39. Vanhaesebroeck B, Welham MJ, Kotani K, Stein R, Warne PH, Zvelebil MJ, et al. P110delta, a novel phosphoinositide 3-kinase in leukocytes. Proc Natl Acad Sci USA 1997;94(9):4330-5.
40. Furman RR, Byrd JC, Brown JR, Coutre SE, Benson JDM, Wagner-Johnston ND, et al. CAL-101, An Isoform-Selective Inhibitor of Phosphatidylinositol 3-Kinase P110, Demonstrates Clinical Activity and Pharmacodynamic Effects In Patients with Relapsed or Refractory Chronic Lymphocytic Leukemia. ASH Annual Meeting 2010. Abstract 55.
41. Kahl B, Byrd JC, Flinn IW, Wagner-Johnston N, Spurgeon S, Benson JDM, et al: Clinical Safety and Activity In a Phase 1 Study of CAL-101, An Isoform-Selective Inhibitor of Phosphatidylinositol 3-Kinase P110, In Patients with Relapsed or Refractory Non-Hodgkin Lymphoma. ASH Annual Meeting 2010. Abstract 1777.
42. Coutre SE, Byrd JC, Furman RR, Brown JR, Benson DM, Wagner-Johnston ND, et al: Phase I study of CAL-101, an isoform-selective inhibitor of phosphatidylinositol 3-kinase P110d, in patients with previously treated chronic lymphocytic leukemia. ASCO Annual Meeting 2011. Abstract 6631.
43. Furman RR, Barrientos JC, Sharman JP, De Vos S, Leonard J, Coutre SE, et al. A phase I/II study of the selective phosphatidylinositol 3-kinase-delta (PI3K{delta}) inhibitor, GS-1101 (CAL-101), with ofatumumab in patients with previously treated chronic lymphocytic leukemia (CLL). ASCO Annual Meeting 2012. Abstract 6518.
44. Coutre SE, Leonard JP, Furman RR, Barrientos JC, de Vos S, Flinn IW, et al: Combinations of the Selective Phosphatidylinositol 3-Kinase-Delta (PI3Kdelta) Inhibitor GS–1101 (CAL-101) with Rituximab and/or Bendamustine Are Tolerable and Highly Active in Patients with Relapsed or Refractory Chronic Lymphocytic Leukemia (CLL): Results From a Phase I Study. ASH Annual Meeting 2012. Abstract 191.
45. US National Institutes of Health. A Randomized, Double-Blind and Placebo-Controlled Study of Idelalisib in Combination With Rituximab for Previously Treated Chronic Lymphocytic Leukemia (CLL). [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01539512
46. US National Institutes of Health. A Randomized, Controlled Study Evaluating the Efficacy and Safety of GS-1101 (CAL-101) in Combination With Ofatumumab for Previously Treated Chronic Lymphocytic Leukemia. [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01659021
47. US National Institutes of Health. A Randomized, Double-Blind and Placebo-Controlled Study of Idelalisib in Combination With Bendamustine and Rituximab for Previously Treated Chronic Lymphocytic Leukemia (CLL). [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT01569295
48. Bottcher S, Ritgen M, Fischer K, Stilgenbauer S, Busch RM, Fingerle-Rowson G, Fink AM, Buhler A, Zenz T, Wenger MK et al. Minimal residual disease quantification is an independent predictor of progression-free and overall survival in chronic lymphocytic leukemia: a multivariate analysis from the ran-

domizedGCLLSG CLL8 trial. J Clin Oncol. 2012;30(9):980-8.
49. Moreton P, Kennedy B, Lucas G, Leach M, Rassam SM, Haynes A, et al. Eradication of minimal residual disease in B-cell chronic lymphocytic leukemia after alemtuzumab therapy is associated with prolonged survival. J Clin Oncol. 2005;23(13):2971-9.
50. Toze CL, Dalal CB, Nevill TJ, Gillan TL, Abou Mourad YR, Barnett MJ, et al. Allogeneic haematopietic stem cell transplantation for chronic lymphocytic leukaemia: outcome in a 20-year cohort. Br J Haematol. 2012;158(2):174-85.
51. Brown JR, Kim HT, Armand P, Cutler C, Fisher DC, Ho V, et al. Long –term follow-up of reduced-intensity allogeneic stem cell transplantation for chronic lymphocytic leukemia: prognostic model to predict outcome. Leukemia. 2013;27(2):362-9.
52. Richardson SE, Khan I, Rawstron A, Sudak J, Edwards N, Verfuerth S, et al. Risk-stratified adoptive cellular therapy following allogeneic hematopoietic stem cell transplantation for advanced chronic lymphocytic leukaemia. Br J Haematol. 2013;160(5):640-8
53. Jena B, Dotti G, Cooper LJ. Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. Blood. 2010;116(7):1035-44.
54. Hosing C, Kebriaei P, Wierda W, Jena B, Cooper LJ, Shpall E. CARs in Chronic Lymphocytic Leukemia – Ready to Drive. Curr Hematol Malig Rep. 2013;8(1):60-70.
55. Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G, et al. CD28 costimulation improves expansion and persistence of chimeric antigen receptor – modified T cells in lymphoma patients. J Clin Invest. 2011;121(5):1822-6.
56. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, Feldman SA, et al. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. Blood. 2010;116(20):4099-102.
57. Brentjens RJ, Rivière I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood. 2011;118(18):4817-28.
58. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric Antigen Receptor-Modified T Cells in Chronic Lymphoid Leukemia. N Engl J Med. 2011;365(8):725-33.
59. Porter DL, Grupp SA, Kalos M, Loren AW, Lledo L, Gilmore J, et al. Chimeric Antigen Receptor T Cells Directed Against CD19 Induce Durable Responses and Transient Cytokine Release Syndrome in Relapsed, Refractory CLL and ALL. ASH 2012. Abstract 717.

Recent advances in understanding chronic myeloid leukemia biology

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ABSTRACT

Progress has been made towards understanding the biology of chronic myeloid leukemia (CML). Areas of active research include the role of microRNAs in CML pathogenesis and resistance to tyrosine kinase inhibitors (TKIs), the mechanisms of genetic instability in CML cells and the role of autophagy. Next generation sequencing has revealed that multiple different mutations can co-exist with BCR-ABL1 at the time of blastic transformation, but not in the chronic phase (CP), painting a complex molecular picture for advanced CML, while confirming the notion that BCR-ABL1 alone is sufficient to induce CML-CP. Arguably the greatest advances were made in revealing the pathways and conditions that promote the growth and survival of CML stem cells (SCs) upon exposure to TKIs. Evidence is accumulating that the bone marrow microenvironment plays a central role in mediating CML-SC resistance to TKIs and that the combination of extrinsic and intrinsic mechanisms accounts for the persistence of fully leukemogenic SCs despite long-term TKI therapy.

Learning goals

At the conclusion of this activity, participants should be able to:
- acquire an understanding of the basic properties of CML-SCs;
- understand the major pathways involved in CML-SC survival;
- understand the mechanistic basis for residual leukemia in CML and the role of the microenvironment in facilitating CML-SC survival;
- learn about approaches to target CML-SC.

Introduction

The major clinical challenges in CML are related to the two extremes of the response spectrum to TKIs.[1] On one end there is the majority of patients with excellent responses, in whom the CML clone (measured by quantitative PCR for BCR-ABL1) has been suppressed to low, sometimes undetectable levels, a state referred to as a complete molecular response (CMR). Numerous studies have shown that these patients have an excellent long-term outcome. In fact, a multicenter study demonstrated that patients with chronic phase CML (CML-CP) who achieve a complete cytogenetic response (CCyR) and maintain this response for two years have a survival that is identical to that of an age- and sex-matched control population.[2] The challenge is the need to continue therapy indefinitely, sometimes despite significant side effects. Moreover, the growing prevalence of CML poses a significant health-economic problem.[3] At the other extreme are patients with TKI resistant blastic phase CML (CML-BP); most of these patients are incurable even with allogeneic stem cell transplantation. At the molecular level, there is increasing evidence that BCR-ABL1 independence is involved at both ends of the response spectrum. This suggests that the extremes of the clinical disease may be beyond the reach of single-agent BCR-ABL1 TKIs. Progress has been made in several important areas of CML biology, such as the role of autophagy[4,5] and genetic instability[6-8] in disease pathogenesis and as modulators of response to TKIs. Several deep sequencing studies have implicated various mutations reported in myelodysplastic syndromes or acute myeloid leukemia in the progression from CML-CP to CML-BP,[9] although it remains to be seen how precisely they drive disease progression. However, the most important advances from an applied science perspective were made in CML-SC biology as it relates to TKI resistance and the persistence of MRD despite long-term TKI treatment. In this update on the biology of CML we will focus on pathways in CML-SCs that may be involved in resistance and MRD and provide potential targets for therapy.

Persistent CML stem cells

Several studies of patients with CMR have demonstrated BCR-ABL1-positive cells within functionally defined hematopoietic cell compartments, including colony forming units granulocyte macrophage (CFU-GM), long-term culture initiating cells (LTC-IC) and Lineage^{-}CD34^{+}38^{-} cells.[10,11] The SC potential of the latter was elegantly demonstrated by their multilineage engraftment capacity in

immunodeficient mice, which led to the estimate that patients with MMR or CMR on imatinib harbor 0.09-1.61% *BCR-ABL1*+ cells in the CD34+/CD38- fraction, consistent with the very low percentage of BCR-ABL1+ cells detected by fluorescence *in situ* hybridization in CD34+38- cells.[11,12]

Studies of residual human CML are hampered by the lack of markers that distinguish between normal and leukemic cells within the CD34+38- fraction. This might change with recently identified candidates such as IL-1 receptor accessory protein (IL1RAP) and dipeptidylpeptidase IV (CD26).[13,14] At this point, however, it is custom to use Lineage-CD34+38- cells from newly diagnosed patients that survive *ex vivo* TKI exposure, to model residual leukemia cells that persist *in vivo*.[15] Since *in vivo* exposure to TKIs may select cells with characteristics not reflected in the bulk population of primitive Lineage-CD34+38- cells at diagnosis, these cells may not necessarily reflect residual disease in patients on therapy. While it is clear that primary CML cells as a model of residual disease have limitations, the same must be said for murine models. In particular, the retroviral transduction/transplantation system frequently employed produces a disease that behaves more aggressively than chronic phase CML, although on morphological grounds it does not meet blastic transformation criteria.[16] Perhaps the best murine model currently available is the BCR-ABL1 transgenic mouse developed by the Tenen laboratory.[17]

CML progenitor cells or CML-SCs could conceivably survive *in vivo* TKI exposure due to high BCR-ABL1 expression (maintaining some kinase-active protein in the presence of TKIs) or low BCR-ABL1 expression (identifying themselves as less BCR-ABL1 dependent). Several labs showed higher levels of BCR-ABL1 in primitive lineage-CD34+38- cells than lineage-CD34+38+ progenitor cells,[18,19] suggesting primitive cells may be TKI resistant due to maintenance of BCR-ABL1 activity. Conversely, BCR-ABL1 mRNA expression in myeloid colonies cultured from patients with TKI-induced MMR was found to be lower than in colonies cultured from untreated patients, consistent with *in vivo* selection of progenitors that are less reliant on BCR-ABL1.[20] Analogous results were seen in normal human CD34+ cells infected with BCR-ABL1 retrovirus.[21]

There is consensus that TKIs inhibit BCR-ABL1 kinase activity in primitive lineage-CD34+38– cells.[22,23] However, in contrast to progenitor cells, these cells undergo little apoptosis upon TKI exposure, and prolonged survival of clonogenic cells is seen even in the absence of cytokines,[23] indicating that the critical cells are not (or not completely) addicted to BCR-ABL1. At closer view, this is in accordance with some fundamentals of CML biology. The presence of BCR-ABL1 in all hematopoietic lineages is evidence that the initial Philadelphia translocation occurs in a pluripotent HSC.[24] Consistent with this, a murine CML model demonstrated that BCR-ABL1 does not confer self-renewal capacity to committed progenitor cells, suggesting CML originates from a cell with intrinsic self-renewal capacity.[25] At diagnosis, the majority of LTC-ICs is frequently Ph– [26] in contrast to the largely Ph+ myeloid progenitor cell population. Thus, the initial proliferative drive is directed almost exclusively at the progenitor cell compartment, establishing the clinical phenotype with expansion of myeloid cells, but maintaining hierarchy of myeloid differentiation.[26] The situation changes at time of transformation to CML-BP, when granulocyte-macrophage progenitor cells acquire self-renewal capacity, presumably due to activation of β-catenin.[27] These biological fundamentals are reflected in the clinical responses to TKIs. First, inhibition of BCR-ABL1 is predicted to roll back the progenitor cell expansion, clinically evident by the rapid hematologic and profound cytogenetic responses to TKIs. Second, the limited effects of BCR-ABL1 on CML-SCs may explain why they are largely insensitive to BCR-ABL1 inhibition, explaining disease persistence or MRD. Nonetheless responses are often durable, as few residual CML-SCs equate with little opportunity for additional mutations; additionally TKIs may mitigate genetic instability caused by BCR-ABL1 kinase activity.[28] Third, once acquisition of self-renewal capacity at the level of progenitor cells[27] has undermined the hierarchical structure of CML hematopoiesis, the pool of fully leukemogenic cells at risk for resistance mutations multiplies and unsurprisingly CML-BP poorly responds to TKI therapy.

Targeting CML stem cells

Conceptually, one can approach the phenomenon of CML-SC resistance to TKIs by identifying the factors that render CML progenitor cells TKI sensitive. One possible explanation is that simultaneous inhibition of BCR-ABL1 and KIT, the receptor for stem cell factor (SCF), is synergistic toward CML progenitor cells. In support of this effective elimination of BCR-ABL1-expressing murine myeloid progenitor cells requires inhibition of both KIT and BCR-ABL1,[29] and imatinib effects on human CML progenitor cells are mimicked by combining a KIT blocking antibody and BCR-ABL1-specific TKI.[30] Nilotinib effects on CML CD34+ cells at concentrations that do not inhibit KIT are mitigated by SCF. Bosutinib, which lacks direct anti-KIT inhibitory activity, may intercept signaling downstream of the receptor by blocking Src kinases,[31,32] Thus, fortuitous BCR-ABL1/KIT dual inhibitory activity in the same TKI may generate synthetic lethality[33-35] in CML progenitor, but not stem cells, and underlie hematologic and cytogenetic responses. Suppression of BCR-ABL1 and a second pathway other than KIT may be necessary to eliminate CML-SCs and identifying such a pathway is of major therapeutic interest.

Inhibition of BCR-ABL1 activity could convert a CML-SC into a loss-of-function, gain-of-function or neutral variant compared to a normal CML-SC. A loss-of-function variant would result if pharmacologically silenced BCR-ABL1 acted in a dominant-negative manner. In view of the well-documented ability of primitive CML cells to respond to cytokines upon BCR-ABL1 inhibition this seems unlikely.[36] On the other hand, functions of BCR-ABL1 that persist despite effective suppression of kinase activity could confer a gain of function phenotype. For example, it has been shown that a kinase-inactive BCR-ABL1 mutant enhances migration and reduces adhesion,[37] and activation of SRC family kinases can persist in the presence of TKIs.[36,39] Elegant studies in a murine CML model have revealed that BCR-ABL1 enhances the expression of several genes in a kinase independent fashion, including Alox5, IL1R2 and ASPRV1.[40] Another mechanism to consider is epigenetic changes imparted by

active BCR-ABL1 that persist despite subsequent effective kinase inhibition. For example, BMS-214662, a compound originally developed as a farnesyl transferase inhibitor (FTI), selectively induces apoptosis in lineage- $CD34^+38^+$ CML cells in a PKCβ-dependent fashion, with or without concomitant inhibition of BCR-ABL1, and it has been suggested that this particular sensitivity might be due to prior exposure to BCR-ABL1 activity.[41,42] Alternatively, BMS-214667 may impair nuclear-cytoplasmic transport.[43] As a third possibility, inactive BCR-ABL1 could be neutral, producing an CML-SC that is biologically indistinguishable from a normal HSC and making selective elimination of residual leukemia through biochemical means impossible. Although speculative, these considerations point to a sometimes neglected factor that is likely to impact clinical responses in CML. Elimination of leukemic hematopoiesis may ultimately depend on the fitness of the normal HSCs; if the size of the HSC pool is controlled by the number of available niches, then a healthy normal HSC compartment is critical to replace the leukemic cells in the niches.[44]

The bone marrow microenvironment as a protective factor for CML stem cells

The term 'microenvironment' describes a complex assortment of specialized cells (osteoblasts, osteoclasts, endothelial cells, stromal/mesenchymal cells, amongst others), extracellular matrix (e.g. collagen, fibronectin) and diffusible factors (cytokines, chemokines, oxygen) that regulate hematopoiesis.[45] An important concept is the niche, a physicochemical space that protects HSC, controls their numbers and regulates their initial initial steps of differentiation.[46] Current thinking holds that in leukemia normal HSC are replaced by LSC.[47] Significant progress has been made toward understanding the role of the bone marrow microenvironment as a critical factor for CML-SC survival. Diffusible factors generated by CML-SC have profound effects on the bone marrow stroma, resulting in abnormal trafficking as well as less habitable conditions for competing normal HSC. An elegant study using transgenic mice with a tetracycline-repressible BCR-ABL1 transgene showed that long-term HSCs (LT-HSC) are reduced in the bone marrow but enriched in the spleens of leukemic mice. This is caused by reduced CXCL12 expression by bone marrow stromal cells, which in turn is the result of GSCF production by CML cells.[48] CML cell-conditioned medium from leukemic mice or untreated CML patients inhibited the proliferation of normal progenitor cells, indicating that CML-derived factors such as TNF-α, MIP-1β and others influence the competition between leukemic and CML progenitor cells in favor of leukemic cells. This confirms earlier data in CML[49] and is reminiscent of a recent observation in $JAK2^{V617F}$ induced murine myeloproliferative disease[50]. In CML, imatinib partially restored the homing defects; increased but still subnormal CXCL12 expression was seen in CML patients with a complete cytogenetic response to imatinib.[48] The same murine model was used in another study that implicated IL-6 as a mediator of myeloid *versus* lymphoid expansion driven by BCR-ABL1.[51] Lastly stromal derived placental growth factor (PlGF) was shown to support the expansion of CML progenitor cells and absence of this cytokine prolonged survival in a murine leukemia model.[52] Defects in CD44 and β-integrins have also been described in CML and are thought to contribute to abnormal SC trafficking,[53,54] but the CXCR4/CXCL12 axis has attracted most attention due to the availability of clinical antagonists such as plerixafor. Previous studies had shown that BCR-ABL1 kinase activity inhibits CXCR4 expression and interferes with signaling downstream of the receptor, suggesting that in CML this system is impaired at several levels.[55,56] Ironically, restoration of CXCR4/CXCL12 function may not be desirable, since it enhances homing of CML cells to the protective environment of the bone marrow.[57] Intercepting the CXCR4/CXCL12 interaction with plerixafor was shown to sensitize leukemia cells to the effects of TKIs in mice transplanted with 32Dcl3 cells engineered to express BCR-ABL1.[58] On the other hand, prolonged plerixafor treatment in combination with TKIs failed to significantly reduce leukemia burden in a retroviral CML model, but caused an increase in extramedullary hematopoiesis and central nervous system involvement.[59] While it is possible that this adverse effect was due to the aggressive nature of the retroviral model and that beneficial effects would be observed in a state of MRD, this study nonetheless calls for caution when manipulating hematopoietic cell trafficking over prolonged periods of time.

Targeting CML stem cells through synthetic lethality

Combinatorial approaches targeting CML-SCs are implicitly based on induction of a lethal phenotype by simultaneous inhibition of BCR-ABL1 kinase and one or more additional pathways.[33,60] These pathways may be redundant or even inactive in the presence of uninhibited BCR-ABL1, and assume an essential role only in the presence of TKIs. For instance, upon treatment with imatinib, CML $CD34^+$ cells can enhance survival by activating MAP kinases in the presence of cytokines.[61] Similarly, CML cell lines cultured on HS-5 bone marrow stromal cells are partially protected from TKI-induced apoptosis by JAK kinase-dependent upregulation of phosphorylated STAT3.[62,63] A recent study reported that abnormally spliced anti-apoptotic BCL2 family proteins are expressed by quiescent niche-resident CML-BC SCs and confer resistance to TKIs.[64] For obvious reasons, pathways with an essential role in CML as well as normal cells are less preferred as therapeutic targets, although a degree of selectivity toward CML-SCs may still be exploitable. On the other hand, pathways activated as part of a stress response to TKIs should be particularly attractive as therapeutic targets. Here we will focus on four major pathways that are supported by independent studies.

Wnt/β-catenin

β-catenin, the central mediator of canonical Wnt signaling, has a dual function as an adhesion-related tight junction protein and a transcriptional co-activator that recruits cAMP response element binding protein (CBP) to lymphoid enhancer factor/T-cell factor (LEF/TCF) binding sites to activate developmentally regulated transcriptional programs. Without active Wnt signaling, cytoplasmic *β*-catenin outside of tight junctions is phosphorylated by

glycogen synthase kinase 3β (GSK3β) and subsequently degraded by a multimeric destruction complex whose rate-limiting component is Axin.[65] Unlike in several solid tumors, in CML there is no evidence for somatic mutations in proteins involved in the canonical Wnt pathway.[66]

Nuclear β-catenin is required for self-renewal and viability of normal HSCs.[67] Several studies have implicated nuclear β-catenin in aspects of CML pathogenesis and response to TKIs. Lack of β-catenin attenuates disease in a murine CML model by impairing self-renewal of CML-SCs.[68] However, in a recent study, deletion of β-catenin after CML initiation did not significantly increase survival in mice.[69] Rather, pharmacological inhibition of β-catenin through block of prostaglandin signaling resulted in greatly reduced numbers of LSCs. In support of an important role of nuclear β-catenin in TKI resistance, we have found that gene expression in imatinib-naive CD34$^+$ cells from patients with primary cytogenetic resistance may be partially regulated by β-catenin.[70] Lastly, activation of nuclear β-catenin in granulocyte-macrophage progenitor cells is associated with myeloid blastic transformation,[27] which may be due to inactivation of GSK3β by abnormal splicing.[71] or conformational changes of β-catenin as a result of BCR-ABL1-induced tyrosine phosphorylation that prevents Axin binding.[72] Paradoxically pharmacological inhibition of GSK3β in combination with imatinib, but not dasatinib, was effective at targeting CML-SCs.[73] This is, at least on the surface, hard to reconcile with previous data, but could suggest that the intensity of the β-catenin signal must be tightly controlled to support optimal CML-SC survival.

Several pathways have been implicated in Wnt signaling in CML-SCs (Figure 1A). First, ligand-induced CD27 signaling may enhance extrinsic and intrinsic activation of nuclear β-catenin. In support of an important role for this pathway, blockage or the absence of CD27 was shown to prolong survival in a murine CML model.[75] A second non-canonical Wnt/Ca^{2+}/NFAT pathway was recently identified by a synthetic lethal screen; this pathway mediates TKI resistance through upregulation of IL-4, and is inhibited by cyclosporine A.[76] Lastly, BCR-ABL1 was shown to up-regulate arachidonate 5-lipoxygenase (5-LO) (Alox5) in a kinase-independent fashion, which is associated with increased levels of its metabolic product leukotriene B4 (LTB4).[74] Lack of Alox5 prolongs survival in a murine CML model, apparently by promoting gradual depletion of CML-SCs, and this may be due to a failure of *Alox5$^{-/-}$* LSCs, but not *Alox5$^{+/+}$* HSCs, to up-regulate β-catenin. Interestingly, treatment with the 5-LO antagonist zileuton prolonged survival of mice with BCR-ABL1-induced leukemia alone and especially in combination with imatinib. The pivotal role of nuclear β-catenin in the pathogenesis of colon cancer has sparked great interest in development of β-catenin inhibitors, but β-catenin remains a challenging target and no clinical compounds have emerged.[65] While most studies have focused on nuclear β-catenin, there is recent evidence that the cytoplasmic fraction, which is associated with N-Cadherin, is indirectly involved in TKI resistance conferred by the microenvironment. N-Cadherin-mediated adhesion to stroma was associated with increased cytoplasmic N-Cadherin-β-catenin complex formation, but also with enhanced β-catenin nuclear translocation and transcriptional activity.[91] Although the precise mechanism by which N-Cadherin-bound β-catenin up-regulates the nuclear pool is unclear, one could imagine that the degradation complex may become over-saturated by intracytoplasmic β-catenin released from the receptor. β-catenin is an example of the co-operation of extrinsic and intrinsic mechanisms in activating identical cellular TKI resistance programs, and that mechanisms engaged at the extremes of the response spectrum in CML may overlap.

Hedgehog

Hedgehog (HH) signaling is essential for primitive fetal hematopoiesis[77] but seems to be dispensable for adult HSC function.[78] HH binding to its cell surface receptor (Patched, PTCH), induces a conformational change in a downstream intermediate termed Smoothened (SMO), thereby releasing the transcriptional activator GLI1 (Figure 1B). Two independent studies have implicated HH signaling in the self-renewal of CML-SCs and identified SMO as a critical mediator.[79] HH signaling in CML-SCs is inhibited by HH blocking antibodies[80] but not BCR-ABL1 TKIs,[79] consistent with a BCR-ABL1 kinase-independent mechanism of HH activation by ligand. Just as in the case of β-catenin, mutations in HH pathways were found in various malignancies, but have not so far been reported in CML. At present, the bulk of data implicating HH in CML-SC survival is based on mouse models or advanced CML, and its role in CML-CP is less well defined. Compared to Wnt/β-catenin, the HH pathway is a more accessible drug target. Cyclopamine, an alkaloid that stabilizes SMO in an inactive conformation, selectively targets CML-SCs over normal HSCs, alone and in combination with BCR-ABL1 TKIs. The discovery of PTCH mutations in other malignancies has stimulated the development of inhibitors of SMO, including PF-04449913, LDE225 and BMS-833923. Several clinical trials are underway in TKI-resistant CML, with a suggestion of activity.[81]

TGF-β/Foxo3a/BCL6

In CML progenitor cells, BCR-ABL1 activates AKT, which in turn phosphorylates the transcription factor Foxo3a, promoting its cytoplasmic retention and subsequent degradation. TKI-induced Foxo3a activation leads to expression of p27 and Bim, with subsequent cell cycle arrest and apoptosis (Figure 1C).[83] Surprisingly, it was found that Foxo3a is nuclear in lineage$^-$CD34$^+$38$^-$ CML cells even in the absence of TKIs, raising the question why AKT signaling is turned off despite active BCR-ABL1.[84,92] Studies in a murine CML model demonstrated TGF-β inhibition of AKT, suggesting that TGF-β signaling is responsible for maintaining LSC in a quiescent, TKI inaccessible state.[84] Consistent with this, loss of Foxo3a does not impair leukemogenesis in primary transplantations, but impairs leukemogenicity in subsequent transplantations. Inhibitors of TGF-β (*e.g.* LY364947) combined with imatinib prolonged survival of leukemic mice compared to imatinib alone. At this point it is unclear whether TGF-β signaling in CML-SC is cell-autonomous or driven by microenvironmental factors. Foxo3A provides a link to BCL6, another transcription factor with a critical role for CML-SC survival and response to TKIs that may regulate key downstream effects of Foxo3A (Figure 1C).[85] BCL6 seems to play the role of a thermostat that calibrates cellular responses according to the level of BCR-ABL1 activi-

ty. Upon TKI inhibition of BCR-ABL1, AKT inhibition promotes Foxo3a activation, which results in strong upregulation of BCL6. BCL6 in turn suppresses the activation of p53 that would otherwise result in apoptosis. In contrast, under basal conditions even low BCL6 levels are sufficient to repress p53 and ARF. If the TKI-induced upregulation of BCL6 is blocked, CML cells are sensitized to BCR-ABL1 TKIs in a p53/ARF-dependent manner. Thus, BCL6 is central to a stress response by which CML cells escape the execution of a p53/ARF-dependent apoptotic program. Inhibition of AKT has a critical role in activating this pathway, as the PI3K/AKT suppressor PTEN is sufficient to induce BCL6. Additionally, recent reports have implicated the SIRT1 deacetylase in suppression of p53 activity in CML-SCs. SIRT1 deacetylates multiple substrates, including p53, FOXO1 and Ku70. TKIs only partially suppressed SIRT1 activity, but inhibition of SIRT1 with the small molecule tenovin-6 in combination with TKIs led to increased apoptosis in a p53 dependent manner.[60,93] Conversely, lack of SIRT1 attenuated BCR-ABL1 leukemogenesis in a murine model. Similarly absence of BCL6 from lineage$^-$KIT$^+$Sca1$^+$ (LSK) cells (the murine equivalent to human lineage$^-$CD34$^+$38$^-$ HSCs) infected with *BCR-ABL1* retrovirus blocks leukemogene-

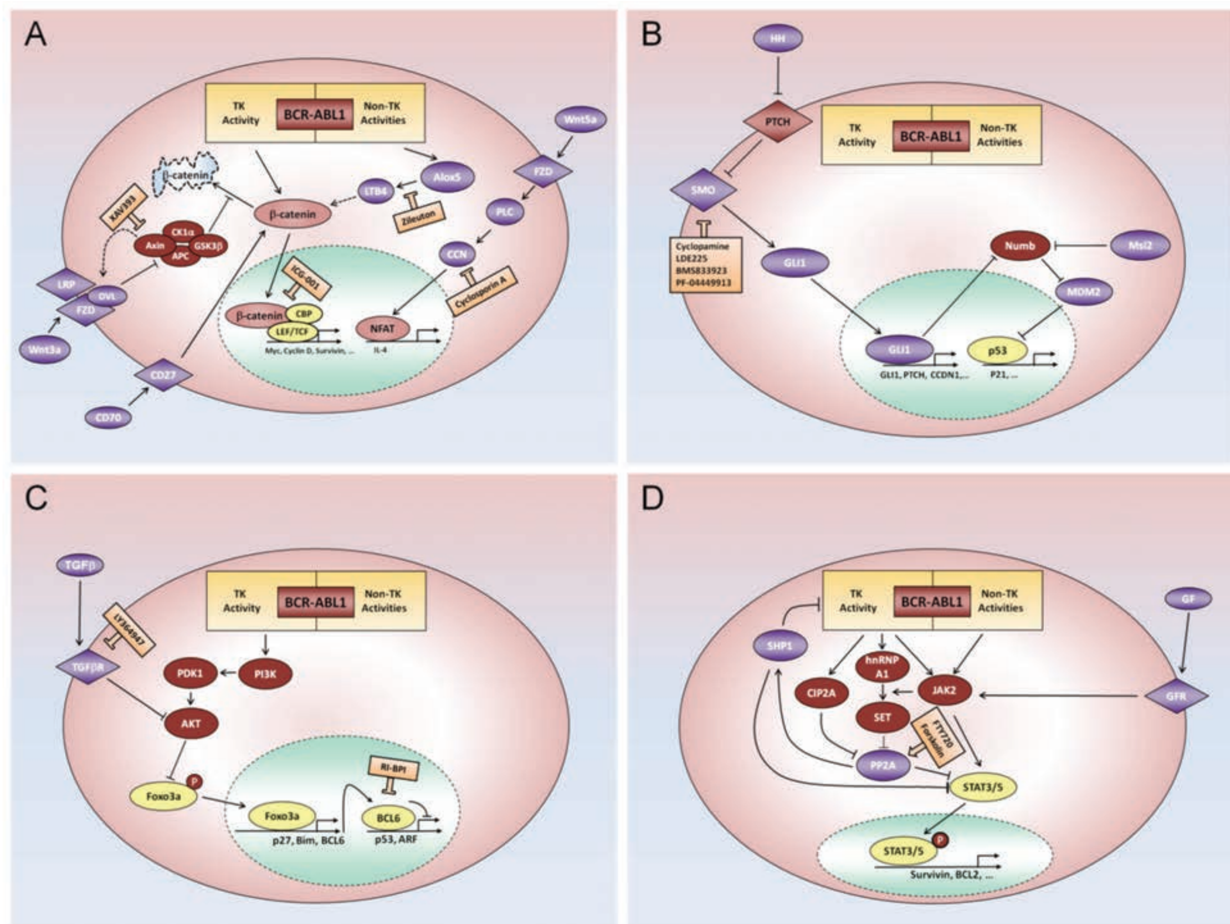

Figure 1. Pathways active in CML-SCs that are possibilities for therapeutic targeting. (A) β-catenin stabilization through Wnt3a binding Frizzled (FZD) and lipoprotein receptor-related protein (LRP) binding Disheveled (DVL) and subsequently sequestering Axin antagonizes destruction complex assembly (red: Axin, APC, casein kinase 1a (CK1a), glycogen synthase kinase 3β (GSK3β)). Extrinsic stabilization through activation of CD27 by CD70 and intrinsic stabilization through tyrosine phosphorylation by BCR-ABL1 preclude Axin binding and promote β-catenin stabilization. Additionally, BCR-ABL1-induced Alox5 upregulation increases β-catenin mRNA.[74] Stabilized, nuclear β-catenin engages lymphoid enhancer factor/T-cell factor (LEF/TCF) and CBP on target gene promoters. Non-canonical Wnt5a signaling activates a phospholipase C (PLC) and calcineurin (CCN) dependent pathway that enhances expression of nuclear factor of activated T cells (NFAT)-regulated genes (e.g. IL-4).[67,75,76] (B) Hedgehog (HH) binding to Patched (PTCH) activates Smoothened (SMO), thereby activating GLI transcription factors, reducing Numb expression and increasing MDM2-induced p53 degradation. Similarly, Msi2 inhibits Numb to suppress p53.[77-82] (C) In CD34$^+$38$^+$ progenitors, BCR-ABL1 activates PI3K, promoting AKT phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1). AKT phosphorylates Foxo3a, preventing its nuclear translocation. In CD34$^+$38$^-$ cells, TGF-β signaling inhibits AKT, permitting Foxo3a translocation and activation of transcriptional targets, including p27 and possibly BCL6.[83-85] (D) BCR-ABL1 suppresses PP2A by activating two negative regulators, SET and CIP2A. SET expression is promoted by hnRNP A1. BCR-ABL1 kinase dependent and independent functions and extrinsic signals regulate JAK2 activity, which in turn controls SET. PP2A, a serine/threonine phosphatase, negatively regulates BCR-ABL1 phosphorylation, activity and stability through SHP1 tyrosine phosphatase. PP2A may dephosphorylate and inactivate additional signaling proteins such as STATs.[86-90] Adapted from O'Hare *et al.*[1]

sis *in vivo* by inducing cell cycle arrest and apoptosis, suggesting that BCL6 may be a viable therapeutic target. A peptide inhibitor of BCL6, known as RI-BPI, prolonged survival in a human CML cell line xenograft model.[85] Several issues regarding the role of BCL6 and its regulation remain incompletely understood. For example, as Foxo3a is active in primitive CML cells but not progenitors,[84] one would predict differential expression of BCL6, which is not the case.[85]

JAK2/PP2A

Several years ago studies from the Perrotti laboratory first implicated the serine/threonine phosphatase PP2A in CML-BP.[86] Mechanistically, high levels of BCR-ABL1 in advanced CML cells enhance the expression of hnRNP A1, an RNA binding protein that increases expression of the PP2A inhibitor SET[132]. Interestingly, this appears to require activation of JAK2.[86,87] Additionally, in patients with a high risk of progression to CML-BP, high levels of cancerous inhibitor of PP2A (CIP2A) may further suppress PP2A activity (Figure 1D).[88] SET knockdown restores PP2A activity, thereby decreasing BCR-ABL1 tyrosine phosphorylation and expression in conjunction with inhibition of downstream effectors such as STAT5, AKT and ERK.[86] These effects are dependent on SHP-1 tyrosine phosphatase, a negative regulator of cytokine signaling in CML cells.[86] The PP2A pathway is an accessible drug target. Reactivation of PP2A in CML $CD34^+$ cells by forskolin induces apoptosis irrespective of BCR-ABL1 activity[86] and independent of adenylate cyclase activation.[86] Another PP2A activator, the immunosuppressant FTY720 (approved for the treatment of multiple sclerosis), has similar PP2A-activating effects on CML progenitor cells.[94] Non-immunosuppressive FTY720 derivatives that activate PP2A are currently being tested.[89] Another option to activate PP2A may be JAK2 inhibition. In lineage $CD34^+38^-$ CML cells, JAK2 is activated by BCR-ABL1 in a kinase-independent fashion, suggesting that combined inhibition of JAK2 and BCR-ABL1 may be synergistic.[90] In contrast, another recent study concluded that JAK2 is not required for disease maintenance and hence not a therapeutic target in CML.[95] Given that different models were used, more experimentation and clinical trials are required to clarify the role of JAK2 as a target in CML.

Conclusion

Due to the efficacy of TKIs, therapeutic objectives in CML have shifted. While preventing blastic transformation for as long as possible, not infrequently at the cost of low quality of life, was the paramount goal in the pre-TKI era, we are now in the privileged position of discussing strategies to eradicate CML-SCs by drug therapy. Incremental yet significant progress has been made in targeting BCR-ABL1 kinase activity. Ponatinib, the recently approved 3rd generation BCR-ABL1 TKI, has activity against all single mutants of BCR-ABL1, including the T315I mutant that is resistant against imatinib, dasatinib, nilotinib and bosutinib.[96,97] Preliminary data from a large phase II study (PACE trial) showed that ponatinib is effective in many patients who failed 3 or more TKIs, including nilotinib and dasatinib.[98] At the other end of the spectrum, i.e. MRD, progress is less obvious. While some patients have maintained CMR after discontinuation of imatinib therapy, for the moment this is only a small minority and we may face the reality that persistent disease is largely beyond the reach of TKIs.[99] Although numerous pathways have been implicated in CML-SC survival in the presence of TKIs, no clear winner has emerged and clinical translation is largely lacking. While the increasing prevalence of CML[3] is testimony to the success of TKI therapy, it has also placed significant strain on health care resources. Developing rational strategies to eliminate residual disease is clearly the most important clinical challenge in CML.

References

1. O'Hare T, Zabriskie MS, Eiring AM, et al. Pushing the limits of targeted therapy in chronic myeloid leukaemia. Nat Rev Cancer. 2012;12:513-26.
2. Gambacorti-Passerini C, Antolini L, Mahon FX, et al. Multicenter independent assessment of outcomes in chronic myeloid leukemia patients treated with imatinib. J Natl Cancer Inst. 2011;103:553-61.
3. Corm S, Roche L, Micol JB, et al. Changes in the dynamics of the excess mortality rate in chronic phase-chronic myeloid leukemia over 1990-2007: a population study. Blood. 2011;118:4331-7.
4. Sheng Z, Ma L, Sun JE, et al. BCR-ABL suppresses autophagy through ATF5-mediated regulation of mTOR transcription. Blood. 2011;118:2840-8.
5. Goussetis DJ, Gounaris E, Wu EJ, et al. Autophagic degradation of the BCR-ABL oncoprotein and generation of antileukemic responses by arsenic trioxide. Blood. 2012;120:3555-62.
6. Slupianek A, Poplawski T, Jozwiakowski SK, et al. BCR/ABL stimulates WRN to promote survival and genomic instability. Cancer Res. 2011;71:842-51.
7. Slupianek A, Dasgupta Y, Ren SY, et al. Targeting RAD51 phosphotyrosine-315 to prevent unfaithful recombination repair in BCR-ABL1 leukemia. Blood. 2011;118:1062-8.
8. Chakraborty S, Stark JM, Sun CL, et al. Chronic myelogenous leukemia stem and progenitor cells demonstrate chromosomal instability related to repeated breakage-fusion-bridge cycles mediated by increased nonhomologous end joining. Blood. 2012;119:6187-97.
9. Makishima H, Jankowska AM, McDevitt MA, et al. CBL, CBLB, TET2, ASXL1, and IDH1/2 mutations and additional chromosomal aberrations constitute molecular events in chronic myelogenous leukemia. Blood. 2011;117:e198-206.
10. Chomel JC, Bonnet ML, Sorel N, et al. Leukemic stem cell persistence in chronic myeloid leukemia patients with sustained undetectable molecular residual disease. Blood. 2011;118:3657-60.
11. Mustjoki S, Rohon P, Rapakko K, et al. Low or undetectable numbers of Philadelphia chromosome-positive leukemic stem cells (Ph(+)CD34(+)CD38(neg)) in chronic myeloid leukemia patients in complete cytogenetic remission after tyrosine kinase inhibitor therapy. Leukemia. 2010;24:219-22.
12. Chu S, McDonald T, Lin A, et al. Persistence of leukemia stem cells in chronic myelogenous leukemia patients in prolonged remission with imatinib treatment. Blood. 2011;118:5565-72.
13. Jaras M, Johnels P, Hansen N, et al. Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. Proc Natl Acad Sciences USA. 2010;107:16280-5.
14. Herrmann H, Sadovnik I, Cerny-Reiterer S, et al. The leukemic stem cell (LSC) in Ph+ CML is a CD34+/CD38-/Lin-cCell that co-expresses dipeptidylpeptidase IV (CD26) and disrupts LSC-niche interactions by degrading the CXCR4 ligand SDF-1{alpha}. Blood. 2011;118:961.
15. Graham SM, Jorgensen HG, Allan E, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood. 2001;99:319-25.
16. Pear WS, Miller JP, Xu L, et al. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood. 1998;92:3780-92.

17. Koschmieder S, Gottgens B, Zhang P, et al. Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL leukemogenesis. Blood. 2005;105:324-34.
18. Jiang X, Zhao Y, Smith C, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. Leukemia. 2007;21:926-35.
19. Copland M, Hamilton A, Elrick LJ, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood. 2006;107:4532-9.
20. Kumari A, Brendel C, Hochhaus A, et al. Low BCR-ABL expression levels in hematopoietic precursor cells enable persistence of chronic myeloid leukemia under imatinib. Blood. 2012;119:530-9.
21. Modi H, McDonald T, Chu S, et al. Role of BCR/ABL gene-expression levels in determining the phenotype and imatinib sensitivity of transformed human hematopoietic cells. Blood. 2007;109:5411-21.
22. Corbin AS, Agarwal A, Loriaux M, et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. J Clin Invest. 2011;121:396-409.
23. Hamilton A, Helgason GV, Schemionek M, et al. Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival. Blood. 2012;119:1501-10.
24. Takahashi N, Miura I, Saitoh K, et al. Lineage involvement of stem cells bearing the Philadelphia chromosome in chronic myeloid leukemia in the chronic phase as shown by a combination of fluorescence-activated cell sorting and fluorescence in situ hybridization. Blood. 1998;92:4758-63.
25. Huntly BJ, Shigematsu H, Deguchi K, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell. 2004;6:587-96.
26. Petzer AL, Eaves CJ, Lansdorp PM, et al. Characterization of primitive subpopulations of normal and leukemic cells present in the blood of patients with newly diagnosed as well as established chronic myeloid leukemia. Blood. 1996;88:2162-71.
27. Jamieson CH, Ailles LE, Dylla SJ, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. New Eng J Med. 2004;351:657-67.
28. Koptyra M, Falinski R, Nowicki MO, et al. BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance. Blood. 2006;108:319-27.
29. Wong S, McLaughlin J, Cheng D, et al. Sole BCR-ABL inhibition is insufficient to eliminate all myeloproliferative disorder cell populations. Proc Natl Acad Sci USA. 2004;101: 17456-61.
30. Corbin AS, O'Hare T, Druker BJ, et al. Suppression of CML progenitor but not CML stem cell growth requires dual inhibition of BCR-ABL and KIT. Blood. 2008;112:190.
31. Lennartsson J, Blume-Jensen P, Hermanson M, et al. Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. Oncogene. 1999;18:5546-53.
32. Ronnstrand L. Signal transduction via the stem cell factor receptor/c-Kit. Cell Mol Life Sci. 2004;61:2535-4.
33. Kaelin WG Jr. The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer 2005;5:689-98.
34. Reinhardt HC, Jiang H, Hemann MT, et al. Exploiting synthetic lethal interactions for targeted cancer therapy. Cell Cycle. 2009;8:3112-9.
35. Packer LM, Rana S, Hayward R, et al: Nilotinib and MEK inhibitors induce synthetic lethality through paradoxical activation of RAF in drug-resistant chronic myeloid leukemia. Cancer Cell. 2011;20:715-27.
36. Zhang B, Konig H, McDonald T, et al. Protection of CML Progenitors from Bcr-Abl Tyrosine Kinase Inhibitor Mediated Apoptosis by the Bone Marrow Stromal Microenvironment. ASH Annual Meeting Abstracts. 2007;110:3378.
37. Ramaraj P, Singh H, Niu N, et al. Effect of mutational inactivation of tyrosine kinase activity on BCR/ABL-induced abnormalities in cell growth and adhesion in human hematopoietic progenitors. Cancer Res. 2004;64:5322-5331.
38. Hu Y, Liu Y, Pelletier S, et al. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. Nat Genet. 2004;36:453-61.
39. Hu Y, Swerdlow S, Duffy TM, et al. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph+ leukemia in mice. Proc Natl Acad Sci USA. 2006;103:16870-5.
40. Chen Y, Hu Y, Zhang H, et al. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. Nat Genet. 2009;41:783-92.
41. Pellicano F, Copland M, Jorgensen HG, et al. BMS-214662 induces mitochondrial apoptosis in chronic myeloid leukemia (CML) stem/progenitor cells, including CD34+38- cells, through activation of protein kinase Cbeta. Blood. 2009;114:4186-96.
42. Copland M, Pellicano F, Richmond L, et al. BMS-214662 potently induces apoptosis of chronic myeloid leukemia stem and progenitor cells and synergizes with tyrosine kinase inhibitors. Blood. 2008;111:2843-53. 8
43. Balabanov S, Evans CA, Abraham SA, et al. Quantitative proteomics analysis of BMS-214662 effects on CD34 positive cells from chronic myeloid leukaemia patients. Proteomics. 2013;13:153-68.
44. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003;425:836-41.
45. Shen Y, Nilsson SK. Bone, microenvironment and hematopoiesis. Curr Opin Hematol. 2012;19:250-5.
46. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells. 1978;4:7-25.
47. Nwajei F, Konopleva M. The bone marrow microenvironment as niche retreats for hematopoietic and leukemic stem cells. Adv Hematol. 2013;2013:953982.
48. Zhang B, Ho YW, Huang Q, et al. Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia. Cancer Cell. 2012;21:577-92.
49. Bhatia R, McGlave PB, Dewald GW, et al. Abnormal function of the bone marrow microenvironment in chronic myelogenous leukemia: role of malignant stromal macrophages. Blood. 1995;85:3636-45.
50. Fleischman AG, Aichberger KJ, Luty SB, et al. TNFalpha facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. Blood. 2011;118:6392-8.
51. Reynaud D, Pietras E, Barry-Holson K, et al. IL-6 controls leukemic multipotent progenitor cell fate and contributes to chronic myelogenous leukemia development. Cancer Cell. 2011;20:661-73.
52. Schmidt T, Kharabi Masouleh B, Loges S, et al. Loss or inhibition of stromal-derived PlGF prolongs survival of mice with imatinib-resistant Bcr-Abl1(+) leukemia. Cancer Cell. 2011;19:740-53.
53. Krause DS, Lazarides K, von Andrian UH, et al. Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. Nat Med. 2006;12:1175-80.
54. Verfaillie CM, Hurley R, Lundell BI, et al. Integrin-mediated regulation of hematopoiesis: do BCR/ABL- induced defects in integrin function underlie the abnormal circulation and proliferation of CML progenitors? Acta Haematol. 1997;97:40-52.
55. Ptasznik A, Urbanowska E, Chinta S, et al. Crosstalk between BCR/ABL oncoprotein and CXCR4 signaling through a Src family kinase in human leukemia cells. J Exp.Med. 2002;196:667-8.
56. Geay JF, Buet D, Zhang Y, et al. p210BCR-ABL inhibits SDF-1 chemotactic response via alteration of CXCR4 signaling and down-regulation of CXCR4 expression. Cancer Res. 2005;65:2676-83.
57. Jin L, Tabe Y, Konoplev S, et al. CXCR4 up-regulation by imatinib induces chronic myelogenous leukemia (CML) cell migration to bone marrow stroma and promotes survival of quiescent CML cells. Mol.Cancer Ther. 2008;7:48-58.
58. Weisberg E, Azab AK, Manley PW, et al. Inhibition of CXCR4 in CML cells disrupts their interaction with the bone marrow microenvironment and sensitizes them to nilotinib. Leukemia. 2012;26:985-90.
59. Agarwal A, Fleischman AG, Petersen CL, et al. Effects of plerixafor in combination with BCR-ABL kinase inhibition in a murine model of CML. Blood. 2012. [Epub ahead of print]
60. Li L, Wang L, Wang Z, et al: Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell. 2012;21:266-81.
61. Chu S, Holtz M, Gupta M, et al: BCR/ABL kinase inhibition by imatinib mesylate enhances MAP kinase activity in chronic myelogenous leukemia CD34+ cells. Blood. 2004;103:3167-74.
62. Traer E, Mackenzie R, Snead J, et al: Blockade of JAK2-mediated extrinsic survival signals restores sensitivity of CML cells to ABL inhibitors. Leukemia. 2012;26:1140-3.
63. Nair RR, Tolentino JH, Argilagos RF, et al: Potentiation of nilotinib-mediated cell death in the context of the bone marrow microenvironment requires a promiscuous JAK inhibitor

in CML. Leuk Res. 2011;36:756-63.
64. Goff DJ, Recart AC, Sadarangani A, et al. A Pan-BCL2 Inhibitor Renders Bone-Marrow-Resident Human Leukemia Stem Cells Sensitive to Tyrosine Kinase Inhibition. Cell Stem Cell. 2013. [Epub ahead of print]
65. Yao H, Ashihara E, Maekawa T. Targeting the Wnt/beta-catenin signaling pathway in human cancers. Expert Opin Ther Targets. 2011;15:873-87.
66. Chomel JC, Villalva C, Sorel N, et al. Evaluation of beta-catenin activating mutations in chronic myeloid leukemia. Leuk.Res. 2008;32:838-9.
67. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 2003;423:409-14.
68. Zhao C, Blum J, Chen A, et al. Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell. 2007;12:528-41.
69. Heidel FH, Bullinger L, Feng Z, et al. Genetic and pharmacologic inhibition of beta-catenin targets imatinib-resistant leukemia stem cells in CML. Cell Stem Cell. 2012;10:412-24.
70. McWeeney SK, Pemberton LC, Loriaux MM, et al. A gene expression signature of CD34+ cells to predict major cytogenetic response in chronic-phase chronic myeloid leukemia patients treated with imatinib. Blood. 2010;115:315-25.
71. Abrahamsson AE, Geron I, Gotlib J, et al. Glycogen synthase kinase 3beta missplicing contributes to leukemia stem cell generation. Proc Natl Acad Sci USA. 2009;106:3925-9.
72. Coluccia AM, Vacca A, Dunach M, et al. Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation. EMBO J. 2007;26:1456-66.
73. Reddiconto G, Toto C, Palama I, et al. Targeting of GSK3beta promotes imatinib-mediated apoptosis in quiescent CD34+ chronic myeloid leukemia progenitors, preserving normal stem cells. Blood. 2012;119:2335-45.
74. Chen Y, Hu Y, Zhang H, et al. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. Nat Genet. 2009;41:783-92.
75. Schurch C, Riether C, Matter MS, et al. CD27 signaling on chronic myelogenous leukemia stem cells activates Wnt target genes and promotes disease progression. J Clin Invest. 2012;122:624-38.
76. Gregory MA, Phang TL, Neviani P, et al. Wnt/Ca2+/NFAT signaling maintains survival of Ph+ leukemia cells upon inhibition of Bcr-Abl. Cancer Cell. 2010;18:74-87.
77. Bhardwaj G, Murdoch B, Wu D, et al. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nat Immunol. 2001;2:172-80.
78. Gao J, Graves S, Koch U, et al. Hedgehog signaling is dispensable for adult hematopoietic stem cell function. Cell Stem Cell. 2009;4:548-58.
79. Dierks C, Beigi R, Guo GR, et al. Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. Cancer Cell. 2008;14:238-249.
80. Zhao C, Chen A, Jamieson CH, et al. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature. 2009;458:776-9.
81. Jamieson C, Cortes JE, Oehler V, et al. Phase 1 dose-escalation study of PF-04449913, an oral Hedgehog (Hh) inhibitor, in patients with select hematologic malignancies. Blood. 2011;118:424.
82. Irvine DA, Copland M. Targeting hedgehog in hematologic malignancy. Blood. 2012;119:2196-204.
83. Essafi A, Fernandez dM, Hassen YA, et al. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. Oncogene. 2005;24:2317-29.
84. Naka K, Hoshii T, Muraguchi T, et al. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. Nature. 2010;463:676-80.
85. Hurtz C, Hatzi K, Cerchietti L, et al. BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia. J Exp Med. 2011;208:2163-74.
86. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. Cancer Cell. 2005;8:355-68.
87. Samanta AK, Chakraborty SN, Wang Y, et al. Jak2 inhibition deactivates Lyn kinase through the SET-PP2A-SHP1 pathway, causing apoptosis in drug-resistant cells from chronic myelogenous leukemia patients. Oncogene. 2009;28:1669-81.
88. Lucas CM, Harris RJ, Giannoudis A, et al. Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukemia is a critical determinant of disease progression. Blood. 2011;117:6660-8.
89. Walker C, Oaks JJ, Neviani P, et al. PP2A Activating Drugs (PAD): Anti-Leukemic and Non-Toxic Activity of Two Novel and Non-Immunosuppressive FTY720 Derivatives. Blood. 2010;116:2901.
90. Neviani P, Harb JG, Oaks JJ, et al. BCR-ABL1 kinase activity but not its expression is dispensable for Ph+ quiescent stem cell survival which depends on the PP2A-controlled Jak2 activation and is sensitive to FTY720 treatment. Blood. 2010;116:515.
91. Zhang B, Li M, McDonald T, et al. Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-Cadherin and Wnt-beta-catenin signaling. Blood. 2013. [Epub ahead of print]
92. Corbin AS, Agarwal A, Loriaux M, et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. J Clin Invest. 2011;121:396-409.
93. Wang Z, Yuan H, Roth M, et al. SIRT1 deacetylase promotes acquisition of genetic mutations for drug resistance in CML cells. Oncogene. 2012. [Epub ahead of print]
94. Neviani P, Santhanam R, Oaks JJ, et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. J Clin Invest. 2007;117:2408-21.
95. Hantschel O, Warsch W, Eckelhart E, et al. BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. Nat Chem Biol. 2012;8:285-93.
96. O'Hare T, Shakespeare WC, Zhu X, et al. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell. 2009;16:401-12.
97. Cortes JE, Kantarjian H, Shah NP, et al. Ponatinib in refractory Philadelphia chromosome-positive leukemias. N Engl J Med. 2012;367:2075-88.
98. Cortes JE, Kim D-W, Pinilla-Ibarz J, et al. Initial Findings From the PACE Trial: A Pivotal Phase 2 Study of Ponatinib in Patients with CML and Ph+ ALL Resistant or Intolerant to Dasatinib or Nilotinib, or with the T315I Mutation. ASH Annual Meeting Abstracts. 2011;118:109.
99. Mahon FX, Rea D, Guilhot J, et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. Lancet Oncol. 2010;11:1029-35.

Molecular monitoring of chronic myeloid leukemia patients

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A B S T R A C T

Standardized molecular monitoring of BCR-ABL mRNA transcripts has been established as the most sensitive technique of diagnosis and follow up in chronic myeloid leukemia (CML) patients. Its prognostic impact on outcome has been demonstrated by independent groups using several tyrosine kinase inhibitors. Nevertheless, laborious harmonization efforts are necessary in order to guarantee comparability of molecular results between different laboratories. Current efforts concentrate on increasing the sensitivity of the results in order to meet the requirements of persistent deep molecular response as a prerequisite for participation in treatment discontinuation trials.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe the benefits and limitations of molecular monitoring in CML patients:
- interpret molecular results in terms of prognostic significance;
- discuss the importance of harmonization of technologies according to the International Scale (IS);
- reasonably use derived techniques in case of resistance or unsatisfying response.

Response assessment in CML

The unique genetic hallmarks of CML, Philadelphia translocation and BCR-ABL gene, allow an accurate quantification of tumor burden and treatment response down to very low levels of leukemia activity. While in numerous hematologic neoplasias the cytomorphological assessment of bone marrow smears is a predominant method to track the course of disease, this classical method of hematology has taken a back seat as far as well-responding CML is concerned. No cytology will tell if an individual CML patient achieves only a superficial or a profound response. More sensitive methods are required to detect minimal residual disease (MRD). They are provided by the cytogenetic analysis of the number of Philadelphia chromosome-positive metaphases and by the quantification of BCR-ABL fusion gene expression in relation to a reference gene. Thereby the measurement of BCR-ABL expression represents not only the most sensitive method by far, but also delivers an absolute value on a ratio scale suited for statistical analyses. In the last two decades, molecular monitoring has become an important tool to track the course of response and is indispensable in state of the art monitoring of CML.

Multiplex PCR

CML is routinely diagnosed by detection of the t(9;22)(q34;q11) translocation via cytogenetic analysis of bone marrow aspirate or by detection of a BCR-ABL fusion gene transcript by qualitative polymerase chain reaction (PCR) from peripheral blood. Approximately 90% of patients presenting with the cytomorphological phenotype of CML are found to be Philadelphia-chromosome positive (Ph^+), approximately 95% are BCR-ABL positive, while the others are termed atypical CML.[1] Molecular diagnosis uses a multiplex PCR with different primer pairs to detect different types of BCR-ABL fusion gene transcripts.[2] The vast majority of patients express either the e13a2 or the e14a2 BCR-ABL transcript or both of them since they are considered splice variants of the same genomic breakpoint.[3]

The routine multiplex PCR assay reported by Cross *et al.*[2] also detects rarer transcripts such as e19a2, e13a3, e14a3 or the e1a2 BCR-ABL transcript which is predominantly associated with Ph^+ acute lymphoblastic leukemia (ALL). If CML is suspected from cytological phenotype and BCR-ABL is negative in routine multiplex PCR it is important to exclude rare transcripts, e.g. e6a2 or e8a2 using specific primer pairs.[4] The identification of the individual BCR-ABL transcript type is a prerequisite for quantitative monitoring and detection of MRD later in the course of disease because a specific assay might be needed. Its identification is mandatory at the onset of treatment since BCR-ABL transcripts might become undetectable for multiplex PCR under treatment of CML.

Quantitative real-time PCR

The quantification of BCR-ABL transcripts using real-time polymerase chain reaction (quantitative RT-PCR) after isolation of total leukocyte RNA and cDNA synthesis represents an accurate method to determine tumor load in patients with CML.[5] Different quantitative RT-PCR assays using different platforms (*e.g.* LightCycler, TaqMan, Rotor-Gene) are established in specific laboratories indicating the need of a standardization to establish comparable reporting of results. Most of the assays apply the absolute quantification method using a dilution series of plasmid standards containing the gene sequence of interest. Therefore, BCR-ABL transcript types differing in length from the used standards, *e.g.* e1a2 or e19a2, will result in biased quantification due to differing PCR efficiencies using a standard PCR assay.[6] For these transcript types, specific PCR assays are available in specialized laboratories.

BCR-ABL transcript levels have been shown to correlate with cytogenetic response: the 10% BCR-ABL level is considered equivalent to partial cytogenetic response (PCyR) given by a reduction to 35% or less Ph+ metaphases,[7] the 1% BCR-ABL transcript level is supposed to be a molecular correlate of complete cytogenetic remission (CCyR), given by the absence of Ph+ metaphases. Therefore, the European LeukemiaNet (ELN) recommends bone marrow aspiration and cytogenetic diagnostics until CCyR is reached. In case of the additional achievement of a major molecular remission (MMR, *i.e.* BCR-ABL transcript level ≤0.1%) no further cytogenetic assessments are needed since they are not expected to provide additional information.[8] However, clonal evolution, *i.e.* the emergence of additional cytogenetic aberrations (ACA) in the Ph+ clone can only be detected by cytogenetics. Hence, in case of suspected resistance or loss of molecular response levels additional cytogenetic diagnostics again becomes necessary.

Routine molecular diagnostics are performed as RT-PCR ('RT' may stand for 'real-time' and 'reverse transcriptase') from cDNA and not from genomic DNA. This approach has been chosen due to the wide range of breakpoints on the genomic DNA level. However, a higher sensitivity can be reached using a patient-specific PCR,[9] which is of growing importance if treatment discontinuation is being considered.

Bone marrow *versus* peripheral blood

PCR for BCR-ABL can be performed using either peripheral blood or bone marrow, while peripheral blood is preferred due to easier access and more exact assessment of the number of leukocytes contained. Furthermore, it has been shown that comparable results were received using parallel measures from bone marrow and peripheral blood in chronic phase CML patients.[10] For other BCR-ABL positive diseases like ALL, it is recommended to use bone marrow instead of peripheral blood which represents a general rule for monitoring other molecular targets in acute leukemias (e.g. acute myeloid leukemia, AML) due to higher tumor load in the marrow.

Reference genes and pre-analytics

Quantification of BCR-ABL has to be complemented by the quantification of an internal control gene that most often is ABL, GUS or BCR. The value of these housekeeping genes is proportional to the amount and integrity of RNA extracted from the blood sample. In order to achieve a sufficient number of housekeeping gene copies, a minimum volume of 10 mL EDTA-anticoagulated PB is recommended.[11] A huge variety of procedures are being performed in molecular laboratories since multiple commercial kits are available for RNA extraction, cDNA synthesis and PCR conditions. Generally commercial easy-to-use RNA extraction kits reach lower amounts of RNA than laborious phenol-chloroform-based methods like TRIzol.[12] Since sample sensitivity is of increasing importance, the main focus of molecular laboratories should be on increasing the RNA yield in order to be able to measure small amounts of BCR-ABL. It should be kept in mind that negative BCR-ABL results are achieved earlier and more frequently if samples do not for any reason meet high sensitivity criteria. A negative BCR-ABL-PCR, either qRT-PCR or nested PCR, can only be interpreted in relation to the expression of a reference gene representing the yield of mRNA transcripts from total leukocyte RNA.

It should be mentioned that BCR-ABL harmonization can be achieved using different housekeeping genes as internal control. Nevertheless, ABL is by far the most commonly performed control gene besides BCR and GUS (beta-glucuronidase) because it has been shown to be comparably expressed in both normal and leukemic samples.[13]

Standardization process, International Scale (IS)

The International Randomized Study of Interferon and STI571 (IRIS) was the first global multicenter study that aimed to follow up patients not only by conventional cytogenetics but also by molecular diagnostics from peripheral blood. Therefore the main three monitoring laboratories (Adelaide, Australia; London, UK; Seattle, USA) shared samples from 30 patients at initial diagnosis and performed quantitative RT-PCR. In order to be able to compare results from different laboratories around the world, the median BCR-ABL expression level of these samples was determined at each site and a conversion factor was derived to transform this median to a 100% level which from there on has been considered as standardized IRIS baseline.[14]

In 2006, an expert panel agreed on continuing to use this baseline as an anchor from which the definition of a MMR as a 3-log reduction was derived.[15] From then on a worldwide harmonization approach was performed, starting from the Australian laboratory in Adelaide, by performing control rounds using spiked cell line dilutions and patient leukocytes.[16] This was extended to other regions, such as the European approach with extension to more than 60 laboratories in 28 European countries.[17] The aim of these sample exchanges is the calculation and validation of conversion factors which can be used to multiply local BCR-ABL expression results and provide BCR-ABL expression results according to the international scale (BCR-ABL^{IS}). This approach has been shown to be a valuable tool to

enable different laboratories to speak the same language.

Nevertheless, the sample exchanges represent a cumbersome procedure which might be substituted within the coming years by the introduction of commercially available secondary reference materials derived from a primary reference material described by White *et al.*[18]

Confounding variables

Even small changes in the procedures, e.g. change of the RT enzyme, use of another RNA extraction kit, or change of PCR primers, can lead to a considerable change in the local conversion factor.[19] Nevertheless, it takes much more to make a good molecular laboratory than renewing their conversion factor every year. Thus, it is of utmost importance that thorough protocols are being followed guaranteeing reproducible steps and enabling false negative or false positive results to be checked by implementing negative and positive controls for several facets of the procedure. The presence of these protocols and rules within the setting of a laboratory certification and/or accreditation represents an important and helpful base for delivering reliable results and minimizing the risk of misinterpretation or misjudgments.

Molecular response landmark according to ELN recommendations

The ELN recommendations on treatment and diagnostics of CML include the proposal to use molecular monitoring every three months on tyrosine kinase inhibitor (TKI) treatment until a MMR is achieved. Maintaining this depth of response might prolong the intervals of measurements up to six months.[8] Achieving MMR by 18 months on imatinib treatment is considered to classify for an optimal response that in turn means that there is no indication that a change of therapy may improve the survival of the patients. In contrast, failure to achieve MMR by 18 months is considered to indicate a suboptimal response that is associated with a risk of not achieving an optimal outcome, and thereby suggests that alternative treatment approaches should be considered.

The prognostic significance of early BCR-ABL transcript levels

In recent years, the prognostic impact of the reduction in BCR-ABL transcript levels at specific treatment periods has become more and more important. Distinct molecular landmarks have been defined that allow the individual treatment situation to be interpreted. The identification of a response situation associated with inferior survival can bring clinicians to change the TKI in use or to evaluate stem cell transplantation. A 7-year update of the IRIS trial revealed that the achievement of a BCR-ABL expression below 1% after 12 months on imatinib therapy is associated with significantly higher rates of event-free (EFS) and progression-free survival (PFS) compared to the cohort of patients which remained above 1% BCR-ABL^{IS}.[20] Furthermore, it has been shown that the achievement of a molecular response of below 1% BCR-ABL^{IS} at 12 months is reassuring for a good risk cohort on imatinib treatment concerning overall survival, which in general is considered the molecular response level corresponding to CCyR.[21] Evaluating molecular response levels after six months on imatinib showed significant benefits in terms of EFS and PFS in patients below 10% BCR-ABL^{IS},[20] or in terms of overall survival (OS) which was even more pronounced having reached a reduction to below 1% BCR-ABL^{IS}.[22] Prognostic data have recently been published from different groups concerning the early 3-month time point on imatinib therapy. Thus, the failure to achieve a molecular response below 10% BCR-ABL^{IS} has been shown to be associated with significantly lower PFS and OS.[22,23] Even though the observation time of the phase III trials DASISION (dasatinib *vs.* imatinib in first-line treatment) and ENESTnd (nilotinib *vs.* imatinib in first-line treatment) have been rather short (3 years), it has been shown that the 10% BCR-ABL^{IS} level after three months has similar prognostic implications. Dasatinib treated patients experienced significantly worse 3-year PFS (68% *vs.* 93%) and OS (86% *vs.* 96%) in case of failing the 3-month criterion. Less patients were in this high-risk group after three months on dasatinib (16%) compared to patients on imatinib (36%).[24] In addition, remaining above 10% BCR-ABL^{IS} after three months led to a significantly lower chance to achieve a MMR within the first two years (~20% *vs.* >50%). Nilotinib-treated patients also showed significantly lower 3-year PFS (91% *vs.* 98%) and overall survival (87% *vs.* 98%) if 10% BCR-ABL^{IS} after three months have not been met.[25] This group of patients only had a chance of 29% to achieve a MMR within two years compared to over 70% in the good risk group. Nevertheless, a marked difference was demonstrated regarding the frequency of ending up in the high-risk group after three months between nilotinib-treated (9%) and imatinib-treated (33%) patients. The cited data clearly indicate that future recommendations will include molecular hallmarks not only for the 18-month time point but also for 12 months, six months, and three months.

Definitions of deep molecular response

The term complete molecular response (CMR) has been critically evaluated and discussed over the last couple of years since there are some intrinsic issues. The ELN recommendations define a CMR as follows: undetectable BCR-ABL mRNA transcripts by real time quantitative and/or nested PCR in two consecutive blood samples of adequate quality (sensitivity $>10^4$).[8] Rates of CMR within trials have been published using multiple criteria of sensitivity and therefore have not been comparable. One essential reason for high rates of CMR in patients measured by certain laboratories is the generally low sensitivity of samples being reflected by low numbers of housekeeping gene transcripts. In such samples, even relatively high numbers of BCR-ABL cannot be detected. Therefore, it is crucial to care about aiming for high-sample quality. Recently, it has been proposed to qualify the depth of the response rather than to use statements like 'complete' molecular response or 'negative' PCR. This concept introduces MR^4, $MR^{4.5}$, and MR^5 as response equal or below 0.01%, 0.0032% and 0.001% on the international scale. These can be achieved by either showing a positive result below the respective

values by quantitative PCR or scoring BCR-ABL negative by quantitative and/or qualitative PCR and achieving a sample quality of above 10000, 32000 or 100000 ABL copies.[5]

Stopping the TKI

The French STIM trial looked at 100 patients for the possibility to stop imatinib treatment in patients who had achieved and maintained a MR^5 for at least two years. Besides the pharmaco-economic implications of this concept, it was shown that approximately 40% of the patients remained in deep molecular response without treatment, whereas about 60% experienced a molecular relapse. None of the patients with a molecular relapse suffered from a disease progression and all were remitted by readministration of imatinib or another TKI.[26] The prerequisite to stop TKIs within current stopping trials (*e.g.* EURO-SKI) are adapted to the definition of a MR^4 in order to evaluate if more conservative levels might be enough to safely stop a TKI.

Achieving a very deep response (MR^4, $MR^{4.5}$, MR^5) is becoming more prevalent on 2nd generation TKIs nilotinib and dasatinib compared to imatinib and this has increased the hope that more patients could qualify for a treatment discontinuation in the future. Stable deep molecular responses without treatment might be called 'operational cure' for a rising number of CML patients, and so help to ease the burden of rising costs for health care systems.

Monitoring of TKI-resistance, BCR-ABL kinase domain mutations

Different genetic mechanisms may contribute to the emergence of TKI-resistant Ph^+ clones resulting in molecular, cytogenetic and, finally, hematologic and clinical relapse. Approximately 50% of resistant patients harbor point mutations of the BCR-ABL kinase domain that abrogate tyrosine kinase inhibition. Over 100 different mutations have been described, most of which can be treated efficiently with 2nd generation TKI.[27] The first herald of resistance is an increase in BCR-ABL transcript levels reflecting the proliferating mutant clone. Different approaches have been made to define the rise in BCR-ABL levels that should trigger a mutation analysis. A 2-fold rise has been shown to be a usable indicator to test patients for BCR-ABL kinase domain mutations.[28] A comparable rise has been confirmed by a more recent analysis that found a 2.6-fold increase in BCR-ABL levels an optimal predictor.[29] According to the current recommendations of the ELN expert panel, mutation analysis should be performed if a landmark defining optimal response is not achieved (*i.e.* CHR at 3 months, PCyR at 6 months, CCyR at 12 months and MMR at 18 months of treatment) or if any of these landmarks is lost during the course of disease.[30] A two-step PCR amplification process allows a specific amplification of the ABL alleles rearranged to the fusion gene. A first PCR step is spanning the BCR-ABL fusion sequence, the subsequent second PCR step targets the ABL kinase domain. While mutation analysis is routinely performed by conventional Sanger sequencing, with a sensitivity of 10-20% mutant alleles, deep sequencing assays using next generation sequencing platforms provide a highly sensitive technique that will allow an earlier mutation detection and selection of an efficient TKI.[31]

References

1. Hehlmann R, Hochhaus A, Baccarani M. Chronic myeloid leukaemia. Lancet. 2007;370(9584):342-50.
2. Cross NC, Melo JV, Feng L, Goldman JM. An optimized multiplex polymerase chain reaction (PCR) for detection of BCR-ABL fusion mRNAs in haematological disorders. Leukemia. 1994;8(1):186-9.
3. Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. Blood. 2000;96(10):3343-56.
4. Hochhaus A, Reiter A, Skladny H, Melo JV, Sick C, Berger U, et al. A novel BCR-ABL fusion gene (e6a2) in a patient with Philadelphia chromosome-negative chronic myelogenous leukemia. Blood. 1996;88(6):2236-40.
5. Cross NC, White HE, Muller MC, Saglio G, Hochhaus A. Standardized definitions of molecular response in chronic myeloid leukemia. Leukemia. 2012;26(10):2172-5.
6. Emig M, Saussele S, Wittor H, Weisser A, Reiter A, Willer A, et al. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Leukemia. 1999;13(11):1825-32.
7. Branford S, Rudzki Z, Harper A, Grigg A, Taylor K, Durrant S, et al. Imatinib produces significantly superior molecular responses compared to interferon alfa plus cytarabine in patients with newly diagnosed chronic myeloid leukemia in chronic phase. Leukemia. 2003;17(12):2401-9.
8. Baccarani M, Cortes J, Pane F, Niederwieser D, Saglio G, Apperley J, et al. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. J Clin Oncol. 2009;27(35):6041-51.
9. Sobrinho-Simoes M, Wilczek V, Score J, Cross NC, Apperley JF, Melo JV. In search of the original leukemic clone in chronic myeloid leukemia patients in complete molecular remission after stem cell transplantation or imatinib. Blood. 2010;116(8):1329-35.
10. Kiss TL, Xu WM, Jamal N, Messner HA. Comparative testing of peripheral blood and bone marrow for BCR-ABL transcripts in patients post allogeneic bone marrow transplantation and during interferon treatment for chronic myeloid leukemia. Leuk Lymphoma. 1999;34(5-6):493-500.
11. Thorn I, Olsson-Stromberg U, Ohlsen C, Jonsson AM, Klangby U, Simonsson B, et al. The impact of RNA stabilization on minimal residual disease assessment in chronic myeloid leukemia. Haematologica. 2005;90(11):1471-6.
12. Muller MC, Hordt T, Paschka P, Merx K, La Rosee P, Hehlmann R, et al. Standardization of preanalytical factors for minimal residual disease analysis in chronic myelogenous leukemia. Acta Haematol. 2004;112(1-2):30-3.
13. Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. Leukemia. 2003;17(12):2474-86.
14. Hughes TP, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley ML, et al. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med. 2003;349(15):1423-32.
15. Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood. 2006;108(1):28-37.
16. Branford S, Fletcher L, Cross NC, Muller MC, Hochhaus A, Kim DW, et al. Desirable performance characteristics for BCR-ABL measurement on an international reporting scale to allow consistent interpretation of individual patient response and comparison of response rates between clinical trials. Blood. 2008;112(8):3330-8.
17. Muller MC, Cross NC, Erben P, Schenk T, Hanfstein B, Ernst T, et al. Harmonization of molecular monitoring of CML therapy in Europe. Leukemia. 2009;23(11):1957-63.

18. White HE, Matejtschuk P, Rigsby P, Gabert J, Lin F, Lynn Wang Y, et al. Establishment of the first World Health Organization International Genetic Reference Panel for quantitation of BCR-ABL mRNA. Blood. 2010;116(22):e111-7.
19. Muller MC, Munjal U, Erben P, Lion T, Mitterbauer-Hohendanner G, El Housni H, et al. Stability of Conversion Factors for BCR-ABL Monitoring — Implications for the Frequency of Validation Rounds. ASH Annual Meeting Abstracts. 2010;116(21):893.
20. Hughes TP, Hochhaus A, Branford S, Muller MC, Kaeda JS, Foroni L, et al. Long-term prognostic significance of early molecular response to imatinib in newly diagnosed chronic myeloid leukemia: an analysis from the International Randomized Study of Interferon and STI571 (IRIS). Blood. 2010;116(19):3758-65.
21. Hehlmann R, Lauseker M, Jung-Munkwitz S, Leitner A, Muller MC, Pletsch N, et al. Tolerability-Adapted Imatinib 800 mg/d Versus 400 mg/d Versus 400 mg/d Plus Interferon-{alpha} in Newly Diagnosed Chronic Myeloid Leukemia. J Clin Oncol. 2011;29(12):1634-42.
22. Hanfstein B, Muller MC, Hehlmann R, Erben P, Lauseker M, Fabarius A, et al. Early molecular and cytogenetic response is predictive for long-term progression-free and overall survival in chronic myeloid leukemia (CML). Leukemia. 2012;26(9):2096-102.
23. Marin D, Ibrahim AR, Lucas C, Gerrard G, Wang L, Szydlo RM, et al. Assessment of BCR-ABL1 transcript levels at 3 months is the only requirement for predicting outcome for patients with chronic myeloid leukemia treated with tyrosine kinase inhibitors. J Clin Oncol. 2012;30(3):232-8.
24. Hochhaus A, Boqué C, Bradley-Garelik MB. Molecular Response Kinetics and BCR-ABL Reduction in Patients With Newly Diagnosed Chronic Myeloid Leukemia in Chronic Phase (CML-CP) Receiving Dasatinib vs Imatinib: DASISION 3-year Follow-Up. Haematologica. 2012;97(Suppl 1):Abstract 0192.
25. Hochhaus A, Guilhot F, Al-Ali K, Rosti G. Early BCR-ABL Transcript Levels Predict Future Molecular Response and Long-term Outcomes in Newly-diagnosed Patients with Chronic Myeloid Leukemia in Chronic Phase: Analysis of ENESTnd 3-year Data. Haematologica. 2012;97(Suppl 1):Abstract 0584.
26. Mahon FX, Rea D, Guilhot J, Guilhot F, Huguet F, Nicolini F, et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. Lancet Oncol. 2010;11(11):1029-35.
27. Hochhaus A, La Rosee P, Muller MC, Ernst T, Cross NC. Impact of BCR-ABL mutations on patients with chronic myeloid leukemia. Cell Cycle. 2011;10(2):250-60.
28. Branford S, Rudzki Z, Parkinson I, Grigg A, Taylor K, Seymour JF, et al. Real-time quantitative PCR analysis can be used as a primary screen to identify patients with CML treated with imatinib who have BCR-ABL kinase domain mutations. Blood. 2004;104(9):2926-32.
29. Press RD, Willis SG, Laudadio J, Mauro MJ, Deininger MW. Determining the rise in BCR-ABL RNA that optimally predicts a kinase domain mutation in patients with chronic myeloid leukemia on imatinib. Blood. 2009;114(13):2598-605.
30. Soverini S, Hochhaus A, Nicolini FE, Gruber F, Lange T, Saglio G, et al. BCR-ABL kinase domain mutation analysis in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors: recommendations from an expert panel on behalf of European LeukemiaNet. Blood. 2011;118(5):1208-15.
31. Parker WT, Lawrence RM, Ho M, Irwin DL, Scott HS, Hughes TP, et al. Sensitive detection of BCR-ABL1 mutations in patients with chronic myeloid leukemia after imatinib resistance is predictive of outcome during subsequent therapy. J Clin Oncol. 2011;29(32):4250-9.

Deciding to continue or discontinue therapy in chronic myeloid leukemia

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A B S T R A C T

In current clinical practice, the treatment of chronic myeloid leukaemia (CML) with tyrosine kinase inhibitors (TKI) is continued indefinitely. Several studies show that TKI therapy yields durable responses and prolongs survival. Despite the outstanding efficacy of TKI in CML, their curative potential remains uncertain. Recently, preliminary results of trials stopping TKI have modified the horizon of CML therapy and so the issue of treatment cessation has become of utmost importance for both patients and physicians. Today, several clinical trials propose the interruption of TKIs for patients in sustained deep molecular response. We know that most TKI-treated patients retain residual leukemic cells detected by means of real time polymerase chain reaction (PCR), depending on the sensitivity of this test. After stopping treatment, more than half of the patients exhibit a molecular relapse, which does not lead automatically to disease relapse of CML. Therefore, the decision to continue or discontinue therapy in CML is a key question. By asking these questions, we set ourselves on the path of finding the answer that encompasses both clinical research and philosophical consideration on the issue of cure.

Learning goals

At the conclusion of this activity, participants should know that:
- the main clinical studies of TKI discontinuation;
- TKI discontinuation requires a sustained deep molecular remission;
- interruption of TKI should only be considered in a clinical trial with strict molecular monitoring.

Discontinuation of TKI treatment is possible in clinical trials

The National Comprehensive Cancer Network (NCCN) guideline on CML and the expert panel of the European LeukemiaNet (ELN) recommend continuation of TKI treatment indefinitely in all responding patients.[1,2] However, in recent years a variety of clinical studies have explored the option to discontinue TKI therapy in patients with sustained molecular responses.[3] First, a pilot study was reported where it was proposed to discontinue imatinib in 12 patients with CML treated and maintained in complete molecular response (CMR) for at least two years. In that study, and at that time, CMR was defined by undetectable molecular response (UMR) with PCR sensitivity between 4.5 and 5 log. After a median follow up of 18 months, 50% of patients remained off therapy without confirmed reappearance of peripheral blood BCR-ABL transcripts.[4] Updated results confirmed that 50% of patients off therapy had an undetectable level of BCR-ABL transcripts after a median follow up of 7.5 years (range 4.4-8.4 years).[5] In that study, the patients who did not exhibit relapse, had been previously treated with interferon (IFN). This pilot study provided a proof-of-concept that imatinib discontinuation could be achieved in selected patients. This pilot study was followed by a multicenter study: the 'Stop Imatinib' (STIM) trial. This was a prospective trial including 100 patients with chronic phase CML on imatinib therapy with the same criteria, i.e, undetectable peripheral blood BCR-ABL transcripts for at least two years (with an assay sensitivity close to a 5 log reduction). Fifty-one per cent of the patients had been previously treated with IFN, and the other half were treated with imatinib only. Molecular relapse, which was arbitrarily defined as two positive RQ-PCR results over a period of one month showing a significant rise (1 log) in BCR-ABL transcripts, was a trigger for imatinib resumption. An interim analysis yielded promising results with a 12-month molecular relapse-free survival rate of 41%.[6] A recent update of that study showed that the overall probability of maintaining CMR at 36 months was 39% (95%CI: 29-48); 3 cases of late relapse were observed at 19, 20 and 22 months, respectively.[7] Most patients who experienced molecular relapse did so within six months of imatinib cessation and remained responsive to re-treatment with imatinib, as we had observed in the pilot study. Similar results were reported in the Australasian Leukaemia and Lymphoma Group (*ALLG*) CML8 study (TWISTER), which used very similar criteria, i.e. UMR with a PCR sensitivity of 4.5 log treated on imatinib for more than two years as an entry criterion.[8,9] After a median of 36 months of follow up, 45% of patients had stable CMR off therapy, while

55% had relapsed.[9] A nationwide survey in Japan identified 50 patients who had discontinued imatinib for at least six months, 43 of whom were analyzed. Molecular recurrence was detected in 19 patients, and the CMR rate following imatinib discontinuation was estimated to be 47%.[10] Several other studies, including the 'According to STIM' and KEIO STIM, have evaluated imatinib discontinuation after sustained, deep molecular response, and in all cases significant percentages of patients have been able to remain relapse-free off therapy.[11,12] It is important to note that 'molecular relapse' and, therefore, the trigger for reintroduction of TKI therapy, in these studies has often been defined differently (Table 1). For example, in the STIM study, molecular relapse was defined as two positive RQ-PCR results over a 1-month period.[6,7] In contrast, in the 'According to STIM' study, molecular relapse was less stringently defined as loss of MMR at any time or a 1-log increase or over in BCR-ABL on two consecutive assessments.[11] Many other studies investigating discontinuation of TKI are in progress or will start soon as discussed in the following paragraphs.[13-15]

The need to achieve a sustained deep molecular response

The criterion of sustained UMR for at least two years is of major importance in planning TKI discontinuation strategies (Figure 1). Other attempts at imatinib discontinuation that did not fill this criterion exhibited rapid molecular relapses.[16-19] Different TKI discontinuation studies confirmed and suggested that the duration of response, especially the duration of CMR was important. Takahashi *et al.* reported that a significant difference in the estimated molecular relapse free survival rates at five years following discontinuation between patients in whom CMR was sustained for more than 24 months prior to imatinib discontinuation and those sustaining a CMR for less than 24 months (78% *vs.* 15%, *P*=0.0002).[10] In the Australasian TWISTER study, a sustained UMR for at least two years was also used as a criterion.[8] The validation of this criterion was reinforced using mathematical models confirming a biphasic dynamic of BCR-ABL transcript decline with a 2-slope model of imatinib response: the α slope corresponded to the rapid initial decrease in BCR-ABL transcript levels (cycling cells) after the start of treatment, and the β slope corresponded to the longer-term BCR-ABL dynamics (less proliferative cells).[20]

Another model recently reported based on the biphasic decline of BCR-ABL transcript levels suggested that 31% of the patients will remain in deep molecular remission after treatment cessation after a fixed period of two years in MR[5], whereas 69% are expected to relapse.[21]

However, in the STIM study, when we analyzed factors which potentially predicted molecular relapse by univariate analysis, the duration of molecular remission was not significant.[6] This may be due to the low power of the statistical analysis. In France, an STIM2 trial has started which includes patients with chronic phase CML treated initially with imatinib as a single agent and with a sustained CMR for at least two years. At the last update, 120 patients have been recruited out of a planned 200.[22] The identification of patients who would benefit most from discontinuation of imatinib remains a key issue and the question of the duration of molecular response before discontinuation is crucial. It is also one of the objectives of the European Stop Kinase Inhibitor (EURO-SKI) trial from the European LeukemiaNet (ELN) that is in progress in several countries. The criteria for discontinuation are less strict than in the STIM studies: duration of TKI treatment before enrolment of at least three years, no PCR-results over 0.01% within the last year. ELN plans to recruit 500 patients to a discontinuation study that will address the following questions. 1) How long should treat-

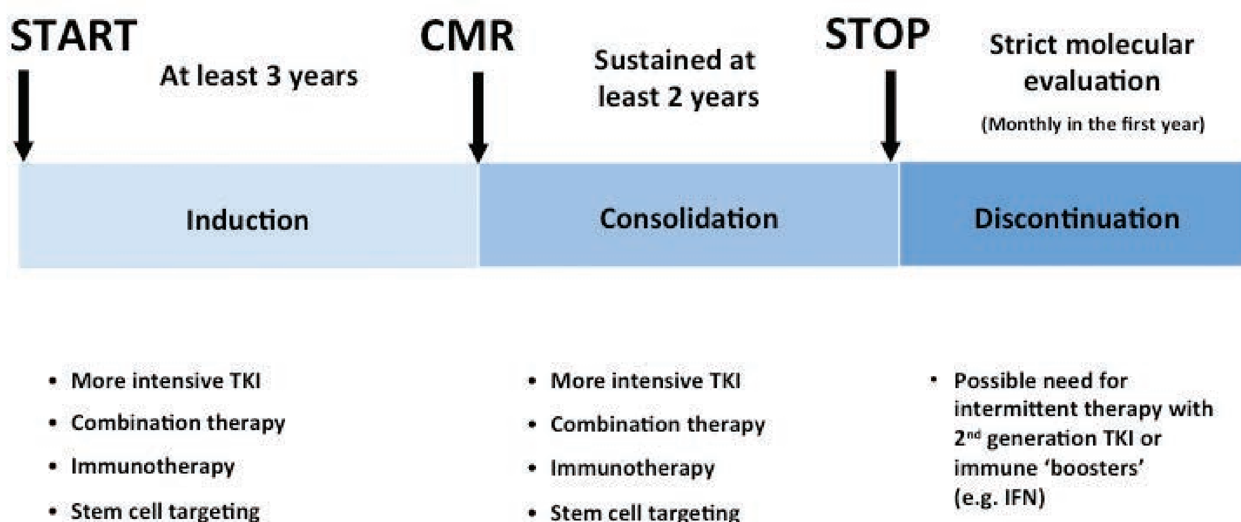

Figure 1.General design for discontinuation of trials.

ment with TKIs last? 2) What level of MR is required? 3) Do gender, combination therapy or the type of TKI influence the chance of relapse after diccontinuation of TKIs? 4) Does the type of TKI used for treatment influence the relapse rate?

The depth of response is an important factor in the decision to discontinue TKI treatment. The definition of molecular response and the standardisation of BCR-ABL transcript measurement remain a concern. For this reason, the CML Working Group of the ELN has recently proposed revised definitions of molecular response (MR) taking into account the sensitivity of molecular test:

MR^4 indicates ≥ 4-log reduction (*BCR-ABL*^{IS}≤ 0·01%);
$MR^{4.5}$ indicates ≥4.5-log reduction (*BCR-ABL*^{IS}≤ 0·0032%); and
MR^5 indicates ≥5-log reduction (*BCR-ABL*^{IS} ≤0·001%).[23]

Like CMR, undetectable levels of minimal residual disease (UMRD) indicates a negative RQ-PCR result and must be associated with a defined PCR assay sensitivity; however, it should be noted that leukemic cells may still be present even if RQ-PCR results are negative.[24] Current RQ-PCR methods can reliably detect up to a 5-log reduction in *BCR-ABL*, but newer techniques, such as DNA-based PCR, RNA-based digital PCR, and replicated PCR have demonstrated increased sensitivities and may enable the assessment of even deeper levels of molecular response.[25] However, it should be noted that using an ultrasensitive PCR technique, a low level of *BCR-ABL* transcripts has been found in the blood of normal individuals, suggesting that a complete absence of transcripts may not be required to eradicate the disease.[26,27] We still do not know the threshold of residual disease which will allow us to safely stop TKI without molecular recurrence.

Analysis of other factors to define the best candidates for stopping TKI

Beside the duration and deep of response, which other factors may be used to suggest the possibility of interrupting TKI treatment? In the STIM study, several potential factors for prediction of molecular relapse were retrospectively assessed.[6] The probability of remaining in stable CMR after discontinuation was favorable in the low Sokal risk group when compared to the intermediate or high Sokal risk groups. Using multivariate analysis and logistical regression at eight months, Sokal risk and imatinib therapy duration were confirmed as two independent prognostic factors for prediction of molecular relapse after imatinib cessation. Despite the low number of patients, Yhim and colleagues also confirmed that high Sokal risk was associated with a higher rate of molecular relapse after imatinib discontinuation.[13] Using univariate analysis Ross and colleagues in the TWISTER study found that high Sokal risk score at diagnosis was the strongest predictor of molecular recurrence.[8] It is of some interest to note that a factor such as the Sokal score illustrating the aggressiveness of the disease at diagnosis is still significant.[28] The identification of other predictive factors of molecular recurrence depends on the power of the statistical analysis which requires the analysis of a larger cohort of patients. As mentioned before, this is one of the goals of the EURO-SKI and the STIM2 studies.

Using the criteria of the STIM and TWISTER studies, it should be possible to predict which patients are ideal candidates for discontinuation of TKIs. Recently Branford and colleagues found in a study of 415 patients treated with imatinib for eight years, that the cumulative rate of stable $MR^{4.5}$ (for at least 2 years) was 43%. In these patients, the time to achieve MMR was correlated with the time to achieve stable $MR^{4.5}$.[29] In addition, the only two independent factors, i.e, female gender and a low level of BCR-ABL1 value at three months were strongly statistically linked to the prediction of sustained $MR^{4.5}$. Factors associated with sustained $MR^{4.5}$ and undetectable transcripts induced by TKI (imatinib, dasatinib and nilotinib) were also analyzed in a multivariable analysis (n=495) by Falchi and colleagues from the MD Anderson Cancer Center (MDACC) in Huston. They showed that older age, higher baseline hemoglobin, higher baseline platelets, TKI modality and response at three months were significant.[30] A larger cohort of patients would be necessary to validate and refine this analysis. This is also one of the aim of the STIM2 study for imatinib and the EURO-SKI for other TKI.

Increase the number of patients who might stop TKI

The possibility of discontinuing IFN treatment for very good responder CML patients was reported ten years ago. At that time, it was shown that IFN α treatment could be stopped after a complete cytogenetic response (CCgR) was achieved and the rate of persistent CCgR depended on time elapsed between CCgR achievement and treatment discontinuation. The authors concluded: "If the cytogenetic responses are confirmed in the future, the issue of curtailing treatment might also become relevant in patients in CCR after STI571".[31] In 2010, stopping treatment in CML patients after imatinib, formerly named STI571, became a reality.

With the STIM study, criteria for discontinuation were defined: a state likely to be stable with undetectable *BCR-ABL1* transcripts for at least 24 months with an appropriate PCR sensitivity below 4.5 and 5 logs. These criteria could be modified particularly if the question of stopping treatment with more powerful drugs such as 2nd generation TKIs is addressed. The more potent 2nd generation TKIs, such as nilotinib, dasatinib, bosutinib, have been shown to induce faster, higher and deeper molecular responses compared with imatinib.[32] Three randomized, phase III clinical studies comparing imatinib with the 2nd generation TKIs nilotinib, dasatinib and bosutinib (ENESTnd, DASISION and BELA, respectively)[33-35] demonstrated that use of 2nd generation TKIs as initial therapy is associated with faster and higher rates of MR compared with imatinib In ENESTnd, patients achieved higher rates of MR^4 and $MR^{4.5}$ by one, two, and three years with nilotinib compared with imatinib.[36] Improved rates of MR^4, and $MR^{4.5}$ by one, two, and three years were also observed with dasatinib compared with imatinib in DASISION.[37] In the BELA phase III study of bosutinib *versus* imatinib, the primary end point (CCgR rate at 12 months) was not met; however, bosutinib did show a higher MMR rate at 12 months (41% *vs.* 27%; P<0·001) compared with imatinib.[35] CMR (in

this study, equivalent to MR[4]) rate at 12 months was also higher with bosutinib (12% *vs.* 3%, *P*<0·001). Several other single-arm trials of 2nd generation TKIs as initial therapy have also assessed deep molecular response end points, including the Italian (GIMEMA) nilotinib phase II study, the MDACC nilotinib phase II study, the MDACC dasatinib phase II study, and ENEST1st.[38-42] It may be also beneficial if patients are switched to 2nd generation TKIs after imatinib. This is one of the aims of the ENESTcmr study. The increasing importance of deep molecular response in CML therapy has prompted the question: should patients with CCyR who do not achieve these deep levels of molecular response be switched to an alternate therapy? In ENESTcmr, patients with detectable *BCR-ABL* after 2 years or over on imatinib were randomized to receive either continued imatinib or nilotinib. After 12 months of follow up, nilotinib demonstrated significantly higher rates of MR[4], MR[4.5], and CMR (≥4·5-log assay sensitivity) compared with continued imatinib.[43] Results after 24 months of follow up continued this trend, with 32.7% and 16.5% of patients in the nilotinib and imatinib arms, respectively, achieving CMR.[44]

All of these studies confirm that 2nd generation TKI are able to induce deeper molecular response in CML patients, which is *sine qua non* condition for discontinuation of treatment. However, so far, only limited experience is available with nilotinib or dasatinib.[45] Rea and colleagues from the FILMC (French CML Group) study reported 34 patients with a minimum follow up of six months (median 14, range 7-33)[46] (Table 1). The criteria of discontinuation were those used in the STIM. Two patients discontinued a 2nd generation TKI in the front-line setting, 29 in the second-line setting, and 3 in the third-line setting. The last reported follow up showed 18 patients with stable MMR remaining off therapy for a median of 16 months (range 7-33); of these patients, 7 had stable undetectable BCR-ABL and 11 had at least one instance of weakly detectable BCR-ABL. The 12-month probability of remaining in stable MMR was 58.3% (95%CI: 41.5%-75%). the rate of relapse according to STIM and CML8. The corresponding 12-month probabilities were 55.8% (95%CI: 39.2%-72.6%) according to the definition of relapse in the STIM (detectable *BCR-ABL* on 2 consecutive tests with at least 1 log increase between the 2) and 44.1% (95%CI: 27.4%-60.8%) according to TWISTER (detectable BCR-ABL on 2 consecutive tests at any level). After a median follow up of 14 months, 13 of 15 patients regained MMR and 10 of 13 patients regained CMR after relapse.[46] Many other studies are now in progress aiming to explore the possibility of safely stopping 2nd generation TKI including the EURO-SKI study. The Pharma industry has defined a path-to-cure or a what is called 'TFR, treatment free remission' program on the basis of reported data, indicating that a sustained deep molecular response may become the next molecular end point in future trials. Novartis Pharma has built a program with nilotinib and extended ENEST to different trials such ENESTPath, ENESTop, ENESTfreedom. Bristol Myer Squibb (BMS) also proposes a phase II study (Dasatinib Functional Cure CA180-406 Study) evaluating dasatinib therapy discontinuation in patients with CML with stable deep molecular responses. Combination studies may increase molecular responses. IFN combined with TKI may have a synergistic effect. In the French SPIRIT trial, the addition of pegylated IFN alfa-2a to imatinib therapy for chronic-phase CML patients resulted in significantly higher rates of MR[4].[47]

Table 1. Clinical studies of TKI discontinuation in patients with CML-CP.

Study	N	Treatment before discontinuation	Response required for discontinuation	Definition of relapse	Patients free from relapse (median follow-up time)	Patients responding to TKI after relapse
Trials of imatinib discontinuation						
STI [6,7]	100	Imatinib for ≥ 3 years	MR5 for ≥ 2 years	Confirmed loss of MR5	39% (30 months)	56/61 regained MR5
ALLG CML8[8,9]	40	Imatinib for ≥ 3 years	MR4.5 for ≥ 2 years	Confirmed loss of MR4.5	45% (3 years)	22/22 regained MMR or better
According to STIM[11]	66	Imatinib for ≥ 3 years	MR4.5 for ≥ 2 years	Loss of MMR or ≥ 1-log	64% (23 months) increase in BCR-ABL[a]	24/24 regained MMR or better
Korean[15]	40	Imatinib for ≥ 3 years	CMR for ≥ 2 years	Confirmed loss of MMR	77% (7.9 months)	4/6 regained MMR or better
Yhim *et al.*[13]	14	Imatinib for median 56.4 months (range 26.2-82.0)	CMR for ≥ 1 years	Confirmed loss of CMR	28.6% at 1 year (23 months)	7/10 regained CMR
KEIO STIM[12]	30	Imatinib for median 92 months (range 32-114)	UMRD for ≥ 2 years (< 100 copies by TMA)	Loss of MMR	46.8% at 6 months (5 months)	6/11 regained UMRD
Trials of nilotinib/dasatinib discontinuation						
STOP 2G-TKI[46]	42	Nilotinib or dasatinib for median 35 months (range 21-72)	CMR for median 29 months (range 21-39)	Loss of MMR	58.3% (12 months)	13/15 regained stable MMR; 10/13 regained UMRD

UMRD: undetectable minimal residual disease.

Burchert and colleagues reported results of IFN maintenance therapy after induction therapy with imatinib plus IFN.[48] This induced stable long-term molecular remissions and may be helpful in increasing the number of patients who can safely discontinue TKIs, particularly if immunological responses are achieved. Despite the fact that the mechanism underlying the anti-leukemic effect of IFN in CML is unknown, a single-arm phase II study started recently to evaluate pegylated IFN in association with nilotinib in newly diagnosed CP-CML patients. The preliminary results with the highest rate of MR4.5 at 12 months (21%) were presented recently but need to be confirmed.[49] A phase III randomized study, the TIGER study, is evaluating nilotinib *versus* nilotinib plus PEG-IFN, and asking the question of discontinuation in newly diagnosed CML patients is in progress in Germany (A Hocchaus *et al.,* personal communication, 2013).

Increase the rate of treatment free remission: targeting leukemic stem cells?

We have learnt from discontinuation studies that the loss of CMR is observed in approximately 60% of patients. In spite of questions about the definition of cure, we should strive to reduce the rate of molecular recurrence after discontinuation of treatment. In the future, CML treatment could be considered in 3 steps: induction, consolidation and sustained CMR (Figure 2). The concept that a patient can only be considered cured of leukemia if every leukemia cell has been eradicated has evolved in recent years. We may cure CML patients, but we may never know whether all leukemic cells have been completely eradicated. In addition, we still do not know if leukemic stem cells (LSC) are really the true enemies because progression to AP may occur at the level of a more committed progenitor cell.[50] In spite of these considerations, if we want to decrease the rate of molecular recurrence after stopping TKI, we need to understand why quiescent LSCs are insensitive to TKIs, which is illustrated by the large number of publications focused on targeting the LSCs.[51,52] Compared to normal stem cells, LSC exhibit aberrant or non-regulated self-renewal, survival and dormancy (Figure 2). Several strategies have been proposed, including inhibiting survival/renewal pathways, sensitizing LSC (cycling or differentiating), immune targeting, or modifying the bone marrow niche (Figure 2). JAK/STAT, JAK2 kinase, the protein phosphatase 2A (PP2A), arachidonate 5-lipoxygenase gene (ALOX5), histone deacetylases (HDACs), Sirtuin 1 (SIRT1), and BCL6 are among the most relevant targets for such a strategy.[53-57] Two of the most important pathways for self-renewal of CML LSCs are the Wnt-b-catenin and the Hedgehog (Hh) pathways.[58,59] Targeting of the Hh pathway in solid tumors has been attempted by smoothened homolog (SMO) inhibitors.[60] Different Phase I or phase II trials have begun in CML with low level of residual disease in combination with TKI, but toxicity may inhibit the development of SMO inhibitors drugs for this indication.

Having shown that RAD52 is necessary to repair numerous reactive oxygen species (ROS)-induced DNA double-strand breaks (DSBs) in LSCs to promote leukemogenesis, Skorski and colleagues demonstrated the possibility of using this specific property to target LSC. Using peptide aptamer to target the DNA binding domain of RAD52, they reported a specific effect on CML LSC in mouse models.[61] One promising development is represented by new data which support selective targeting of LSC by small molecule antagonists of anti-apoptotic BCL2-family proteins.[62,63] It has been reported that LSCs from patients with advanced phase CML are quiescent,TKI-resistant in the marrow niche and Sabutoclax, a pan-BCL2 inhibitor, enhances TKI sensitivity of bone marrow BC LSCs.[64]

Other considerations and other questions for the future

Until recently, discontinuing imatinib has been limited to a few CML patients with undetectable BCR-ABL1 transcripts. In the STIM trial in France, we estimated that 10% of patients might be candidates for discontinuation of TKIs. We know that the depth of molecular response to imatinib increases with time. Recently, the Australian

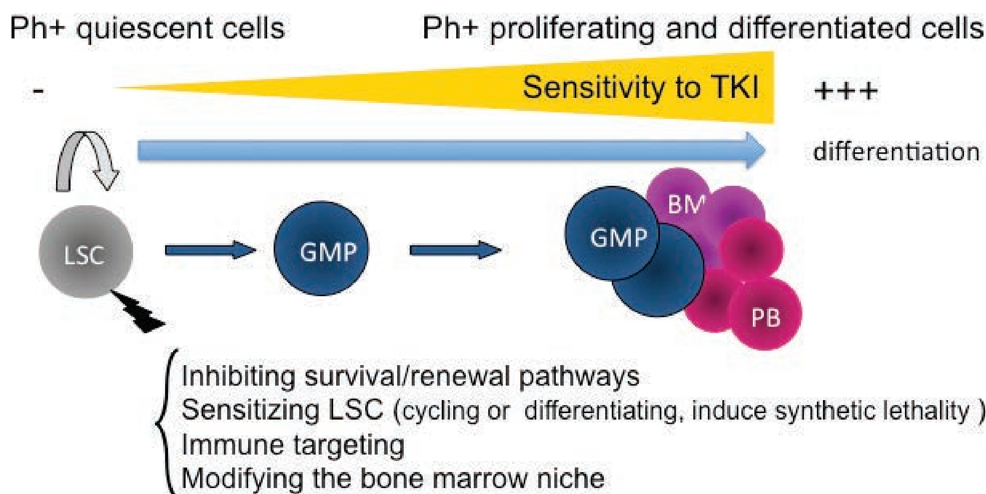

Figure 2. Strategies of CML stem cell targeting. LSC: Leukemic stem cell; GMP: granulocyte macrophage progenitor, BM Bone marrow differentiated cells; PB: peripheral blood cells.

group examined 415 patients with *de novo* CML in chronic phase enrolled in consecutive clinical trials of imatinib since July 2000, and estimated that after eight years of imatinib therapy the cumulative incidence of stable $UMR^{4.5}$ would be 43%.[29]

The achievement of deep molecular responses with 2nd generation TKI may provide a hope of increasing the number of candidate who can safely stop TKI. We still do not know if the rate of molecular relapse will be improved with 2nd generation TKI. Studies with less stringent definitions of molecular relapse have reported lower relapse rates so it is very important to use the same criteria to compare the different studies. Other questions remain, such as how can we treat molecular recurrence?

Legros and colleagues described 16 patients with CML in $MR^{4.5}$ to imatinib who underwent a second trial of treatment discontinuation after the first attempt had failed (i.e. they had experienced a molecular relapse and regained CMR).[65] Twelve of the patients had a rapid return of detectable disease and were successfully re-treated, 2 patients maintained CMR, and 2 had a rise of transcripts but maintained MMR. The kinetic of the first relapse generally differed to that of the second. The authors conclude that while the success rate of this maneuver is only 25% it is safe to discontinue therapy for a second time once CMR is re-established. It would be interesting to know what would happen if we treat patients with 2nd generation TKI for a second time. Such studies with nilotinib have started in France (NilopostSTIM).

The economic implications of TKI treatment cessation are very important. In the STIM, the savings were estimated to be over 5 million Euros at the latest analysis. A formal and rationally designed medico-economic study taking into account different aspects of dealing with CML as a chronic disease, including the quality-of-life parameters, is currently in progress.

Treatment-free remission for patients with CML is a new topic which has been developing over the last few years, re-enforcing the notion that CML is a model for other cancers and, more particularly, for hematologic disorders. While the subset of patients achieving molecular remission leading to cessation of treatment is heterogeneous, CML remains an extraordinary model both for raising awareness about 'curability' as well as thought provoking questions about medicine as a whole. What is the definition of 'cure'? Is it necessary to eradicate the last leukemic (stem) cell? Deciding to continue or discontinue therapy in patients with CML is a Shakespearean question reflecting what is in every patient's mind: to be or not to be cured. However, from a physician's perspective, "to thine own self be true" is truer more than ever.

References

1. National Comprehensive Cancer Network. Chronic Myelogenous Leukemia. NCCN Clinical Practice Guidelines in Oncology. 2012;v1.2013.
2. Baccarani M, Cortes J, Pane F, Niederwieser D, Saglio G, Apperley J, et al. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. J Clin Oncol .2009;27:6041-51.
3. Rea D, Rousselot P, Guilhot J, Guilhot F, Mahon FX. Curing chronic myeloid leukemia. Curr Hematol Malig Rep. 2012;7:103-8.
4. Rousselot P, Huguet F, Rea D, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. Blood. 2007;109:58-60.
5. Mahon FX. Is going for cure in chronic myeloid leukemia possible and justifiable? Hematology Am Soc Hematol Educ Program. 2012;2012:122-8.
6. Mahon FX, Rea D, Guilhot J, Guilhot F, Huguet F, Nicolini F, et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. Lancet Oncol. 2010;11:1029-35.
7. Mahon F, Rea D, Guilhot J, Guilhot F, Huguet F, Nicolini FE, et al. Discontinuation of Imatinib in Patients with Chronic Myeloid Leukemia Who Have Maintained Complete Molecular Response: Update Results of the STIM Study. ASH Annual Meeting Abstracts. Blood. 2011;118:603.
8. Ross DM, Branford S, Seymour JF, et al. Patients with chronic myeloid leukemia who maintain a complete molecular response after stopping imatinib treatment have evidence of persistent leukemia by DNA PCR. Leukemia. 2010;24:1719-24.
9. Ross DM, Branford S, Seymour JF, Arthur C, Schwarer AP, Dang P, et al. Frequent and sustained drug-free remission in the Australasian CML8 trial of imatinib withdrawal. Haematologica. 2012;97: 74.
10. Takahashi N, Kyo T, Maeda Y, Sugihara T, Usuki K, Kawaguchi T, et al. Discontinuation of imatinib in Japanese patients with chronic myeloid leukemia. Haematologica. 2012;97:903-6.
11. Rousselot P, Charbonnier A, Cony-Makhoul P, Agape P, Nicolini F, Varet B, et al. Loss of major molecular response is accurate for restarting imatinib after imatinib discontinuation in CP-CML patients with long lasting CMR: Importance of fluctuating values of MRD and interferon pre-treatment. Haematologica. 2012;97:77.
12. Matsuki E, Ono Y, Tonegawa K, Sakurai M, Kunimoto H, Ishizawa J, et al. Detailed Investigation On Characteristics of Japanese Patients with Chronic Phase CML Who Achieved a Durable CMR After Discontinuation of Imatinib – an Updated Result of the Keio STIM Study. ASH Annual Meeting Abstracts. Blood. 2012;120:2788.
13. Yhim HY, Lee NR, Song EK, Yim CY, Jeon SY, Shin S, et al. Imatinib mesylate discontinuation in patients with chronic myeloid leukemia who have received front-line imatinib mesylate therapy and achieved complete molecular response. Leuk Res. 2012;36:689-93.
14. Goh HG, Kim YJ, Kim DW, Kim HJ, Kim SH, Jang SE, et al. Previous best responses can be re-achieved by resumption after imatinib discontinuation in patients with chronic myeloid leukemia: implication for intermittent imatinib therapy. Leuk Lymphoma. 2009;50:944-51.
15. Lee S-, Choi SY, Bang J-, Kim S-, Jang E-, Choi M, et al. BCR-ABL kinetics after discontinuation of imatinib in CML patients with MR4.5 or undetectable molecular residual disease. Haematologica. 2012;97:80.
16. Cortes J, O'Brien S, Kantarjian H. Discontinuation of imatinib therapy after achieving a molecular response. Blood. 2004;104:2204-5.
17. Mauro MJ, Druker BJ, Marziaz RT. Divergent clinical outcome in two CML patients who discontinued imatinib therapy after achieving a molecular remission. Leuk Res. 2004;28S1:S71-S73.
18. Merante S, Orlandi E, Bernasconi P, et al. Outcome of four patients with chronic myeloid leukemia after imatinib mesylate discontinuation. Haematologica. 2005;90:979-81.
19. Michor F, Hughes TP, Iwasa Y, et al. Dynamics of chronic myeloid leukaemia. Nature. 2005;435:1267-70.
20. Stein AM, Bottino D, Modur V, et al. BCR-ABL transcript dynamics support the hypothesis that leukemic stem cells are reduced during imatinib treatment. Clin Cancer Res. 2011;21:6812-21.
21. Horn M, Glauche I, Müller MC, Hehlmann R, Hochhaus A, Loeffler M, et al. Model-based decision rules reduce the risk of molecular relapse after cessation of tyrosine kinase inhibitor therapy in chronic myeloid leukemia. Blood. 2013;121:378-84.
22. Mahon FX, personal communication.
23. Cross NCP, White H, Müller MC, Saglio G, Hochhaus A. Standardized definitions of molecular response in chronic myeloid leukemia. Leukemia. 2012;26(10):2172-5.
24. Branford S, Seymour JF, Grigg A, Arthur C, Rudzki Z, Lynch K, et al. BCR-ABL messenger RNA levels continue to decline in patients with chronic phase chronic myeloid leukemia treated with imatinib for more than 5 years and approximately half of all first-line treated patients have stable undetectable BCR-

ABL using strict sensitivity criteria. Clin Cancer Res. 2007;13:7080-5.

25. Melo JV, Ross DM. Minimal residual disease and discontinuation of therapy in chronic myeloid leukemia: can we aim at a cure? Hematology Am Soc Hematol Educ Program. 2011;2011:136-42.
26. Biernaux C, Loos M, Sels A, et al. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. Blood. 1995;8:3118-22.
27. Bose S, Deininger M, Gora-Tybor J, et al. The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. Blood. 1998;92:3362-7.
28. Deininger M. Hematology: curing CML with imatinib—a dream come true? Nat Rev Clin Oncol. 2011;3:127-8.
29. Branford S, Ross D, Prime J, Field C, Altamura H, Yeoman A, et al. Early molecular response and female sex strongly predict achievement of stable undetectable BCR-ABL1, a criterion for imatinib discontinuation in patients with CML. ASH Annual Meeting Abstracts. Blood. 2012;120.
30. Falchi L, Kantarjian HM, Quintas-Cardama A, O'Brien S, Jabbour EJ, Ravandi F, et al. Clinical significance of deeper molecular responses with four modalities of tyrosine kinase inhibitors as frontline therapy for chronic myeloid leukemia. ASH Annual Meeting Abstracts. Blood. 2012;120:164.
31. Mahon FX, Delbrel X, Cony-Makhoul P, et al. Follow-up of complete cytogenetic remission in patients with chronic myeloid leukemia after cessation of interferon alfa. J Clin Oncol. 2002;20:214-20.
32. O'Hare T, Zabriskie MS, Eiring AM, Deininger MW. Pushing the limits of targeted therapy in chronic myeloid leukaemia. Nat Rev Cancer. 2012;12:513-26.
33. Saglio G, Kim DW, Issaragrisil S, le Coutre P, Etienne G, Lobo C, et al. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. N Engl J Med. 2010;362:2251-9.
34. Kantarjian H, Shah NP, Hochhaus A, Cortes J, Shah S, Ayala M, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med. 2010;362:2260-70.
35. Cortes JE, Kim DW, Kantarjian HM, Brummendorf TH, Dyagil I, Griskevicus L, et al. Bosutinib Versus Imatinib in Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia: Results From the BELA Trial. J Clin Oncol. 2012;30:3486-92.
36. Kantarjian HM, Hochhaus A, Saglio G, De Souza C, Flinn IW, Stenke L, et al. Nilotinib versus imatinib for the treatment of patients with newly diagnosed chronic phase, Philadelphia chromosome-positive, chronic myeloid leukaemia: 24-month minimum follow-up of the phase 3 randomised ENESTnd trial. Lancet Oncol. 2011;12:841-51.
37. Kantarjian HM, Shah NP, Cortes JE, Baccarani M, Agarwal MB, Undurraga MS, et al. Dasatinib or imatinib in newly diagnosed chronic phase chronic myeloid leukemia: 2-year follow-up from a randomized phase 3 trial (DASISION). Blood. 2012;119:1123-9.
38. Rosti G, Palandri F, Castagnetti F, Breccia M, Levato L, Gugliotta G, et al. Nilotinib for the frontline treatment of Ph(+) chronic myeloid leukemia. Blood. 2009;114:4933-8.
39. Gugliotta G, Castagnetti F, Breccia M, Levato L, Capucci A, Tiribelli M, et al. Early CP CML, Nilotinib 400 mg Twice Daily Frontline: Beyond 3 Years, Results Remain Excellent and Stable (A GIMEMA CML Working Party Trial). Blood. 2011;116.
40. Cortes JE, Jones D, O'Brien S, Jabbour E, Konopleva M, Ferrajoli A, et al. Nilotinib as front-line treatment for patients with chronic myeloid leukemia in early chronic phase. J Clin Oncol. 2010;28:392-7.
41. Cortes JE, Jones D, O'Brien S, Jabbour E, Ravandi F, Koller C, et al. Results of dasatinib therapy in patients with early chronic-phase chronic myeloid leukemia. J Clin Oncol. 2010;28: 398-404.
42. Hochhaus A, Rosti G, le Coutre PD, Ossenkoppele G, Griskevicius L, Rea D, et al. Nilotinib in patients with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP): A European and EUTOS clinical initiative for standardization of molecular response. Haematologica. 2012;97:74.
43. Hughes TP, Lipton JH, Leber B, Spector N, Cervantes F, Pasquini R, et al. Complete molecular response (CMR) rate with nilotinib in patients (pts) with chronic myeloid leukemia in chronic phase (CML-CP) without CMR after >= 2 years on imatinib: preliminary results from the randomized ENESTcmr trial of nilotinib 400 mg twice daily (BID) vs imatinib. Blood. 2011;118:278.
44. Hughes TP, Lipton JH, Spector N, Leher B, Pasquini R, Clementino N, et al. Switching to nilotinib is associated with continued deeper molecular responses in CML-CP patients with minimal residual disease after ≥ 2 years on imatinib: ENESTcmr 2-year follow-up results. ASH Annual Meeting Abstracts. Blood. 2012;2012:694.
45. Ross DM, Bartley PA, Goyne J, Morley AA, Seymour JF, Grigg AP. Durable complete molecular remission of chronic myeloid leukemia following dasatinib cessation, despite adverse disease features. Haematologica. 2011;96:1720-2.
46. Rea D, Rousselot P, Guilhot F, Tulliez M, Nicolini FE, Guerci-Bresler A, et al. Discontinuation of second generation (2G) tyrosine kinase inhibitors (TKI) in chronic phase (CP)-chronic myeloid leukemia (CML) patients with stable undetectable BCR-ABL transcripts. ASH Annual Meeting Abstracts. Blood. 2012;120:916.
47. Preudhomme C, Guilhot J, Nicolini FE, Guerci-Bresler A, Rigal-Huguet F, Maloisel F, et al. Imatinib plus peginterferon alfa-2a in chronic myeloid leukemia. N Engl J Med. 2010; 363:2511-21.
48. Burchert A, Wolfl S, Schmidt M, et al. Interferon-alpha, but not the ABL-kinase inhibitor imatinib (STI571), induces expression of myeloblastin and a specific T-cell response in chronic myeloid leukemia. Blood. 2003;101259-64.
49. Nicolini FE, Etienne G, Dubruille V, Roy L, Huguet F, Legros L, et al. Pegylated interferon-α 2a in combination to nilotinib as first line therapy in newly diagnosed chronic phase chronic myelogenous leukemia provides high rates of MR4.5. Preliminary results of a phase II study. ASH Annual Meeting Abstracts. Blood. 2012;120:166.
50. Jamieson CH, Ailles LE, Dylla SJ, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. New Eng J Med. 2004;351:657-667
51. Gallipoli P, Abraham SA, Holyoake TL. Hurdles toward a cure for CML: the CML stem cell. Hematol Oncol Clin North Am.2011;25:951-966.
52. Rea D, Rousselot P, Guilhot J, et al. Curing chronic myeloidleukemia. Curr Hematol Malig Rep. 2012;7:103-8.
53. Hantschel O, Warsch W, Eckelhart E, et al. BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. Nat Chem Biol. 2012;8:285-293.
54. Chen Y, Peng C, Sullivan C, et al. Novel therapeutic agents against cancer stem cells of chronic myeloid leukemia. Anticancer Agents Med Chem. 2010;10:111-5.
55. Zhang B, Strauss AC, Chu S, et al. Effective targeting of quiescent chronic myelogenous leukemia stem cells by histone deacetylase inhibitors in combination with imatinib mesylate. Cancer Cell. 2010;17:427-42.
56. Li L, Wang L, Li L, et al. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell. 2012;21:266-81.
57. Hurtz C, Hatzi K, Cerchietti L, et al. BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia. J Exp Med. 2011;208:2163-74
58. Kleppe M, Levine RL. Targeting beta-catenin in CML: leukemia stem cells beware! Cell Stem Cell. 2012;10:351-3.
59. Dierks C, Beigi R, Guo GR, et al. Expansion of Bcr-Ablpositive leukemic stem cells is dependent on Hedgehog pathway activation. Cancer Cell. 2008;14:238-49.
60. Von Hoff DD, LoRusso PM, Rudin CM, et al. Inhibition of the hedgehog pathway in advanced basal cell carcinoma. N Engl J Med. 2009;361:1164-72.
61. Cramer K, Nieborowska-Skorska M, Slupianek A, Sliwinski T, Irvine D, Copland M, et al. TRAD52-Dependent Synthetic Lethality Eradicates Leukemia Stem Cells. ASH Annual Meeting Abstracts. Blood. 2012;120.
62. Hogdal LJ, Letai A. BCL-2 inhibition: stemming the tide of myelod malignancies. Cell Stem Cell. 2013;12:269-70.
63. Airiau K, Mahon F.X, Josselin M, Jeanneteau M, Turcq B, Belloc F. ABT-737 increases tyrosine kinase inhibitor-induced apoptosis in chronic myeloid leukemia cells through XIAP downregulation and sensitizes CD34(+) CD38(-) population to imatinib. Exp Hematol. 2012, 5:367-78.
64. Goff DJ, et al. A Pan-BCL2 Inhibitor Renders Bone-Marrow-Resident Human Leukemia Stem Cells Sensitive to Tyrosine Kinase Inhibition. Cell Stem Cell. 2013;12:316-28.
65. Legros L, Rousselot P, Giraudier S, Tulliez M, Huguet F, Nicolini FE, et al. Second attempt to discontinue imatinib in CP-CML patients with a second sustained complete molecular response. Blood. 2012;120:1959-60.

Molecular pathology of diffuse large B-cell lymphomas

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A B S T R A C T

Diffuse large B-cell lymphomas (DLBCL) are clinically and biologically heterogeneous. Despite improved therapeutic options, a significant proportion of DLBCL patients cannot be cured with current treatment regimens. On the molecular basis, several DLBCL subtypes can be discerned including the germinal center B-like (GCB) and activated B-like (ABC) DLBCL. GCB and ABC DLBCL are associated with varying underlying genetic alterations. GCB DLBCL carry BCL2 translocations in a subset of cases as well as alterations affecting the PTEN/PI3K pathway, whereas BCL6 translocations, deletions of the CDKN2A locus and deregulation of PRDM1 are more frequent in ABC DLBCL. Activation of NFkB, a hallmark feature of ABC DLBCL, is accomplished through mutations of positive and negative regulators of this pathway (A20, TRAF2, TRAF5) and through deregulation of Toll-like receptor signaling (MyD88 mutations). CARD11 mutations as well as mutations affecting B-cell receptor signaling also contribute to the pathogenesis of DLBCL. Next-generation sequencing studies highlight a novel pathogenetic aspect in DLBCL, the perturbation of chromatin biology, since frequent mutations in MLL2, CREBBP and MEF2B affect epigenetic features (methylation, acetylation). Finally, deregulation of MYC on the genetic or protein level plays an important role in a subset of DLBCL and is associated with an inferior prognosis.

Learning goals

At the conclusion of this activity, participants should:
- be aware of the complexity of the molecular pathogenesis of diffuse large B-cell lymphomas (DLBCL);
- be aware of the most frequent genetic alterations, mutations and some of the affected molecular pathways in DLBCL;
- be aware of the role of MYC deregulation in DLBCL.

Introduction

Diffuse large B-cell lymphomas (DLBCL) constitute the most common type of adult non-Hodgkin's lymphomas (NHL) and account for 30-40% of cases.[1] When discussing the molecular pathology of DLBCL, it is important to realize that the World Health Organization (WHO) classification defines a relatively large number of diverse DLBCL subgroups that all differ with regard to their morphological, genetic, immunophenotypic and, importantly, also their clinical features. For example, T-cell/histiocyte-rich large B-cell lymphomas, cutaneous forms of DLBCL, central nervous system (CNS) DLBCL, EBV-associated DLBCL of the elderly or plasmablastic lymphomas all have their own morphological characteristics, differ in underlying biological, immunophenotypic and molecular features, and present in different clinical settings.[1] Thus, all of these subgroups have their 'own molecular pathology'. Moreover, the biological and clinical borders between DLBCL and Burkitt's lymphoma (BL) and between DLBCL and classic Hodgkin's lymphoma (cHL) are not clear resulting in provisional gray zone categories termed B-cell lymphoma unclassifiable with features intermediate between DLBCL and Burkitt's lymphoma and B-cell lymphoma unclassifiable with features intermediate between DLBCL and Hodgkin's lymphoma, respectively.[2] Nevertheless, the vast majority of DLBCL belong to the category of DLBCL not otherwise specified (NOS) and this subgroup will be the subject of this educational review.

DLBCL NOS, however, are not a homogeneous lymphoma entity which is highlighted by the fact that DLBCL remains incurable in at least 30% of patients, while in the remainder a long-term remission or cure can be achieved. This clinical heterogeneity is attributed to the diversity of underlying genetic and molecular features of this neoplasm that, at present, cannot easily be recognized in the routine diagnostic setting at the time of diagnosis.

This review will summarize genetic features of DLBCL, the current view on the gene expression-based distinction into the germinal center B-cell like (GCB) and activated B-cell like (ABC) DLBCL, the role of the NFkB-pathway, and results from recent, high-throughput next-generation sequencing studies.

Basic genetic alterations in DLBCL

During various stages of B-cell development, DNA modifications of the genes that encode the variable regions of the heavy and light chains forming part of the B-cell receptor (BCR) occur that enhance the risk of the introduction of potentially oncogenic DNA recombinations. Specifically, the recombination activating genes 1 and 2 (RAG1, RAG 2) are involved in the V(D)J recombination process of the heavy chain genes at early stages of B-cell development by inducing double strand breaks.[3,4] During the germinal center reaction, a specific enzyme termed activation-induced cytidine deaminase (AID) mediates the somatic hypermutation (SHM) and class switch recombination (CSR) processes that lead to increased affinity of produced antibodies.[5,6] Errors in these DNA recombination processes are thought to result in several recurrent chromosomal translocations that can be found in DLBCL. The translocation t(14;18), the hallmark translocation of follicular lymphoma leading to upregulation of BCL2, can be detected in approximately 20% of DLBCL[7] and occurs almost exclusively in the GCB DLBCL subtype.[8] Less than 10% of DLBCL carry the Burkitt's-typical translocation t(8;14) involving the MYC oncogene.[7,9,10] The most common translocation in DLBCL involves BCL6 (30% of DLBCL).[7,11] Of note, this translocation is more frequent in the ABC DLBCL subtype.[12] In these translocation events, the BCL6 locus is juxtaposed downstream of alternative promoters or enhancers, usually including the immunoglobulin loci, that prevent the physiological downregulation of BCL6 when germinal center B cells exit the germinal center reaction to mature into plasma cells. Thus, deregulated expression of BCL6 is believed to block terminal B-cell differentiation and to maintain a pro-proliferative phenotype of the neoplastic B-cell clone that is characteristic of normal germinal center B cells.[7] Accordingly, in a mouse model, deregulated BCL6 expression leads to a B-cell lymphoma that appears to recapitulate the pathogenesis of human DLBCL.[13] Additional genetic alterations that are repeatedly detected in DLBCL include amplifications of the *BCL2* locus (18q21), amplification of the *REL* locus (2p13), homozygous *CDKN2A* deletions, gains of the *SPIB* locus (19q13), PRDM1 (Blimp-1) mutations/deletions and trisomies of chromosome 3.[12,14-18] PRDM1 mutations or deletions affect approximately 25% of ABC DLBCL, as do SPIB gains/amplifications. Since PRDM1 is a master regulator of plasma cell differentiation and SPIB represses PRDM1, both genetic events lead to a block of terminal plasma cell differentiation in the neoplastic DLBCL cells thus contributing significantly to the pathogenesis.

Gene expression profiling

Analyses of global gene expression profiles have defined two major subtypes of DLBCL, the germinal center B-cell like (GCB) and the activated B-cell like (ABC) subtypes.[19] GCB and ABC DLBCL are molecularly distinct and their gene expression profiles suggest that they are pathogenetically linked to different stages of B-cell differentiation. Specifically, GCB DLBCL expresses many genes that are physiologically expressed in normal germinal center B cells including CD10 and BCL6. Moreover, the process of somatic hypermutation of the immunoglobulin genes appears to be ongoing in GCB DLBCL that constitutes another characteristic feature of germinal center B cells.[20] In contrast, ABC DLBCL displays a gene expression phenotype that is closely linked to a post-germinal center stage of B-cell differentiation. In this subtype, key regulators of plasma cell differentiation including IRF4 and XBP-1 are highly expressed, and the potent oncogenic NFkB pathway is activated (*see below*).[19] There is no ongoing somatic hypermutation of the immunoglobulin genes and immunoglobulin class switch recombination shows aberrant features.[21]

It is important to note that the gene expression-based distinction into GCB and ABC DLBCL is supported by several other genetic and molecular features. As mentioned before, the translocation t(14;18) involving BCL2 occurs almost exclusively in GCB DLBCL.[14] Likewise, gains or amplifications of the REL locus in 2p, a feature of germinal center-derived lymphomas, are also predominantly detected in the GCB DLBCL subtype.[14] Genetic alterations involving the phosphatase and tensin homolog (PTEN) pathway also preferentially affect the GCB DLBCL subtype. Approximately 10% of GCB DLBCL show genomic deletions in the PTEN locus and a negative regulator of PTEN, the microRNA (miR)-17-92, is amplified at the genomic level in another 10-15% of this subgroup. Taken together, the PTEN- and, therefore, the phosphatidylinositol 3-kinase (PI3K)-pathway appears to be deregulated in roughly 30% of GCB DLBCL.[16] Finally, recent high throughput sequencing studies identified somatic mutations of the histone methyltransferase EZH2 in a small subset of follicular lymphomas, but also in a considerable proportion (~20%) of GCB DLBCL.[22] EZH2, the catalytic subunit of the polycomb repressive complex 2, plays a role in the regulation of the chromatin structure and, thus, gene expression by catalyzing trimethylation of histone H3 lysine 27 (H3K27). Subsequently, point mutations in EZH2 in a subset of GCB DLBCL lead to H3K27 hypertrimethylation. Remarkably, recent reports suggest that small molecule inhibitors of EZH2 may reduce H3K27 hypertrimethylation in various lymphoma cell lines and inhibit the growth of EZH2-mutated cell lines or DLBCL xenografts in mice.[23,24]

ABC DLBCL is also characterized by distinct genetic abnormalities. While BCL2-translocations, as mentioned before, are a hallmark feature of GCB DLBCL, gains or amplifications of the BCL2 locus in 18q are observed in approximately 30% of ABC DLBCL. The *CDKN2A* (*INK4A-ARF*) locus is homozygously deleted in a subset of ABC DLBCL, and gains/amplifications of SPIB, a member of the E26 transformation-specific family of transcription factors, in concert with deregulation of PRDM1 (Blimp-1) contributes to the pathogenesis of ABC DLBCL by blocking terminal differentiation.[16] The important role of the NFkB-pathway in ABC DLBCL will be discussed in a separate paragraph (*see below*).

The aforementioned biological and genetic features that distinguish GCB and ABC DLBCL also influence the clinical course of DLBCL patients. By multi-agent chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone, CHOP), but also with combined immunochemotherapy including the anti-CD20 antibody rituximab (R-CHOP), the overall survival of GCB

DLBCL patients is much more favorable compared to ABC DLBCL patients and, even with current treatment regimens, 50% of ABC DLBCL patients will succumb to their disease.[14,19,25] Thus, innovative therapeutic approaches in DLBCL will have to specifically address the comparably poor clinical outcome of ABC DLBCL patients. It should be noted that gene expression profiling studies also revealed an important role for the composition of the microenvironment in predicting survival times. Specifically, stromal signatures encompassing gene expression of macrophages, extracellular matrix deposition and endothelial/vascular elements, as well as a host of inflammatory responses, are associated with clinical outcome in DLBCL patients.[25,26]

Given the fundamental differences in the biology of GCB and ABC DLBCL with strong clinical impact on overall survival, many attempts have been made to establish a robust immunohistochemical test that can be reliably used in everyday practice. Since the description of the Hans-classifier in 2004[27] that uses antibodies against CD10, BCL6 and MUM1/IRF-4 to distinguish between GCB and non-GCB DLBCL, this algorithm, with or without modifications, has been applied in many DLBCL cohorts treated in the CHOP and R-CHOP treatment eras with vastly different results. While some studies establish an association between an immunohistochemical algorithm and clinical outcome, others fail to do so.[28-41] Thus, eight years after the initial description of the Hans algorithm, one has to conclude that we still have no robust immunohistochemistry-based classifier that can reliably identify the gene expression-based GCB and ABC DLBCL subgroups. This may, at least in part, be due to the, at most, semi-quantitative nature of immunohistochemical approaches (in contrast to more quantitative measurements of gene expression in array-based technologies) and intra- and inter-laboratory technical variations in the measurement of the antibodies used.[42] Interestingly, a simple morphological feature, namely the immunoblastic appearance of DLBCL tumor cells, has been associated with inferior outcome in DLBCL.[41]

The role of NFkB in DLBCL

As mentioned above, constitutive activation of the NFkB-pathway is a hallmark feature of ABC DLBCL, and a large set of NFkB target genes is highly expressed in this subtype.[43] The NFkB family of transcription factors includes five members (*NFKB1*, *NFKB2*, *RELA*, *RELB* and *REL*). In quiescent B cells, NFkB transcription factors are located in the cytoplasm and retained by an inhibitor termed IkBα. Upon stimulation of the B-cell receptor (BCR), NFkB translocates to the nucleus thereby up-regulating expression of a large number of target genes including pro-survival genes such as BCL-XL, c-IAP1, c-IAP2 and c-FLIP.[44] Moreover, one of the master regulators of plasma cell differentiation, IRF-4,[45] is highly expressed in ABC DLBCL as a result of NFkB activation.

A number of genetic alterations directly affect positive or negative regulators of NFkB thus explaining constitutive activation of this signaling pathway. Specifically, the TNFAIP3 gene, a negative regulator of NFkB encoding for A20, shows biallelic mutations or deletions in a significant subset of ABC DLBCL.[46,47] Likewise, a variety of other components of NFkB can be affected on the genetic level including TRAF2, TRAF5, MAP3K7 (TAK1) and TNFRSF11A (RANK).[46]

Additional major insights into mechanisms leading to constitutive NFkB activation in ABC DLBCL were obtained by shRNA library screens aiming to identify potential oncogenes. One such screen identified the CBM complex consisting of CARD11, MALT1 and BCL10 as essential for the survival of cell lines derived from ABC DLBCL tumors, and subsequent resequencing revealed mutations of CARD11 in approximately 10% of ABC DLBCL.[48] While CARD11 is inactive in resting B cells, it becomes phosphorylated upon BCR-crosslinking and presumably stabilizes the CBM complex, thereby inducing downstream activation of NFkB. Mutations of CARD11 that preferentially occur in the coiled-coil domain of the gene are gain-of-function in nature and exert oncogenic effects.[48]

In a subset of ABC DLBCL tumors, mutations affecting components of the B-cell receptor (BCR) itself were described leading to abnormal BCR signaling termed 'chronic active BCR signaling'.[49] Specifically, mutations in the two BCR signaling subunits CD79A and CD79B that can be detected in approximately 20% of ABC DLBCL contribute to enhanced expression of the BCR by diminishing negative regulation by the Lyn kinase.[49] As a result, 'chronic active' signaling through the BCR leads to enhanced downstream NFkB activation.

Finally, it has been demonstrated that aberrant Toll-like receptor (TLR) signaling contributes to NFkB activation in ABC DLBCL.[50] TLR family members are part of the innate immune system and include receptors that recognize structurally conserved molecules from external pathogens. Cytoplasmic adapters that further transmit receptor signals include MyD88, TIRAP and TRF, amongst others.[51] Twenty-nine percent of ABC DLBCL carry mutations in MyD88, most commonly at the position 265 (L265P). Functional studies demonstrated the oncogenic potential of this mutation that induces NFkB, likely through the involvement of the kinases IRAK4 and IRAK1.[50]

Results of recent high-throughput sequencing approaches

With the availability of current next-generation sequencing technologies, it has become possible to define the genomic landscape of DLBCL tumors on the global scale.[52-56] These studies have yielded important novel information that underlines the complexity and heterogeneity of the biology of DLBCL. Morin and colleagues[53] performed genome/exome sequencing of 13 DLBCL cases and RNA-sequencing of an additional 83 DLBCL. While previously identified recurrent mutations in DLBCL (see above) were easily identified by this approach, the perturbation of chromatin biology emerged as a novel aspect of lymphomagenesis in DLBCL. Specifically, an increased number of somatic mutations in genes with a role in histone modification were detected. These include frequent mutations in the histone methyltransferase MLL2 that showed mutations in 32% of investigated DLBCL. In addition, 11% of DLBCL carried mutations in MEF2B that encodes for a transcription factor involved in the

acetylation of histones. MLL2 mutations were also detected in 24% of DLBCL studied in another series.[54] This study also provided an estimate of the average number of numerical and structural genomic alterations in DLBCL (combining non-silent somatic mutations, copy number gains/losses and translocations per DLBCL) that appears to be relatively low (around 50) compared to, for example, many solid cancers.[54] The histone and non-histone acetyltransferases CREBBP and EP300 are also affected by mutations or genomic deletions in a significant subset of DLBCL, as shown by Pasqualucci and co-workers.[56] The authors provide evidence that impaired inactivation of BCL6 by acetylation or defective activation of the tumor suppressor p53 likely contributes to the pathogenesis of DLBCL.[56] A novel class of drugs that target epigenetic modulation of genes including methylation and acetylation/deacetylation might, therefore, hold promise in future therapeutic approaches in DLBCL patients. Additional DLBCL sequencing results are available from two very recent studies.[52,55] Lohr and colleagues performed whole-exome sequencing of 55 DLBCL cases confirming previously reported and functionally relevant mutations in MYD88, CARD11, EZH2, CREBBP, MLL2 and MEF2B. However, they also identified a large number of additional, yet infrequent mutations some of which might be of functional relevance (NOTCH1, BRAF).[52] The report by Zhang and co-workers[55] extends these findings in a series of 73 DLBCL tumors. Interestingly, occasional mutations in PIK3CD appear to dysregulate the PI3 kinase pathway, as might do rare mutations in the PI3KR1 and MTOR genes. In conclusion, while some mutations in DLBCL appear to occur at higher frequencies, there is an enormous variation of mutated genes between different individuals with DLBCL, showing the extensive underlying genetic complexity of this disease.

The role of MYC in DLBCL

MYC, a nuclear phosphoprotein, is a global transcription factor affecting gene expression of a large number of genes in the genome and constitutes one of the most important proto-oncogenes in cancer in general.[57,58] Thus MYC, not surprisingly, also plays a major role in lymphomagenesis.[59] Translocation of MYC, usually through the translocation t(8;14)(q24;q32), is the hallmark genetic alteration of Burkitt's lymphoma, but MYC deregulation is considered an important aspect of the pathogenesis in a subset of DLBCL as well. MYC translocations have been described to occur in approximately 10% of DLBCL in various patient cohorts.[10,60-66] It is noteworthy that, in roughly 30% of MYC-translocated DLBCL, MYC is rearranged with a non-Ig partner, for example BCL6, PAX5 and IKAROS.[63,67] The presence of MYC translocations in DLBCL was associated with inferior survival times in the CHOP-like treatment era,[61,68,69] but more recently, the negative prognostic impact of MYC-rearrangements has also been demonstrated in DLBCL patients treated with R-CHOP.[10,60,64,70] In the largest cohort reported to date, including 407 lymphoma specimens from DLBCL patients treated prospectively in the RICOVER-60 trial of the German High Grade Non-Hodgkin Lymphoma Study Group (DSHNHL), 8.8% of the investigated tumors carried an MYC-rearrangement, and multivariate analysis identified this feature as a significant genetic factor influencing clinical outcome.[10] It is also evident, however, that MYC protein in DLBCL can be significantly up-regulated by mechanisms other than translocations, since with a newly available MYC antibody[71] high MYC expression on the protein level can be detected in a subset of DLBCL cases lacking an MYC-translocation.[10,72-74] Several studies have shown that high MYC expression on the protein level, independent from an underlying MYC-rearrangement on the genetic level, is highly predictive of outcome in DLBCL patients.[10,72-74] Thus, measurement of MYC expression using immunohistochemistry, in concert with the evaluation of additional markers (e.g. BCL2 expression[73] or in a model combining MYC/BCL6/BCL2 expression plus MYC translocation[10]) might become a useful adjunct in the diagnostic workup of DLBCL tumors in the future (Figure 1). Additional studies will also have to clarify the underlying cause of MYC upregulation in DLBCL tumors that do not carry an MYC rearrangement.

Conclusion

The complexity of the pathogenesis of DLBCL, that has been evident for a long time from morphological and genetic features, as well as from gene expression profiling studies, is also highlighted by results from a larger number of sequenced genomes, exomes and transcriptomes of DLBCL tumors. At the same time, the overall picture of the landscape of genomic alterations in DLBCL becomes more and more complete. Even though there is a wide variation of mutated genes between individuals with DLBCL, new aspects of the pathogenesis of DLBCL have

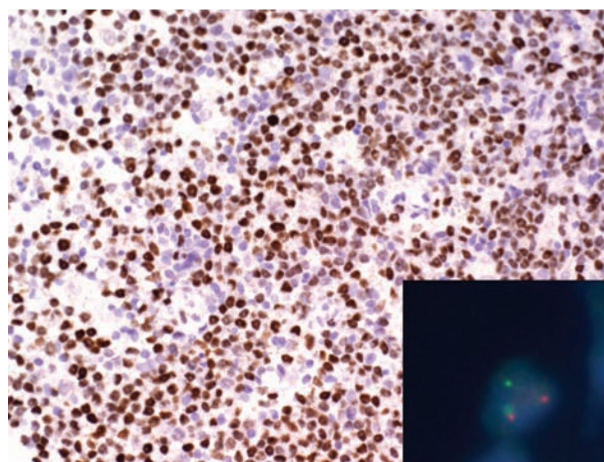

Figure 1. Diffuse large B-cell lymphoma showing strong MYC expression in the majority of tumor cells by immunohistochemistry (400x). In this case, upregulation of MYC expression is the result of an underlying MYC-translocation. (Inset) Fluorescence *in situ* hybridization (FISH) using an MYC break apart-assay showing a fusion signal (normal MYC locus) and a split signal (translocated MYC allele). MYC protein expression can, however, also occur without the presence of an MYC-translocation.

come to light, e.g. the relevance of epigenetic features that appear perturbed by mutations in genes affecting methylation and acetylation. These findings offer new opportunities to employ innovative therapeutic agents in DLBCL that have not been considered previously in this lymphoma subtype.

References

1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri S, Stein H, et al. (eds.) WHO classification of tumors of haematopoietic and lymphoid tissues. Lyon, IARC, 2008.
2. Menon MP, Pittaluga S, Jaffe ES. The histological and biological spectrum of diffuse large B-cell lymphoma in the World Health Organization classification. Cancer J. 2012;18:411-20.
3. Fugmann SD, Lee AI, Shockett PE, et al. The RAG proteins and V(D)J recombination: complexes, ends, and transposition. Annu Rev Immunol. 2000;18:495-527.
4. Rajewsky K. Clonal selection and learning in the antibody system. Nature. 1996;381:751-8.
5. Goossens T, Klein U, Kuppers R. Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. Proc Natl Acad Sci USA. 1998;95:2463-8.
6. Muramatsu M, Kinoshita K, Fagarasan S, et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000;102:553-63.
7. Lossos IS. Molecular pathogenesis of diffuse large B-cell lymphoma. J Clin Oncol. 2005;23:6351-7.
8. Huang JZ, Sanger WG, Greiner TC, et al. The t(14;18) defines a unique subset of diffuse large B-cell lymphoma with a germinal center B-cell gene expression profile. Blood. 2002;99:2285-90.
9. Akasaka T, Akasaka H, Ueda C, et al. Molecular and clinical features of non-Burkitt's, diffuse large-cell lymphoma of B-cell type associated with the c-MYC/immunoglobulin heavy-chain fusion gene. J Clin Oncol. 2000;18:510-8.
10. Horn H, Ziepert M, Becher C, et al. MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. Blood. 2013 Jan 18 [Epub ahead of print].
11. Offit K, Lo Coco F, Louie DC, et al. Rearrangement of the bcl-6 gene as a prognostic marker in diffuse large-cell lymphoma. N Engl J Med. 1994;331:74-80.
12. Iqbal J, Greiner TC, Patel K, et al. Distinctive patterns of BCL6 molecular alterations and their functional consequences in different subgroups of diffuse large B-cell lymphoma. Leukemia. 2007;21:2332-43.
13. Cattoretti G, Pasqualucci L, Ballon G, et al. Deregulated BCL6 expression recapitulates the pathogenesis of human diffuse large B cell lymphomas in mice. Cancer Cell. 2005; 7:445-55.
14. Rosenwald A, Wright G, Chan WC, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002;346:1937-47.
15. Bea S, Zettl A, Wright G, et al. Diffuse large B-cell lymphoma subgroups have distinct genetic profiles that influence tumor biology and improve gene-expression-based survival prediction. Blood. 2005;106:3183-90.
16. Lenz G, Wright GW, Emre NC, et al. Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. Proc Natl Acad Sci USA. 2008;105:13520-5.
17. Pasqualucci L, Compagno M, Houldsworth J, et al. Inactivation of the PRDM1/BLIMP1 gene in diffuse large B cell lymphoma. J Exp Med. 2006;203:311-7.
18. Nogai H, Dorken B, Lenz G. Pathogenesis of non-Hodgkin's lymphoma. J Clin Oncol. 2011;29:1803-11.
19. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000;403:503-11.
20. Lossos IS, Alizadeh AA, Eisen MB, et al. Ongoing immunoglobulin somatic mutation in germinal center B cell-like but not in activated B cell-like diffuse large cell lymphomas. Proc Natl Acad Sci USA. 2000;97:10209-13.
21. Lenz G, Nagel I, Siebert R, et al. Aberrant immunoglobulin class switch recombination and switch translocations in activated B cell-like diffuse large B cell lymphoma. J Exp Med. 2007;204:633-43.
22. Morin RD, Johnson NA, Severson TM, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat Genet. 2010;42:181-5.
23. Knutson SK, Wigle TJ, Warholic NM, et al. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. Nat Chem Biol. 2012;8:890-6.
24. McCabe MT, Ott HM, Ganji G, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature. 2012;492:108-12.
25. Lenz G, Wright G, Dave SS, et al. Stromal gene signatures in large-B-cell lymphomas. N Engl J Med. 2008;359:2313-23.
26. Monti S, Savage KJ, Kutok JL, et al. Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. Blood. 2005;105:1851-61.
27. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004;103:275-82.
28. Amen F, Horncastle D, Elderfield K, et al. Absence of cyclin-D2 and Bcl-2 expression within the germinal centre type of diffuse large B-cell lymphoma identifies a very good prognostic subgroup of patients. Histopathology. 2007;51:70-9.
29. Berglund M, Thunberg U, Amini RM, et al. Evaluation of immunophenotype in diffuse large B-cell lymphoma and its impact on prognosis. Mod Pathol. 2005;18:1113-20.
30. Colomo L, Lopez-Guillermo A, Perales M, et al. Clinical impact of the differentiation profile assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. Blood. 2003;101:78-84.
31. De Paepe P, Achten R, Verhoef G, et al. Large cleaved and immunoblastic lymphoma may represent two distinct clinicopathologic entities within the group of diffuse large B-cell lymphomas. J Clin Oncol. 2005;23:7060-8.
32. Dupuis J, Gaulard P, Hemery F, et al. Respective prognostic values of germinal center phenotype and early (18)fluorodeoxyglucose-positron emission tomography scanning in previously untreated patients with diffuse large B-cell lymphoma. Haematologica. 2007;92:778-83.
33. Haarer CF, Roberts RA, Frutiger YM, et al. Immunohistochemical classification of de novo, transformed, and relapsed diffuse large B-cell lymphoma into germinal center B-cell and nongerminal center B-cell subtypes correlates with gene expression profile and patient survival. Arch Pathol Lab Med. 2006;130:1819-24.
34. Meyer PN, Fu K, Greiner TC, et al. Immunohistochemical methods for predicting cell of origin and survival in patients with diffuse large B-cell lymphoma treated with rituximab. J Clin Oncol. 2011;29:200-7.
35. Muris JJ, Meijer CJ, Vos W, et al. Immunohistochemical profiling based on Bcl-2, CD10 and MUM1 expression improves risk stratification in patients with primary nodal diffuse large B cell lymphoma. J Pathol. 2006;208:714-23.
36. Natkunam Y, Farinha P, Hsi ED, et al. LMO2 protein expression predicts survival in patients with diffuse large B-cell lymphoma treated with anthracycline-based chemotherapy with and without rituximab. J Clin Oncol. 2008;26:447-54.
37. Nyman H, Adde M, Karjalainen-Lindsberg ML, et al. Prognostic impact of immunohistochemically defined germinal center phenotype in diffuse large B-cell lymphoma patients treated with immunochemotherapy. Blood. 2007;109:4930-5.
38. Sjo LD, Poulsen CB, Hansen M, et al. Profiling of diffuse large B-cell lymphoma by immunohistochemistry: identification of prognostic subgroups. Eur J Haematol. 2007;79:501-7.
39. van Imhoff GW, Boerma EJ, van der Holt B, et al. Prognostic impact of germinal center-associated proteins and chromosomal breakpoints in poor-risk diffuse large B-cell lymphoma. J Clin Oncol. 2006;24:4135-42.
40. Veelken H, Vik Dannheim S, Schulte Moenting J, et al. Immunophenotype as prognostic factor for diffuse large B-cell lymphoma in patients undergoing clinical risk-adapted therapy. Ann Oncol. 2007;18:931-9.
41. Ott G, Ziepert M, Klapper W, et al. Immunoblastic morphology but not the immunohistochemical GCB/nonGCB classifier predicts outcome in diffuse large B-cell lymphoma in the RICOVER-60 trial of the DSHNHL. Blood. 2010;116:4916-25.
42. de Jong D, Rosenwald A, Chhanabhai M, et al. Immunohistochemical prognostic markers in diffuse large B-cell lymphoma: validation of tissue microarray as a prerequisite for broad clinical applications—a study from the Lunenburg Lymphoma Biomarker Consortium. J Clin Oncol. 2007;25:805-12.

43. Davis RE, Brown KD, Siebenlist U, et al. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med. 2001;194:1861-74.
44. Lam LT, Wright G, Davis RE, et al. Cooperative signaling through the signal transducer and activator of transcription 3 and nuclear factor-{kappa}B pathways in subtypes of diffuse large B-cell lymphoma. Blood. 2008;111:3701-13.
45. Shaffer AL, Emre NC, Lamy L, et al. IRF4 addiction in multiple myeloma. Nature. 2008;454:226-31.
46. Compagno M, Lim WK, Grunn A, et al. Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. Nature. 2009;459:717-21.
47. Kato M, Sanada M, Kato I, et al. Frequent inactivation of A20 in B-cell lymphomas. Nature. 2009;459:712-6.
48. Lenz G, Davis RE, Ngo VN, et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. Science. 2008;319:1676-9.
49. Davis RE, Ngo VN, Lenz G, et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature. 2010;463:88-92.
50. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. Nature. 2011;470: 115-9.
51. Lim KH, Yang Y, Staudt LM. Pathogenetic importance and therapeutic implications of NF-kappaB in lymphoid malignancies. Immunol Rev. 2012;246:359-78.
52. Lohr JG, Stojanov P, Lawrence MS, et al. Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. Proc Natl Acad Sci USA. 2012;109:3879-84.
53. Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature. 2011;476:298-303.
54. Pasqualucci L, Trifonov V, Fabbri G, et al. Analysis of the coding genome of diffuse large B-cell lymphoma. Nat Genet. 2011;43:830-7.
55. Zhang J, Grubor V, Love CL, et al. Genetic heterogeneity of diffuse large B-cell lymphoma. Proc Natl Acad Sci USA. 2013;110:1398-403.
56. Pasqualucci L, Dominguez-Sola D, Chiarenza A, et al. Inactivating mutations of acetyltransferase genes in B-cell lymphoma. Nature. 2011;471:189-95.
57. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol. 2005;6:635-45.
58. Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer. 2008;8:976-90.
59. Klapproth K, Wirth T. Advances in the understanding of MYC-induced lymphomagenesis. Br J Haematol. 2010; 149:484-97.
60. Barrans S, Crouch S, Smith A, et al. Rearrangement of MYC is associated with poor prognosis in patients with diffuse large B-cell lymphoma treated in the era of rituximab. J Clin Oncol. 2010;28:3360-5.
61. Klapper W, Stoecklein H, Zeynalova S, et al. Structural aberrations affecting the MYC locus indicate a poor prognosis independent of clinical risk factors in diffuse large B-cell lymphomas treated within randomized trials of the German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL). Leukemia. 2008;22:2226-9.
62. Kramer MH, Hermans J, Wijburg E, et al. Clinical relevance of BCL2, BCL6, and MYC rearrangements in diffuse large B-cell lymphoma. Blood. 1998;92:3152-62.
63. Nitsu N, Okamoto M, Miura I, et al. Clinical significance of 8q24/c-MYC translocation in diffuse large B-cell lymphoma. Cancer Sci. 2009;100:233-7.
64. Savage KJ, Johnson NA, Ben-Neriah S, et al. MYC gene rearrangements are associated with a poor prognosis in diffuse large B-cell lymphoma patients treated with R-CHOP chemotherapy. Blood. 2009;114:3533-7.
65. Snuderl M, Kolman OK, Chen YB, et al. B-cell lymphomas with concurrent IGH-BCL2 and MYC rearrangements are aggressive neoplasms with clinical and pathologic features distinct from Burkitt lymphoma and diffuse large B-cell lymphoma. Am J Surg Pathol. 2010;34:327-40.
66. Yoon SO, Jeon YK, Paik JH, et al. MYC translocation and an increased copy number predict poor prognosis in adult diffuse large B-cell lymphoma (DLBCL), especially in germinal centre-like B cell (GCB) type. Histopathology. 2008;53:205-17.
67. Bertrand P, Bastard C, Maingonnat C, et al. Mapping of MYC breakpoints in 8q24 rearrangements involving non-immunoglobulin partners in B-cell lymphomas. Leukemia. 2007;21:515-23.
68. Dave SS, Fu K, Wright GW, et al. Molecular diagnosis of Burkitt's lymphoma. N Engl J Med. 2006;354:2431-42.
69. Hummel M, Bentink S, Berger H, et al. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med. 2006;354:2419-30.
70. Akyurek N, Uner A, Benekli M, et al. Prognostic significance of MYC, BCL2, and BCL6 rearrangements in patients with diffuse large B-cell lymphoma treated with cyclophosphamide, doxorubicin, vincristine, and prednisone plus rituximab. Cancer. 2012;118:4173-83.
71. Ruzinova MB, Caron T, Rodig SJ. Altered subcellular localization of c-Myc protein identifies aggressive B-cell lymphomas harboring a c-MYC translocation. Am J Surg Pathol. 2010;34:882-91.
72. Green TM, Young KH, Visco C, et al. Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol. 2012;30:3460-7.
73. Johnson NA, Slack GW, Savage KJ, et al. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol. 2012;30:3452-9.
74. Kluk MJ, Chapuy B, Sinha P, et al. Immunohistochemical detection of MYC-driven diffuse large B-cell lymphomas. PLoS One. 2012;7:e33813.

Diffuse large B-cell lymphoma: classic and novel prognostic factors and their impact on therapeutic decisions

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A B S T R A C T

The International Prognostic Index, originally established to predict outcome of patients with aggressive lymphoma treated in the pre-rituximab era, has been confirmed to be a valid prognosticator for patients receiving rituximab, with the differences between the four risk groups (low, low-intermediate, high-intermediate and high) being smaller, yet significant compared to the pre-rituximab era. While many IPI risk groups have now a cure rate of over 80%, young high-risk patients and all elderly patients except for those with low risk fare worse, warranting further improvement. Apart from the IPI (and independent of it), there are other subsets of diffuse large B-cell lymphoma (DLBCL) that are characterized by criteria not included in the IPI or are too rare to be recognized in multivariable analyses. This applies to very old patients (>80 years), histological subgroups like DLBCL with immunoblastic or plasmablastic morphology, and Epstein-Barr (BV)-positive B-cell diffuse large B-cell lymphoma of the elderly, the germinal center *versus* the non-germinal center subgroups, DLBCL with MYC breakpoints (including double- and triple hit DLBCL), and expression of MYC together with BCL2 protein. Finally, patients presenting with skeletal involvement or developing central nervous system (CNS) involvement during the course of disease, represent a subpopulation with an almost always fatal course. Strategies to improve the outcome of these prognostically very poor subgroups will be discussed.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe relevant clinical, morphological and molecular risk factors associated with a worse outcome in the rituximab era;
- select appropriate up-front therapy based upon the presence of certain risk factors;
- discuss treatment options for subgroups of DLBCL for which standard therapy is inappropriate.

Introduction

Prognostic factors are (usually) pre-therapeutically identifiable parameters of the tumor and/or the patient that affect the patient's outcome. They emerge and are valid only in the context of a given therapy and are likely to change with different therapies. Numerous factors that affect the prognosis of patients with diffuse large B-cell lymphomas (DLBCL) have been claimed in recent years, and very few have survived scrutiny. In the following review, we will discuss those risk factors which are valid in the rituximab era, i.e. under a treatment with CHOP[1] or CHOP-like chemotherapy in combination with the anti-CD20 antibody rituximab.

The International Prognostic Index

The International Prognostic Index (IPI) is the widely accepted prognostic factor index for patients with aggressive lymphomas. It was introduced by Shipp *et al.*[2,3] in the 1990s and was based on an individual case-based prognostic factor analysis of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP)–like regimens[1] with overall survival (OS) as the end point. The IPI considered five factors: age (≤60 years *vs.* >60 years), lactate dehydrogenase (LDH) value (≤ upper limit of normal [ULN] *vs.* > ULN), performance status (Eastern Cooperative Oncology Group [ECOG] 0, 1 *vs.* >1), Ann Arbor stage (I/II *vs.* III/IV), and the number of extranodal involvements (0, 1 *vs.* >1). The age-adjusted IPI (aaIPI) for younger patients includes the factors LDH, performance status, and stage. The IPI score separates four prognostic groups based on the number of factors present (0, 1: low risk group; 2: low-intermediate risk group; 3: high-intermediate risk group; and 4, 5: high-risk group). The IPI has been widely used and reproduced to analyze various conventional, high-dose, and dose-dense regimens.[1,4-6] Recently, a major improvement in treatment outcome has been achieved by adding rituximab to CHOP-like regimens.[7-12] The revised IPI or "R-IPI" with only three risk groups as suggested by Sehn *et al.*[13] was based on only 365 patients treated with R-CHOP (rituximab plus CHOP) and this suffered initial technical problems (*e.g.* no method to protect against errors of misclassifying ordered risk strata due to its low statistical power, no multivariable model approach, no independent validation set) and did not

hold up to scrutiny when appropriately tested. Rituximab significantly improved treatment outcome within each IPI group resulting in a quenching of the Kaplan-Meier estimators. While the differences became smaller between the four risk groups under R-CHOP, the IPI retained its highly significant prognostic power with respect to all three end points and the ordering of the IPI groups remained valid, demonstrating that the IPI is still valid in the R-CHOP era.[14] In the Mega-CHOEP trial, young patients with aaIPI of 2 had a 3-year survival of 90%, and aaIPI of 3 73% after 8 x R-CHOEP-14.[11] Therefore, in young patients, only the high-risk group with a 3-year survival of less than 75% definitely represents a clinically relevant risk group, while for patients with aaIPI of 2 it will be difficult to achieve and demonstrate further improvement. Since both CHOP-14 and CHOEP-14 leave room for further toxicity, combinations with targeted therapies like bortezomib, lenalidomide or ibrutinib are currently being evaluated in this population of young patients with high-risk DLBCL.

The situation is different in elderly (age 61-80 years) DLBCL patients, with a 3-year overall survival of 88% for low-risk, 78% for low-intermediate, 67% for high-intermediate and 58% for the high-risk group,[10] all but the low-risk group have a high risk of failure and must be improved. The increased toxicity in elderly patients leaves little room for additional hematotoxicity, and strategies pursued include dose-dense application of rituximab, adding other CD20 monoclonal antibodies or antibodies directed against targets other than CD20, addition of lenalidomide to R-CHOP, or lenalidomide or enzastaurin for maintenance therapy.

Morphological subtype

Immunoblastic subtype

In a study of morphological and immunohistochemical biomarkers in elderly patients treated both with and without rituximab within the RICOVER-60 of the German High-Grade Non-Hodgkin Lymphoma Study Group (DSHNHL), immunoblastic morphology emerged as a robust, significantly adverse prognostic factor,[15] confirming a previous study in DBLC.[16] Patients with the immunoblastic subtype had a significantly lower CR/CRu and an inferior 3-year event-free survival (EFS) (*P*=0.013) and OS (54% *vs.* 78%; *P*=0.004), while the survival curves for all other subtypes of DLBCL closely matched the curve of centroblastic lymphomas.[15] This also applies to primary mediastinal B-cell lymphoma, which did not differ from other DLBCL in the MInT trial when treated with rituximab.[17] In multivariate analysis adjusted for the factors of the IPI, the immunoblastic subtype was an independent predictor for EFS (relative risk [RR] 1.5; *P*=0.034) and OS (RR 1.7; *P*=0.007). So far no specific therapeutic approaches have been developed for immunoblastic DLBCL.

Plasmablastic subtype

This subtype has been recently characterized as an aggressive lymphoma, most frequently arising in the oral cavity of HIV-infected or elderly patients, with a male predominance. In the RICOVER-60, after a median follow up of 72 months, 2 of 7 patients with plasmablastic subtype are alive in complete remission for more than six years, and the median overall survival of these patients was 13 months. In another series of 12 patients, 6 of whom were HIV-positive, 8 are alive after a median follow up of more than 11 months.[18-20] Obviously, the outcome of plasmablastic lymphomas is not as dismal as originally reported.

Age-related EBV-associated B-cell lymphoproliferative disorders

EBV-positive B-cell diffuse large B-cell lymphoma of the elderly (also known as senile EBV-associated B-cell lymphoproliferative disorder) is an EBV-positive clonal B-cell lymphoid proliferation that occurs in patients over 50 years of age and predominantly in elderly patients without any known immunodeficiency or prior lymphoma. It accounts for 8%-10% of DLBCL in Asia, and for 20%-25% of DLBCL in patients over 90 years of age. These patients are diagnosed at older age, present more often with elevated LDH, poor performance status, B symptoms, and frequent skin and lung involvement.[21] B symptoms and age over 70 years, but not IPI, appear to be reliable prognostic factors. Patients with 0, 1 or 2 of these risk factors have a median overall survival of 56, 25 and 9 months. The 5-year survival in a series of 96 patients was 25%.[21,22]

Age

Age is one of the strongest prognostic factors in the IPI. This is not only due to increasing comorbidities of elderly patients, but also because adverse biological features like the ABC-type and MYC breaks are enriched in the elderly population. While the IPI discriminates between patients aged 60 years or under and those over 60 years, a modification of the IPI, the IPI for elderly patients or E-IPI, was suggested using 70 instead of 60 years as a cut-off point to delineate older age as a risk factor.[23] However, the prognostic discrimination provided by the E-IPI for elderly DLBCL patients needs validation by other datasets. The results of the RICOVER-60 trial suggest that 75 years is a cut-off above which the outcome of patients with DLBCL shows the sharpest decline, with more therapy-associated deaths in this population and more primary progressions. Best results in patients over 80 years of age have been reported with a combination of rituximab and dose-reduced CHOP,[24] the 2-year survival rate of 59% representing an acceptable compromise between efficacy and toxicity, but further prospective trials in this population are badly needed.

The underrepresentation of patients over 70 years of age in studies designed for ‘elderly’ patients often prohibits meaningful multivariate analyses adjusting for higher age ranges. Even fewer prospective data are available for octogenarians or nonagenarians, even though this population of DLBCL patients is increasing fast. In a retrospective analysis of 205 NHL patients, most of them with DLBCL, who were treated at a single institution from one center, death was shown to be mainly due to lymphoma, justifying and warranting treatment of NHL patients over 80 years of age.[25]

Gender

Male gender is a negative prognostic factor in (elderly) patients treated with rituximab,[26] because female patients have a considerably higher benefit from the addition of rituximab to CHOP chemotherapy than male patients.[27] This is most likely due to the slower rituximab clearance in elderly females that results in higher serum levels, longer serum half-life elimination time and larger area under the curve data.[27] As a consequence, the DSHNHL performed the SEXIER-R CHOP-14 study with more than 250 elderly DLBCL patients, dosing female patients at standard 375 mg/m^2, and male patients at 500 mg/m^2. This resulted in slightly higher serum levels in elderly males compared to females. Efficacy data from this study will not be available until 2014.

A historical comparison of the RICOVER-60 results with the SMARTE-R-CHOP-14 study, a phase-II pharmacokinetic-based study with R-CHOP-14, in which 8 administrations of rituximab at standard dose were given dose-dense at the beginning with increasing intervals and the last application on Day 239, showed an improved outcome of elderly high-risk (IPI 3-5) patients with this extended rituximab exposure time.[28] This better outcome was due to a 20% improvement in 3-year PFS and OS of high-risk elderly males with their faster rituximab clearance who benefited more from the extended exposure time than females: indeed, with the SMARTE-R rituximab schedule, the differences between males and females disappeared.[29] The OPTIMAL>60 study is currently comparing 8 × 2-week administrations of rituximab with a pharmacokinetic-based schedule in elderly DLBLC patients in a randomized fashion.

No pharmacokinetic data are available for young DLBCL patients and results according to gender in young patients have not been published.

Bulky disease

Bulky disease was an independent risk factor in the MInT study in young patients with an aaIPI of 0 or 1 and bulky disease, despite the fact that nearly all patients with bulky disease had received radiotherapy to the respective area.[30,31] A comparison of MInT patients with aaIPI of 1 and patients with this aaIPI score in a French trial[32] in which bulky disease was also an independent risk factor and R-ACVBP was shown to be superior to 8 x R-CHOP-21, strongly suggests that 6 x R-CHOP-21 with radiotherapy to bulky disease is considerably better than 8 x R-CHOP-21 without radiotherapy. The comparison also suggests that 6 x R-CHOP-21 with radiotherapy is indeed equally effective as the more toxic R-ACVBP without radiotherapy. This led to the recommendation in the European Society for Medical Oncology (ESMO) 2012 guidelines[33] that either 6 x R-CHOP-21 with radiotherapy to bulky disease or R-ACVBP (without) should be given to young patients with aaIPI of 1. Moreover, the two arms without radiotherapy of the UNFOLDER study, which compares R-CHOP-14 with R-CHOP-21 in young patients with bulky disease and/or aaIPI of 1, with and without radiotherapy to bulky and extralymphatic disease, had to be closed after a planned interim analysis due to the predefined superiority criteria of the two arms with radiotherapy (C Zwick *et al.,* personal communication, 2013). For elderly patients with bulky disease, the results of the RICOVER-noRX study also suggest a benefit of additional radiotherapy, at least in patients achieving a PR or less.[34] Whether radiotherapy to bulky disease can be skipped in patients with a negative PET scan after chemoimmunotherapy is currently under investigation.

Skeletal involvement

While skeletal involvement (whether localized or diffuse) was not a risk factor in the pre-rituximab era, it evolved as such when rituximab was given. Indeed, the addition of rituximab failed to improve the outcome of patients with skeletal involvement in the RICOVER-60 and MInT studies,[35] while radiotherapy to sites of skeletal involvement did. Therefore, for the time being, radiotherapy to sites of skeletal involvement is recommended.

CNS disease

Involvement of the central nervous system (CNS) is a serious and mostly fatal complication of DLBCL and remains to be so in the rituximab era. Risk models have been developed derived from analyses of prospective studies.[36-40] A multivariate analysis of elderly DLBCL patients treated with R-CHOP identified 3 independent risk factors for development of CNS disease: elevated LDH, >1 extranodal site, and ECOG performance status >1. Patients presenting with all three risk factors made up 4.8% of the 610 patients treated with R-CHOP and they had a 33.5% risk of developing CNS disease compared to only 2.8% in the remaining patients receiving R-CHOP.[40] While intrathecal prophylaxis with MTX appeared to have some effect on the incidence of CNS disease in patients not receiving rituximab, this prophylaxis had no effect in patients receiving R-CHOP in the RICOVER-60 trial or the MInT study. Several retrospective studies[41,42] suggest that intravenous high-dose methotrexate can reduce the incidence of CNS involvement in patients at increased risk. The DHNHL is currently evaluating intermediate-dose methotrexate (1.5 g/m^2) in elderly patients presenting with elevated LDH, ECOG over 1 and more than one extranodal site, which is given and well tolerated before the first and after the last cycle of R-CHOP.

The situation is less clear in younger patients for whom a group at significant risk for CNS involvement (elevated LDH plus advanced stage) develops CNS disease in only 6.5% of the cases.[40] A strategy to limit spinal tab to these 6.5% young patients and treat only those with signs of CNS involvement by sensitive flow cytometric analysis of spinal fluid and/or cranial NMR is currently being pursued by the German High-Grade Non-Hodgkin Lymphoma Study Group (DSHNHL) for young patients.

Other clinical presentations

Concordant bone marrow involvement (with large, but not with small cells) was shown in a retrospective register study to be a risk factor independent of the IPI,[43] as were

elevated serum levels of free light chains,[44] VEGF,[45] soluble IL-2 receptors[46] and interferon-inducible protein 10 (CXCL10)[47] as well as vitamin D,[48] and selenium.[49] Whether substitution of vitamin D or selenium can compensate the worse outcome of these patients still has to be shown.

Interim FDG-PET positivity

Early studies of DLBCL patients not (yet) receiving rituximab suggested that a PET after 1, 2, 3, or 4 cycles of CHOP was highly predictive for patient outcome.[50-53] However, this was not confirmed in larger and more recent studies of patients receiving rituximab[54-56] who showed a good negative predictive value (NPV) of approximately 80%, but a positive predictive value (PPV) of 33% or under. A French group reported that the reduction in SUV_{max} at the interim PET compared to the pre-therapy PET resulted in a much better predictive power (PPV 81%, NPV 75%) than a visual analysis.[57,58] However, using the French criteria for SUV_{max} reduction in a prospective study of 212 patients resulted in a PPV of 37% (U Dührsen, personal communication, 2010). Similar results were recently presented by the Groupe Ouest Est d'Etude des Leucémies et Autres Maladies du Sang (GOELAMS)[59] and an Italian[60] study. In summary, in the rituximab era, a positive interim PET appears to be unable to identify patients with high-risk DLBCL.

Molecular prognostic factors

Diffuse large B-cell lymphomas (DLBCL) constitute a heterogeneous category of aggressive lymphomas. Chromosomal instability and changes confer a worse prognosis,[61] and the expression of certain microRNAs[62] and proteins has been reported to be associated with a favorable (BCL6, CD10, HIF-1α, HLA-DR, IRF4/MUM1, LMO2; CD30) or an adverse (BCL2, CD5, indolamine 2,3-dioxygenase, high Ki-61, mutated p53, VEGFR2, Skp2) outcome. However, none of these reports have been confirmed in prospective studies. In contrast to single molecules, the analysis of the entire exome by gene expression profiling (GEP) studies identified three biologically and prognostically relevant subtypes of DLBCL: the activated B cell (ABC)-like DLBCL, the germinal center (GC)-like and the mediastinal large B-cell lymphoma[63,64] based on cell-of-origin (COO) gene signatures, with the activated B-cell (ABC) type being associated with an inferior outcome compared to the germinal center (GC) type.[64-66] ABC- and GC-like DLBCL differ with respect to the cell of origin, pathogenetic mechanisms and prognosis: the GC/non-GC was shown to be a prognostic factor independent of the IPI in patients treated with CHOP only, and the gene-expression-based model added to the predictive power of the IPI, and the IPI added to the predictive power of the gene-expression–based model in patients treated with CHOP plus rituximab.[64] Only the combined stromal-1/GC groups of patients fared significantly better than the ABC-type independent of the IPI.

DLBCL of the ABC type are characterized by NFkB activation that contributes to the high proliferative capacity of this subtype. Therefore, drugs interfering with this signaling pathway are attractive candidates for targeted therapy. A better response of the ABC-type to bortezomib,[67] lenalidomide[68] and the Bruton tyrosine kinase inhibitor ibrutinib[69] has been reported, but needs to be confirmed prospectively. In contrast, in relapsed DLBCL, the GC type had a better outcome with R-DHAP than with R-ICE chemoimmunotherapy in the CORAL study.[70] Nevertheless, for the time being, there is no justification for a differential treatment approach to GC and non-GC DLBCL outside prospective trials.

Because classical gene expression studies require fresh (-frozen) biopsy material, the impact of GEP on daily lymphoma practice is still rather limited, more than 12 years after Alizadeh *et al.,*'s pivotal publication.[65] Surrogate markers for the assignment to the ABC- and GC-like subtypes are warranted which are applicable to formalin-fixed-paraffin-embedded (FFPE) biopsies. However, the translation of complex GEP predictors into immunohistochemical algorithms such as the "Hans"[71] or "Choi"[72] classifiers that assign a COO subtype based on the expression of subtype-related proteins has been difficult, and prognostic and predictive accuracy of such algorithms have been shown to be quite variable, even in the hands of expert hematopathologists.[15,73-76] While immunohistology of FFPE was reported to allow the assignation of DLBCL to the GC- and non-GC subtype based on an algorithm using a limited number of antibodies suitable for FFPE biopsies,[71] a multivariate analysis by the Groupe D'Etude des Lymphomes de L'Adulte (GELA) confirmed that only the International Prognostic Index (IPI) and treatment arm influenced the outcome, but not the immunohistochemically assigned GC/non-GC phenotype.[77] Moreover, the Lunenburg consortium, made up of the most experienced hematopathologists worldwide observed unexpectedly highly variable results among the leading immunohistochemistry (IHC) laboratories in the world and very poor reproducibility in scoring for almost all markers.[78] Thus, it is not surprising that the largest TMA study performed to date in elderly DLBCL patients did not confirm the "Hans classifier",[15] the most popular algorithm used as surrogate for gene expression profiling. Whether novel algorithms show a better concordance with the GC/ABC subtyping by gene expression profiling, remains to be confirmed.[76] In summary, studies that evaluated the reliability of immunohistochemical algorithms as a surrogate for gene expression profiling yielded controversial results and studies that relied on immunohistochemistry for the assignment of GC and non-CC type DLBCL must be interpreted with caution. This also applies to immunohistochemical algorithms that tried to simulate a stromal-1[76] and stromal-2[79] signature, respectively. Besides these, multiple individual biomarkers as well as prognostic models incorporating several parameters have been evaluated in DLBCL using different techniques. Some of these models are based on mRNA expression by gene expression profiling or by real-time polymerase chain reaction (RT-PCR) and provide a quantitative measurement of gene expression.[80] Other bioprognostic models that have been proposed include a paraffin-based 6-gene prognostic model that distinguished low- and high-risk patients independent of the IPI,[81] and a 2-gene model based on an MYC and HLA-DR expression.[82] Recently, another 2-gene model based on the expression of LMO2 by the lymphoma cells and TNFRFS9 by the microenvironment has been published claiming to be an

independent factor for survival,[83] but none of these models have been confirmed in prospective studies, making it difficult to interpret their value. This also holds true for another 'bioprognostic marker' that was based on microvessel density, non-GCB subtype and low (<5%) expression of SPARC (secreted protein, acidic and rich in cysteine) in the stroma.[84] While all these novel bioprognostic markers are simplified compared to gene expression profiling, the technologies used in these models are not simple, standardized or commercially available, most likely precluding their widespread use.

c-myc breaks, double and triple hits

In many B-cell lymphomas, chromosomal translocations are biological and diagnostic hallmarks of the disease. A subset of these lymphomas has structural aberrations affecting the myc locus that is associated with a poor prognosis independent of clinical risk factors.[85] MYC-break positive DLBCL cases may also co-express high levels of BCL2, and up to half of these cases have a concurrent translocation involving BCL-2. These double-hit (DH) lymphomas are defined by a chromosomal breakpoint affecting the MYC/8q24 locus in combination with another recurrent breakpoint, *e.g.* a t(14;18)(q32;q21) involving BCL2. Recently, these lymphomas have been introduced as a novel category of lymphomas in the 2008 WHO classification[86] and were designated as "B cell lymphoma unclassifiable with features intermediate between DLBCL and Burkitt's lymphoma". DH lymphomas have been classified heterogeneously, but mostly as DLBCL, the majority having a GC phenotype and expressing BCL2. Patients with DH lymphomas often present with a poor prognosis profile including elevated LDH, bone marrow and CNS involvement, and a high IPI score. In a review of the published literature,[87] MYC breakpoints in general had a wide range of frequency (3-16%) and DH lymphomas a frequency of 0-12%. Of 689 MYC breakpoint-positive lymphomas, 47% were DH lymphomas, and from 804 cases diagnosed as DLBCL, 139 (17%) cases had an MYC breakpoint, demonstrating that MYC rearrangements in DLBCL are not rare. BCL2/MYC lymphomas form the vast majority of DH lymphomas (63%); BCL6/MYC DH lymphomas were relatively rare (8%) and triple-hit lymphomas involving MYC, BCL2 and BCL6 (16%) were, in fact, more frequent than BCL6/MYC DH. Other rarer forms of DH lymphomas involve MYC/CCND1, and MYC/BCL3. Most DH lymphomas have a GC phenotype with expression of CD10 and BCL6, a lack of MUM1/IRF, nearly always express BCL2 protein, and have a high Ki67/MIB1 proliferation rate. Therefore, aggressive lymphomas with co-expression of CD10, BCL6, BCL2 and high Ki67 proliferation index should always be checked for DH.

The DH DLBCL have been reported to have a dismal prognosis,[87,88] but a recent study from the GELA found no independent negative impact of MYC-double hits in contrast to MYC single hits[89] on survival. It has been suggested that an MYC translocation, with or without concurrent BCL2 translocation, was associated with inferior survival only, if MAC had immunoglobulin translocation partner gene.[90]

DH lymphomas show heterogeneous morphologies, the majority being morphologically classified as DLBCL. Of note, the category of "mature B cell neoplasms NOS", was in the past often called "Burkitt-like lymphoma"[91] and, therefore, often put with Burkitt's lymphoma. The median age at diagnosis of DH lymphomas ranges from 51-65 years and thus younger than in DLBCL,[87] but is rare in children. The bone marrow and CNS are frequently involved, and pleural effusions are often reported. DH lymphomas have a poor prognosis: both with CHOP and high-dose chemotherapy regimens the median survival is less than one year. The addition of rituximab appears to improve the outcome. However, even with rituximab the median survival rarely exceeds 1.5 years.[91-96] Whether regimens designed for and effective in Burkitt's lymphomas, that typically incorporate high-dose methotrexate such as the CODOX-M/IVAC regimen,[97] will improve the outcome of DH lymphomas still has to be shown. The rarity of DH lymphomas and their poor prognosis call for joint international efforts and prospective clinical phase II studies evaluating new chemotherapy regimens and targeted therapies for these prognostically poor DLBCL.

Expression of MYC and BCL2 proteins

While the prognostic impact of BCL2 and BCL6 breaks has been disputed,[85,98-102] there is a consensus that MYC translocations confer a worse prognosis in DLBCL patients treated with CHOP, both in combination with and without rituximab.[103,104]

In addition to translocations, MYC can also be deregulated by amplifications, mutations, or by microRNA-dependent mechanisms,[105-107] and it has recently been reported[108] that tumors with increased MYC protein expression have co-ordinate upregulation of MYC target genes, providing molecular confirmation of the IHC results. While MYC translocations can be detected by fluorescence *in situ* hybridization (FISH), FISH fails to detect MYC deregulation caused by mechanisms other than translocation. The recent availability of a robust monoclonal antibody (concordance for the ICH scoring was 94% for MYC[109]) that targets the N-terminus of the MYC protein has been shown to predict MYC rearrangements and has been validated for use in formalin-fixed paraffin-embedded (FFPE) tissues,[106] and allows for the study of large series of archived DLBCL samples for nuclear MYC protein expression by immunohistochemistry. Johnson and colleagues[109] found MYC translocations, high MYC mRNA and MYC protein expression in 11%, 11% and 33% of samples, respectively. In contrast to MYC translocations, which were observed in approximately 5% of the cases and had a median overall survival of less than one year, MYC protein expression was associated with an inferior progression-free and overall survival only when BCL2 protein was co-expressed. MYC/BCL2 protein co-expression was observed in 21% of the DLBCL cases, and the negative impact on prognosis remained significant after adjusting for the presence of high-risk features in a multivariable model that included elevated IPI score. The results of Johnson *et al.* confirm similar observations reported by Green and colleagues[110,111] and a German study confirmed the prognostic value of MYC/BC2 double-protein expression in a population treated uniformly within a prospective trial.[112] Since MYC protein expres-

sion is associated with MYC translocation, all MYC protein-positive patients should be tested for MYC translocations by FISH. MYC/BCL2 IHC was possible in 96% of the cases, demonstrating that the vast majority of FFPE tissue samples processed in the community are of satisfactory quality for this type of IHC.[110] However, while MYC IHC appears to be quite robust, it should be kept in mind that BCL2 IHC has been reported to be more variable, even among international experts in the field. In the pivotal validation study of IHC on tissue microarrays, the concordance rate was only 70%,[78] similar to that achieved by a group of German hematopathologists.[15] Data on how reproducible BCL2 IHC is in the community are not available. Therefore, for the time being, the diagnosis of MYC/BCL2 double-protein expressing DLBCL should be made only by internationally recognized hematopathologists.

With repeated and convincing evidence that patients with DLCBL co-expressing MYC and BCL2-proteins by IHC have a poor prognosis, the question arises as to which therapeutic strategies should be pursued for these patients. So far, there are no results from trials that specifically addressed MYC/BCL2 double protein-positive patients, but some information can be drawn from the analysis of DH lymphomas, since the two populations are overlapping. DH DLBCL do even worse than double protein-positive DLBCL when treated with R-CHOP,[103,104] and, in the case of failing during or after primary treatment, can rarely be salvaged by standard approaches like R-ICE or DHAP followed by high-dose BEAM and autologous stem cell transplantation.[113] The very obvious assumption that these patients should fare better with regimens that have been shown to work well in patients with Burkitt's lymphoma, could not be confirmed for the CODOX-M/IVAC regimen,[97] and the numbers of DH patients treated with aggressive regimens that included high-dose chemotherapy and autologous stem cell transplantation[94-96,114] are too small to allow for any conclusion. This also applies to a study from the NCI where MYC+ DLBCL had an event-free survival of 83% after four years with dose-adjusted EPOCH-R, by far the best treatment results reported for this subgroup of DLBCL.[115] Since patients with DH and MYC/BCL2 double-protein expression are rare, it can only be through international joint efforts that new therapeutic approaches for these patients can be tested and validated. With the ease and speed that these patients can now be identified by IHC, an important logistical obstacle has been eliminated. After the pathologists have paved the way, it is now up to clinical investigators to make use of this opportunity and develop better treatments for these patients.

References

1. McKelvey EM, Gottlieb JA, Wilson HE, et al. Hydroxyldaunomycin (Adriamycin) combination chemotherapy in malignant lymphoma. Cancer. 1976;38:1484-93.
2. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med. 1993;329:987-94.
3. Shipp MA. Prognostic factors in aggressive non-Hodgkin's lymphoma: who has "high-risk" disease? Blood. 1994;83:1165-73.
4. Pfreundschuh M, Trumper L, Kloess M, et al. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of elderly patients with aggressive lymphomas: results of the NHL-B2 trial of the DSHNHL. Blood. 2004;104:634-41.
5. Pfreundschuh M, Trumper L, Kloess M, et al. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of young patients with good-prognosis (normal LDH) aggressive lymphomas: results of the NHL-B1 trial of the DSHNHL. Blood. 2004;104:626-33.
6. Milpied N, Deconinck E, Gaillard F, et al. Initial treatment of aggressive lymphoma with high-dose chemotherapy and autologous stem-cell support. N Engl J Med. 2004;350:1287-95.
7. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002;346:235-42.
8. Pfreundschuh M, Truemper L, Osterborg A, et al. CHOP-like chemotherapy plus rituximab compared with CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large B-cell lymphoma: a randomized controlled trial by the Mabthera International Trial (MInT) Group. Lancet Oncology. 2006;7:379-91.
9. Habermann TM, Weller EA, Morrison VA, et al. Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. J Clin Oncol. 2006;24:3121-7.
10. Pfreundschuh M, Schubert J, Ziepert M, et al. Six versus eight cycles of bi-weekly CHOP-14 with or without rituximab in elderly patients with aggressive CD20+ B-cell lymphomas: a randomised controlled trial (RICOVER-60). Lancet Oncol. 2008;9:105-16.
11. Schmitz N, Nickelsen M, Ziepert M, et al. Conventional chemotherapy (CHOEP-14) with rituximab or high-dose chemotherapy (MegaCHOEP) with rituximab for young, high-risk patients with aggressive B-cell lymphoma: an open-label, randomised, phase 3 trial (DSHNHL 2002-1). Lancet Oncol. 2012;13:1250-9.
12. Sehn LH, Donaldson J, Chhanabhai M, et al. Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia. J Clin Oncol. 2005;23:5027-33.
13. Sehn LH, Berry B, Chhanabhai M, et al. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. Blood. 2007;109:1857-61.
14. Ziepert M, Hasenclever D, Kuhnt E, et al. Standard International Prognostic Index remains a valid predictor of outcome for patients with aggressive CD20+ B-cell lymphoma in the rituximab era. J Clin Oncol. 2010;28:2373-80.
15. Ott G, Ziepert M, Klapper W, et al. Immunoblastic morphology but not the immunohistochemical GCB/nonGCB classifier predicts outcome in diffuse large B-cell lymphoma in the RICOVER-60 trial of the DSHNHL. Blood. 2010;116:4916-25.
16. Engelhard M, Brittinger G, Huhn D, et al. Subclassification of diffuse large B-cell lymphomas according to the Kiel classification: distinction of centroblastic and immunoblastic lymphomas is a significant prognostic risk factor. Blood. 1997;89:2291-7.
17. Rieger M, Osterborg A, Pettengell R, et al. Primary mediastinal B-cell lymphoma treated with CHOP-like chemotherapy with or without rituximab: results of the Mabthera International Trial Group study. Ann Oncol. 2011;22:664-70.
18. Colomo L, Loong F, Rives S, et al. Diffuse large B-cell lymphomas with plasmablastic differentiation represent a heterogeneous group of disease entities. Am J Surg Pathol. 2004;28:736-47.
19. Teruya-Feldstein J, Chiao E, Filippa DA, et al. CD20-negative large-cell lymphoma with plasmablastic features: a clinically heterogenous spectrum in both HIV-positive and -negative patients. Ann Oncol. 2004;15:1673-9.
20. Valera A, Balague O, Colomo L, et al. IG/MYC rearrangements are the main cytogenetic alteration in plasmablastic lymphomas. Am J Surg Pathol. 2010;34:1686-94.
21. Oyama T, Yamamoto K, Asano N, et al. Age-related EBV-associated B-cell lymphoproliferative disorders constitute a distinct clinicopathologic group: a study of 96 patients. Clin Cancer Res. 2007;13:5124-32.
22. Park S, Lee J, Ko YH, et al. The impact of Epstein-Barr virus status on clinical outcome in diffuse large B-cell lymphoma. Blood. 2007;110:972-8.
23. Advani RH, Chen H, Habermann TM, et al. Comparison of conventional prognostic indices in patients older than 60

years with diffuse large B-cell lymphoma treated with R-CHOP in the US Intergroup Study (ECOG 4494, CALGB 9793): consideration of age greater than 70 years in an elderly prognostic index (E-IPI). Br J Haematol. 2010;151:143-51.
24. Peyrade F, Jardin F, Thieblemont C, et al. Attenuated immunochemotherapy regimen (R-miniCHOP) in elderly patients older than 80 years with diffuse large B-cell lymphoma: a multicentre, single-arm, phase 2 trial. Lancet Oncol. 2011;12:460-8.
25. Thieblemont C, Grossoeuvre A, Houot R, et al. Non-Hodgkin's lymphoma in very elderly patients over 80 years. A descriptive analysis of clinical presentation and outcome. Ann Oncol. 2008;19:774-9.
26. Carella AM, De Souza CA, Luminari S, et al. Prognostic role of gender in diffuse large B-cell lymphoma treated with rituximab containing regimens: a Fondazione Italiana Linfomi/Grupo de Estudos em Molestias Onco-Hematologicas retrospective study. Leuk Lymphoma. 2013; 54:53-7.
27. Muller C, Murawski N, Wiesen MH, et al. The role of sex and weight on rituximab clearance and serum elimination half-life in elderly patients with DLBCL. Blood. 2012;119:3276-84.
28. Pfreundschuh M, Held G, Zeynalova S, et al. Improved outcome of elderly poor-prognosis DLBCL patients with 6xCHOP-14 and 8 applications Grade of rituximab given over an extended period: Results of the SMARTE-R-CHOP-14 trial of the German High-Grade Non-Hodgkin Lymphoma Study Group (DSHNHL). Blood. 2011;118:272(Abstract).
29. Murawski N, Held G, Zeynalova S, et al. Outcome of elderly DLBCL patients with 6xCHOP-14 and 8 rituximab applications given over an extended period. J Clin Oncol. 2012;20:516s.
30. Pfreundschuh M, Ho AD, Cavallin-Stahl E, et al. Prognostic significance of maximum tumour (bulk) diameter in young patients with good-prognosis diffuse large-B-cell lymphoma treated with CHOP-like chemotherapy with or without rituximab: an exploratory analysis of the MabThera International Trial Group (MInT) study. Lancet Oncol. 2008;9:435-44.
31. Pfreundschuh M, Kuhnt E, Trumper L, et al. CHOP-like chemotherapy with or without rituximab in young patients with good-prognosis diffuse large-B-cell lymphoma: 6-year results of an open-label randomised study of the MabThera International Trial (MInT) Group. Lancet Oncol. 2011; 12:1013-22.
32. Recher C, Coiffier B, Haioun C, et al. Intensified chemotherapy with ACVBP plus rituximab versus standard CHOP plus rituximab for the treatment of diffuse large B-cell lymphoma (LNH03-2B): an open-label randomised phase 3 trial. Lancet. 2011;378:1858-67.
33. Ghielmini M, Vitolo U, Kimby E, et al. ESMO Guidelines consensus conference on malignant lymphoma 2011 part 1: diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL). Ann Oncol. 2013;24:561-76.
34. Held G, Murawski M, Ziepert M, et al. Role of radiotherapy for elderly DLBCL patients in the rituximab era: Final results of the RICOVER-noRX study of the DSHNHL. J Clin Oncol. 2012;30:515s.
35. Held G, Zeynalova S, Murawski N, et al. The impact of rituximab and radiotherapy on treatment outcome of patients with DLBCL and skeletal involvement. Blood. 2012;120:690 (Abstract).
36. Boehme V, Zeynalova S, Kloess M, et al. Incidence and risk factors of central nervous system recurrence in aggressive lymphoma—a survey of 1693 patients treated in protocols of the German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL). Ann Oncol. 2007;18:149-57.
37. Boehme V, Schmitz N, Zeynalova S, Loeffler M, Pfreundschuh M. CNS events in elderly patients with aggressive lymphoma treated with modern chemotherapy (CHOP-14) with or without rituximab: an analysis of patients treated in the RICOVER-60 trial of the German High-Grade Non-Hodgkin Lymphoma Study Group (DSHNHL). Blood. 2009;113:3896-902.
38. Hollender A, Kvaloy S, Nome O, et al. Central nervous system involvement following diagnosis of non-Hodgkin's lymphoma: a risk model. Ann Oncol. 2002;13:1099-107.
39. Bjorkholm M, Hagberg H, Holte H, et al. Central nervous system occurrence in elderly patients with aggressive lymphoma and a long-term follow-up. Ann Oncol. 2007;18:1085-9.
40. Schmitz N, Zeynalova S, Glass B, et al. CNS disease in younger patients with aggressive B-cell lymphoma: an analysis of patients treated on the Mabthera International Trial and trials of the German High-Grade Non-Hodgkin Lymphoma Study Group. Ann Oncol. 2012;23:1267-73.
41. Herbert K, Wolf M, Carney D, et al. The addition of systemic high-dose methotrexate (HD-MTX) to intrathecal chemotherapy (IT) for central nervous system prophylaxis substantially reduces CNS recurrence rates in patients with at-risk aggressive lymphoma: a historically controlled prospective study. Blood. 2008;112:1230(Abstract).
42. Abramson JS, Hellmann M, Barnes JA, et al. Intravenous methotrexate as central nervous system (CNS) prophylaxis is associated with a low risk of CNS recurrence in high-risk patients with diffuse large B-cell lymphoma. Cancer. 2010;116:4283-90.
43. Sehn LH, Scott DW, Chhanabhai M, et al. Impact of concordant and discordant bone marrow involvement on outcome in diffuse large B-cell lymphoma treated with R-CHOP. J Clin Oncol. 2011;29:1452-7.
44. Maurer MJ, Micallef IN, Cerhan JR, et al. Elevated serum free light chains are associated with event-free and overall survival in two independent cohorts of patients with diffuse large B-cell lymphoma. J Clin Oncol. 2011;29:1620-6.
45. Riihijarvi S, Nurmi H, Holte H, et al. High serum vascular endothelial growth factor level is an adverse prognostic factor for high-risk diffuse large B-cell lymphoma patients treated with dose-dense chemoimmunotherapy. Eur J Haematol. 2012;89:395-402.
46. Goto N, Tsurumi H, Goto H, et al. Serum soluble interleukin-2 receptor (sIL-2R) level is associated with the outcome of patients with diffuse large B cell lymphoma treated with R-CHOP regimens. Ann Hematol. 2012;91:705-14.
47. Ansell SM, Maurer MJ, Ziesmer SC, et al. Elevated pretreatment serum levels of interferon-inducible protein-10 (CXCL10) predict disease relapse and prognosis in diffuse large B-cell lymphoma patients. Am J Hematol. 2012;87:865-9.
48. Drake MT, Maurer MJ, Link BK, et al. Vitamin D insufficiency and prognosis in non-Hodgkin's lymphoma. J Clin Oncol. 2010;28:4191-8.
49. Last KW, Cornelius V, Delves T, et al. Presentation serum selenium predicts for overall survival, dose delivery, and first treatment response in aggressive non-Hodgkin's lymphoma. J Clin Oncol. 2003;21:2335-41.
50. Spaepen K, Stroobants S, Dupont P, et al. Early restaging positron emission tomography with (18)F-fluorodeoxyglucose predicts outcome in patients with aggressive non-Hodgkin's lymphoma. Ann Oncol. 2002;13:1356-63.
51. Mikhaeel NG, Hutchings M, Fields PA, O'Doherty MJ, Timothy AR. FDG-PET after two to three cycles of chemotherapy predicts progression-free and overall survival in high-grade non-Hodgkin lymphoma. Ann Oncol. 2005;16:1514-23.
52. Kostakoglu L, Goldsmith SJ, Leonard JP, et al. FDG-PET after 1 cycle of therapy predicts outcome in diffuse large cell lymphoma and classic Hodgkin disease. Cancer. 2006;107:2678-87.
53. Haioun C, Itti E, Rahmouni A, et al. [18F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) in aggressive lymphoma: an early prognostic tool for predicting patient outcome. Blood. 2005;106:1376-81.
54. Cashen AF, Dehdashti F, Luo J, et al. 18F-FDG PET/CT for early response assessment in diffuse large B-cell lymphoma: poor predictive value of international harmonization project interpretation. J Nucl Med. 2011;52:386-92.
55. Han HS, Escalon MP, Hsiao B, Serafini A, Lossos IS. High incidence of false-positive PET scans in patients with aggressive non-Hodgkin's lymphoma treated with rituximab-containing regimens. Ann Oncol. 2009;20:309-18.
56. Moskowitz CH, Schoder H, Teruya-Feldstein J, et al. Risk-adapted dose-dense immunochemotherapy determined by interim FDG-PET in advanced-stage diffuse large B-Cell lymphoma. J Clin Oncol. 2010;28:1896-903.
57. Lin C, Itti E, Haioun C, et al. Early 18F-FDG PET for prediction of prognosis in patients with diffuse large B-cell lymphoma: SUV-based assessment versus visual analysis. J Nucl Med. 2007;48:1626-32.
58. Casasnovas RO, Meignan M, Berriolo-Riedinger A, et al. SUVmax reduction improves early prognosis value of interim positron emission tomography scans in diffuse large B-cell lymphoma. Blood. 2011;118:37-43.
59. Milpied N-J, Legouill S, Lamy T, et al. No benefit of rituximab (R)-high dose therapy (R-HDT) over R-CHOP14 for young adults with diffuse large B-cell lymphoma: preliminary results of the GOELAMS 075 prospective multicentre randomized trial. Blood. 2010;116:301(Abstract).

60. Pregno P, Chiappella A, Bello M, et al. Interim 18-FDG-PET/CT failed to predict the outcome in diffuse large B-cell lymphoma patients treated at the diagnosis with rituximab-CHOP. Blood. 2012;119:2066-73.
61. Bakhoum SF, Danilova OV, Kaur P, Levy NB, Compton DA. Chromosomal instability substantiates poor prognosis in patients with diffuse large B-cell lymphoma. Clin Cancer Res. 2011;17:7704-11.
62. Alencar AJ, Malumbres R, Kozloski GA, et al. MicroRNAs are independent predictors of outcome in diffuse large B-cell lymphoma patients treated with R-CHOP. Clin Cancer Res. 2011;17:4125-35.
63. Rosenwald A, Wright G, Chan WC, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002;346:1937-47.
64. Lenz G, Wright G, Dave SS, et al. Stromal gene signatures in large-B-cell lymphomas. N Engl J Med. 2008;359:2313-23.
65. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000;403:503-11.
66. Habara T, Sato Y, Takata K, et al. Germinal center B-cell-like versus non-germinal center B-cell-like as important prognostic factor for localized nodal DLBCL. J Clin Exp Hematop. 2012;52:91-9.
67. Dunleavy K, Pittaluga S, Czuczman MS, et al. Differential efficacy of bortezomib plus chemotherapy within molecular subtypes of diffuse large B-cell lymphoma. Blood. 2009;113:6069-76.
68. Hernandez-Ilizaliturri FJ, Deeb G, Zinzani PL, et al. Higher response to lenalidomide in relapsed/refractory diffuse large B-cell lymphoma in nongerminal center B-cell-like than in germinal center B-cell-like phenotype. Cancer. 2011;117:5058-66.
69. Wilson WH, Gerecitano JF, Goy A, et al. The Bruton´s tyrosine kinase inhibitor ibrutinib (PCI-32765) has preferential activity in the ABS subtype of rleapsed/refractory de novo diffuse large B-cell lymphoma: Interim results of a multicenter, open-label phase 2 study. Blood. 2012;120:686(Abstract).
70. Thieblemont C, Briere J, Mounier N, et al. The germinal center/activated B-cell subclassification has a prognostic impact for response to salvage therapy in relapsed/refractory diffuse large B-cell lymphoma: a bio-CORAL study. J Clin Oncol. 2011;29:4079-87.
71. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004;103:275-82.
72. Choi WW, Weisenburger DD, Greiner TC, et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. Clin Cancer Res. 2009;15:5494-502.
73. Salles G, de Jong D, Xie W, et al. Prognostic significance of immunohistochemical biomarkers in diffuse large B-cell lymphoma: a study from the Lunenburg Lymphoma Biomarker Consortium. Blood. 2011;117:7070-8.
74. Nyman H, Adde M, Karjalainen-Lindsberg ML, et al. Prognostic impact of immunohistochemically defined germinal center phenotype in diffuse large B-cell lymphoma patients treated with immunochemotherapy. Blood. 2007;109:4930-5.
75. Gutierrez-Garcia G, Cardesa-Salzmann T, Climent F, et al. Gene-expression profiling and not immunophenotypic algorithms predicts prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. Blood. 2011;117:4836-43.
76. Meyer PN, Fu K, Greiner TC, et al. Immunohistochemical methods for predicting cell of origin and survival in patients with diffuse large B-cell lymphoma treated with rituximab. J Clin Oncol. 2011;29:200-7.
77. Molina TJ, Gaulard P, Jais J-P, et al. Germinal center phenotype determined by immunohistochemistry on tissue microarray does not correlate with outcome in diffuse large B-cell lymphoma patients treated with immunochemotherapy in the randomized trial LNH98-5. Blood. 2007;110:24(Abstract).
78. de Jong D, Rosenwald A, Chhanabhai M, et al. Immunohistochemical prognostic markers in diffuse large B-cell lymphoma: validation of tissue microarray as a prerequisite for broad clinical applications—a study from the Lunenburg Lymphoma Biomarker Consortium. J Clin Oncol. 2007;25:805-12.
79. Cardesa-Salzmann TM, Colomo L, Gutierrez G, et al. High microvessel density determines a poor outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus chemotherapy. Haematologica. 2011;96:996-1001.
80. Lossos IS, Morgensztern D. Prognostic biomarkers in diffuse large B-cell lymphoma. J Clin Oncol. 2006;24:995-1007.
81. Malumbres R, Chen J, Tibshirani R, et al. Paraffin-based 6-gene model predicts outcome in diffuse large B-cell lymphoma patients treated with R-CHOP. Blood. 2008;111:5509-14.
82. Rimsza LM, LeBlanc ML, Unger JM, et al. Gene expression predicts overall survival in paraffin-embedded tissues of diffuse large B-cell lymphoma treated with R-CHOP. Blood. 2008;112:3425-33.
83. Alizadeh AA, Gentles AJ, Alencar AJ, et al. Prediction of survival in diffuse large B-cell lymphoma based on the expression of 2 genes reflecting tumor and microenvironment. Blood. 2011;118:1350-8.
84. Perry AM, Cardesa-Salzmann TM, Meyer PN, et al. A new biologic prognostic model based on immunohistochemistry predicts survival in patients with diffuse large B-cell lymphoma. Blood. 2012;120:2290-6.
85. Klapper W, Stoecklein H, Zeynalova S, et al. Structural aberrations affecting the MYC locus indicate a poor prognosis independent of clinical risk factors in diffuse large B-cell lymphomas treated within randomized trials of the German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL). Leukemia. 2008;22:2226-9.
86. WHO classification of tumours of Haematopoietic and lymphoid tissues. Geneva: WHO Press; 2008.
87. Aukema SM, Siebert R, Schuuring E, et al. Double-hit B-cell lymphomas. Blood. 2011;117:2319-31.
88. Kobayashi T, Tsutsumi Y, Sakamoto N, et al. Double-hit lymphomas constitute a highly aggressive subgroup in diffuse large B-cell lymphomas in the era of rituximab. Jpn J Clin Oncol. 2012;42:1035-42.
89. Copie-Bergman C, Culliere-Dartigue P, Baia M, et al. MYC gene simple hit is a strong independent predictive factor of survival in diffuse large B-Cell lymphomas in contrast to MYC double-hit gene alterations: A study by the Groupe d'Etude Des Lymphomes De l'Adulte. Blood. 2012;120:541(Abstract).
90. Ølgod Pedersen M, Ortved Gang A, Svenstrup Poulsen T, et al. MYC Translocation Partner Gene Determines Survival in Large B-Cell Lymphoma with MYC- or Double Hit MYC/BCL2 Translocation. Blood. 2012;120:542(Abstract).
91. MacPherson N, Lesack D, Klasa R, et al. Small noncleaved, non-Burkitt's (Burkitt-Like) lymphoma: cytogenetics predict outcome and reflect clinical presentation. J Clin Oncol. 1999;17:1558-67.
92. Johnson NA, Savage KJ, Ludkovski O, et al. Lymphomas with concurrent BCL2 and MYC translocations: the critical factors associated with survival. Blood. 2009;114:2273-9.
93. Kanungo A, Medeiros LJ, Abruzzo LV, Lin P. Lymphoid neoplasms associated with concurrent t(14;18) and 8q24/c-MYC translocation generally have a poor prognosis. Mod Pathol. 2006;19:25-33.
94. Le Gouill S, Talmant P, Touzeau C, et al. The clinical presentation and prognosis of diffuse large B-cell lymphoma with t(14;18) and 8q24/c-MYC rearrangement. Haematologica. 2007;92:1335-42.
95. Niitsu N, Okamoto M, Miura I, Hirano M. Clinical features and prognosis of de novo diffuse large B-cell lymphoma with t(14;18) and 8q24/c-MYC translocations. Leukemia. 2009;23:777-83.
96. Snuderl M, Kolman OK, Chen YB, et al. B-cell lymphomas with concurrent IGH-BCL2 and MYC rearrangements are aggressive neoplasms with clinical and pathologic features distinct from Burkitt lymphoma and diffuse large B-cell lymphoma. Am J Surg Pathol. 2010;34:327-40.
97. Mead GM, Barrans SL, Qian W, et al. A prospective clinicopathologic study of dose-modified CODOX-M/IVAC in patients with sporadic Burkitt lymphoma defined using cytogenetic and immunophenotypic criteria (MRC/NCRI LY10 trial). Blood. 2008;112:2248-60.
98. Barrans SL, O'Connor SJ, Evans PA, et al. Rearrangement of the BCL6 locus at 3q27 is an independent poor prognostic factor in nodal diffuse large B-cell lymphoma. Br J Haematol. 2002;117:322-32.
99. van Imhoff GW, Boerma EJ, van der Holt B, et al. Prognostic impact of germinal center-associated proteins and chromosomal breakpoints in poor-risk diffuse large B-cell lymphoma. J Clin Oncol. 2006;24:4135-42.
100. Hummel M, Bentink S, Berger H, et al. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med. 2006;354:2419-30.
101. Iqbal J, Sanger WG, Horsman DE, et al. BCL2 translocation defines a unique tumor subset within the germinal center B-

cell-like diffuse large B-cell lymphoma. Am J Pathol. 2004;165:159-66.
102. Yoon SO, Jeon YK, Paik JH, et al. MYC translocation and an increased copy number predict poor prognosis in adult diffuse large B-cell lymphoma (DLBCL), especially in germinal centre-like B cell (GCB) type. Histopathology. 2008;53:205-17.
103. Savage KJ, Johnson NA, Ben-Neriah S, et al. MYC gene rearrangements are associated with a poor prognosis in diffuse large B-cell lymphoma patients treated with R-CHOP chemotherapy. Blood. 2009;114:3533-7.
104. Barrans S, Crouch S, Smith A, et al. Rearrangement of MYC is associated with poor prognosis in patients with diffuse large B-cell lymphoma treated in the era of rituximab. J Clin Oncol. 2010;28:3360-5.
105. Leucci E, Cocco M, Onnis A, et al. MYC translocation-negative classical Burkitt lymphoma cases: an alternative pathogenetic mechanism involving miRNA deregulation. J Pathol. 2008;216:440-50.
106. Gurel B, Iwata T, Koh CM, et al. Nuclear MYC protein overexpression is an early alteration in human prostate carcinogenesis. Mod Pathol. 2008;21:1156-67.
107. Ruzinova MB, Caron T, Rodig SJ. Altered subcellular localization of c-Myc protein identifies aggressive B-cell lymphomas harboring a c-MYC translocation. Am J Surg Pathol. 2010;34:882-91.
108. Kluk MJ, Chapuy B, Sinha P, et al. Immunohistochemical detection of MYC-driven diffuse large B-cell lymphomas. PLoS One. 2012;7:e33813.
109. Johnson NA, Slack GW, Savage KJ, et al. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol. 2012;30:3452-9.
110. Green TM, Nielsen O, de SK, et al. High levels of nuclear MYC protein predict the presence of MYC rearrangement in diffuse large B-cell lymphoma. Am J Surg Pathol. 2012;36:612-9.
111. Green TM, Young KH, Visco C, et al. Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol. 2012;30:3460-7.
112. Horn H, Ziepert M, Becher C, et al. MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. Blood. 2013;121(12):2253-63.
113. Cuccuini W, Briere J, Mounier N, et al. MYC+ diffuse large B-cell lymphoma is not salvaged by classical R-ICE or R-DHAP followed by BEAM plus autologous stem cell transplantation. Blood. 2012;119:4619-24.
114. Tomita N, Tokunaka M, Nakamura N, et al. Clinicopathological features of lymphoma/leukemia patients carrying both BCL2 and MYC translocations. Haematologica. 2009;94:935-43.
115. Dunleavy K, Wayne AS, Shovlin M, et al. MYC+ aggressive B-cell lymphomas: novel therapy of untreated Burktit lymphoma and MYC+ diffuse large B-cell lymphoma with DA-EPOCH-R. Ann Oncol. 2011;22(4 Suppl):iv106.

Beyond R-CHOP treatment of diffuse large B-cell lymphoma

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A B S T R A C T

The last 30 years have seen a plethora of treatments for diffuse large B-cell lymphoma (DLBCL) but none proved better than CHOP, the first curative treatment. In the recent era, however, there is convincing evidence for superior chemotherapy platforms. A randomized study from GELA showed R-ACVBP was superior to R-CHOP in patients under 60 years of age, but toxicity limits its use to younger patients. Studies also suggest dose-adjusted EPOCH-R may represent an advance, but a randomized comparison with R-CHOP is only now nearing completion. However, the simplicity and safety of R-CHOP and long history of failed contenders has set a lofty bar for other approaches. We have now entered the era of targeted therapy, propelled by a rapidly increasing knowledge of tumor biology, driver pathways and clinical successes. The first targeted treatment, rituximab, has been an unqualified albeit empirical success. Rational drug discovery now leverages our understanding of tumor pathogenesis and tumor-host interactions. The discovery of new signaling pathways through gene expression profiling (GEP), transcriptome sequencing, RNA interference screens and DNA sequencing has identified an array of new targets for DLBCL. The division of DLBCL into at least three distinct molecular diseases, germinal center B-cell, activated B-cell, and primary mediastinal B-cell DLBCL, is essential for advancing treatment.

Learning goals

At the conclusion of this activity, participants should have:
- obtained an understanding of DLBCL pathobiology;
- gained an overview of current therapy approaches in DLBCL;
- understood potential therapeutic targets.

Introduction

The application of empiricism over scientific rigor, inadequate translational end points in clinical trials, scientific politics and the entrenchment of R-CHOP have hampered the development of new standards for DLBCL. Fortunately, insights into the molecular taxonomy of DLBCL has led to the identification of ‘driver’ pathways, drugable targets and more effective immunochemotherapy regimens which highlight the importance of conducting studies within the molecular DLBCL subtypes. Nonetheless, progress is slow and many extant clinical trials continue to combine all DLBCL cases, and to focus on clinical end points and not on molecularly driven end points.

Pathobiology: the first essential element

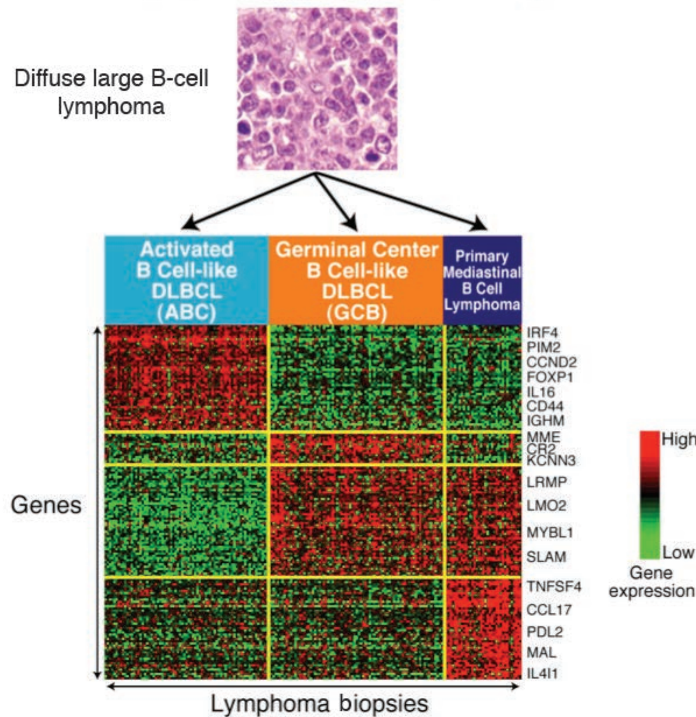
Conceptual therapeutic advances are built on biological foundations and are infrequently the product of empiricism. While this has been long recognized, the recent era has witnessed benefits from biologically-based pathology classifications, which are needed for optimal clinical research. It is important to recall that the National Cancer Institute *Working Formulation*, used in the United States until the early 1990s, was biologically agnostic and categorized lymphomas by morphology and clinical behavior.[1] In contrast, the Kiel Classification, used in Europe, had some biological foundation.[2] It was not until the Revised European-American Lymphoid (REAL) classification, published in 1994, that a clinical-biological foundation was formally incorporated into the classification of lymphomas.[3] Since then, major genetic and biological insights have been codified into the diagnostic criteria of the World Health Organization (WHO) classification of tumors of the lymphoid tissues.[4-6]

The classification of DLBCL has been among the greatest beneficiaries of recent biological discoveries in lymphoid tumors. While it has long been recognized that DLBCL is clinically and biologically diverse, it was not possible to subdivide it into distinct disease entities because of overlapping morphology and pathogenetic features.[7] As a result, treatment strategies have and still depend on clinical features such as stage and age etc. as validated by the International Prognostic Index (IPI) score.[8] However, with the application of large-scale gene expression profiling, DLBCL is now divided into at least three molecular

subtypes. Though still retaining the histological description of a neoplasm of large B-lymphoid cells with a diffuse growth pattern, these subtypes derive from B cells at different stages of differentiation with distinctive molecular and clinical characteristics.[4] When considering treatment, either in the research or clinical setting, it is essential to understand these pathobiological distinctions.

Presently, DLBCL is divided into 4 major groups within the WHO, which are further divided along molecular, pathological and/or clinical grounds. Of these, the most common group is DLBCL not otherwise specified (NOS), which can be further subdivided into the germinal center B-cell like (GCB) and activated B-cell like (ABC) molecular subgroups by GEP (Figure 1A).[9,10] In the initial GEP studies of DLBCL, arrays were performed on a variety of lymphoma types and cell lines, as well as normal lympho-

A



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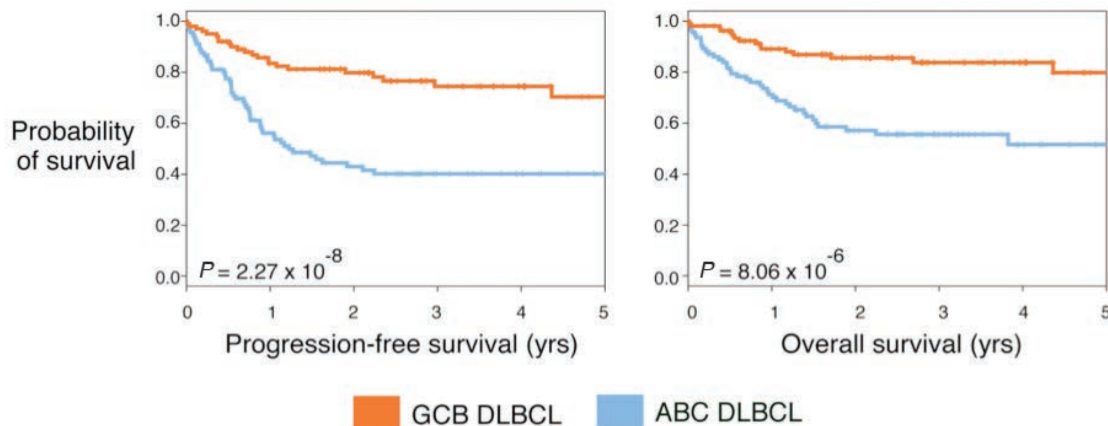


Figure 1. Diagnosis and outcome of DLBCL subtypes by gene expression profiling subtypes. (A) Heat map showing differential expression of genes in GCB, ABC and PMBL DLBCL subtypes. (B) Kaplan-Meier estimates of progression-free and overall survival are shown according to GCB or ABC DLBCL subtype in patients treated with R-CHOP based therapy. Median follow up is approximately two years.

cyte subpopulations obtained under a variety of activation conditions to provide a comparative basis for analysis of DLBCL gene expression.[11] Genes associated with cellular proliferation showed a clear distinction among the lymphoma types with DLBCL generally showing higher albeit variable expression.[12] The proliferation signature genes were a diverse group and included cell-cycle control and and myc genes. Another prominent feature of DLBCL was a group of genes that defined a 'lymph-node' signature that appeared to reflect the non-malignant cells in the biopsy samples. Genes that distinguished germinal center (GC) B cells from other stages of B-cell differentiation were also differentially expressed in the DLBCL cases, and were independent of other expression signatures, suggesting that they could be used to define different subsets.[11,13] Genes associated with GCB DLBCL included known markers of germinal center differentiation such as CD10 and the *bcl-6* gene, which may be translocated or mutated in DLBCL.[14] In contrast, most genes that defined ABC DLBCL were not expressed by normal germinal center B cells, but instead were induced during *in vitro* activation of peripheral B cells. The ABC DLBCL signature also included the IRF4 (MUM1) gene that is transiently induced during normal lymphocyte activation and is necessary for antigen receptor driven B-cell proliferation.[15,16] A noteworthy feature of ABC DLBCL was the expression of *bcl-2* that is induced over 30-fold during peripheral B-cell activation.[17] These results suggested that the GCB and ABC DLBCL subtypes are derived from B cells at different stages of differentiation. GCB DLBCL appears to arise from germinal center B cells whereas ABC DLBCL likely arises from post-germinal center B cells that are blocked during plasmacytic differentiation. Clinically, GCB DLBCL has a higher overall survival compared to ABC DLBCL with R-CHOP based treatment (Figure 1B).[18]

Primary mediastinal B-cell lymphoma (PMBL) is the third molecular subtype of DLBCL, which occurs mostly in young patients (Figure 1A).[19,20] PMBL is pathologically defined by a combination of clinical and histological features, and some cases may have features reminiscent of Hodgkin's lymphoma, all of which can confound an accurate diagnosis.[7,21] Two studies using GEP have confirmed the unique biological identity of PMBL and have shown a strong relationship between PMBL and nodular sclerosis Hodgkin's lymphoma.[9,22] Both diseases arebelieved to be derived from a thymic B cell. Most PMBL cases show *bcl-6* gene mutations, usually along with somatic mutations of the immunoglobulin heavy chain, suggesting a late stage of germinal center differentiation.[23,24] Unlike other types of DLBCL, PMBL tumors have defective immunoglobulin production despite expression of B-cell transcription factors; OCT-2, BOB.1 and PU.1. Over half of PMBL cases have amplification of the *REL* proto-oncogene and the *JAK2* tyrosine kinase genes, which are frequently found in NSHL, suggesting these diseases are pathogenetically related.[25,26]

Chemotherapy: the foundation of treatment

The addition of doxorubicin to CVP in the early 1970s ushered in the first curative regimen (CHOP) for DLBCL. Since that time, anthracyclines have been established as an essential drug class for DLBCL, and are included in all up-front curative regimens. The empiric addition of drugs to CHOP did not improve the cure rate of DLBCL, as shown by the landmark randomized study comparing CHOP to 2nd and 3rd generation regimens in 1993.[27] Later attempts had only qualified success. The Deutsche Studiengruppe für Hochmaligne Non-Hodgkin Lymphome (DSHNHL) four arm studies of CHOP every 14 or 21 days, with or without etoposide (CHOEP), in patients over 60 years of age and low-risk patients aged 60 years or under showed a benefit of CHOEP-21 in younger patients and CHOP-14 in older patients.[28,29] These benefits, however, were lost when similar trials were done with rituximab.[30,31] They also performed a randomized study of 6 *versus* 8 cycles of CHOP-14, with or without rituximab, in elderly patients with DLBCL.[31] In that study, termed RICOVER-60, there was no difference between 6 and 8 cycles of treatment, but the authors suggested that R-CHOP-14 should be the new standard.[31] This conclusion was based on a historical comparison of R-CHOP-14 with R-CHOP-21, but when assessed in two randomized trials (GELA and UK), was found to be incorrect.[32] Hence, R-CHOP-21 continues to be the standard.

The GELA group recently reported a randomized study of dose intense R-ACVBP *versus* R-CHOP-21 in patients under 60 years of age with low-risk International Prognostic Index (IPI).[33] At three years, the PFS of R-ACVBP was 87% compared to 73% for R-CHOP, and this difference was significant; though significant hematologic toxicity limits its use to younger patients. While this study confirms that the R-CHOP platform can be improved, the clinical limitations of R-ACVBP and the absence of information on its performance within the molecular subtypes of DLBCL restricts its use as a universal platform to replace R-CHOP. Other dose intensity approaches have also been studied as initial therapy in DLBCL. A dose intensified R-CHOP showed a failure free survival of 65% at three years in high-risk DLBCL but with several toxic deaths, suggesting it is not an optimal approach.[34] Autologous transplant showed some benefit in the pre-rituximab era, but the lack of substantive evidence for benefit in the rituximab era and its considerable toxicities make it an unacceptable standard of care.[35] The DA-EPOCH-R regimen was developed from *in vitro* modeling of drug resistance and drug pharmacodynamics, and employs infusional drug scheduling, topoisomerase II targeting, and pharmacodynamic dosing.[36-38] In a National Cancer Institute study of 72 patients, 80% and 79% of patients were progression-free and alive, respectively, at the median follow up of 5 years.[39] More recently, a multi-center Cancer and Leukemia Group B (CALGB) co-operative group study of 69 patients reported a 5-year time to progression (TTP) and overall survival of 81% and 88%, respectively.[40] Notably, DA-EPOCH-R showed superior outcome within all IPI and age groups compared to published reports of R-CHOP, suggesting the overall outcomes were not due to accrual of low-risk patients. The outcome of DA-EPOCH-R was also assessed within the GCB and post-GCB molecular subtypes; TTP was 100% in GCB and 67% in non-GCB DLBCL at 62 months.[38] Other phase II trials have reported similarly promising results with DA-EPOCH-R.[41-43] A randomized comparison of DA-EPOCH-R and R-CHOP with analysis of outcome within the molecular subtypes of DLBCL is nearing completion. While the DA-EPOCH-R regimen presents more logistical administration issues than R-CHOP, its serious toxicity profile is similar to R-CHOP, allowing its use in all age groups.

The modern era: germinal center B-cell and activated B-cell DLBCL pathobiology

The establishment of ABC and GCB DLBCL as pathogenetically distinct diseases with different outcomes raises the need for dedicated clinical studies (Figure 1B).[10] GCB DLBCL is exclusively associated with the t(14;18) translocation involving *bcl-2* and amplification of the *c-rel* locus and frequent abnormalities of Bcl-6.[10,44-46] In contrast, ABC DLBCL is characterized by constitutive activation of the NF-kB.[47-49] Activation of NF-kB is downstream of a number of B-cell receptor (BCR) pathway proteins including Bruton's Tyrosine Kinase (Btk) and CARD11, which activate IkB kinase and NF-kB (Figure 2A).[47-50] For the majority of ABC DLBCL cases, NF-kB activation can be observed in the absence of activating CARD11 mutations, suggesting it may be linked to chronic active BCR signaling.

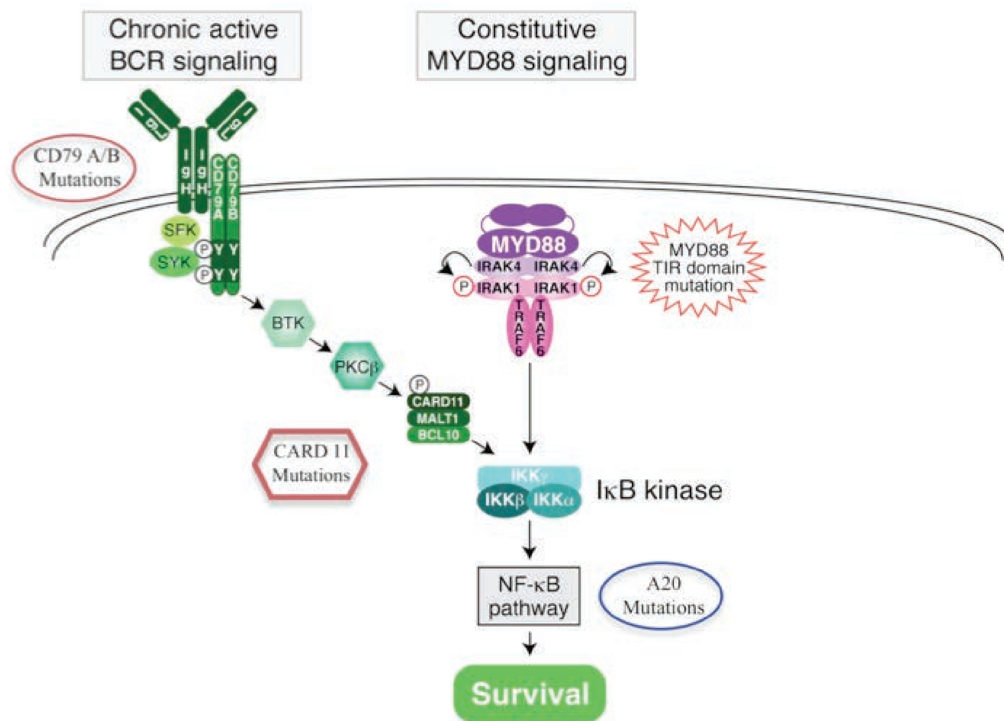
Using RNA interference screening (shRNAi), Staudt *et al.* showed that targeting BTK resulted in significant *in vitro* antiproliferative activity in ABC but not GCB DLBCL (Figure 2B). Furthermore, shRNA-targeting Btk was ineffective in ABC DLBCL cell lines that contained mutant CARD11, which is downstream of Btk. To provide genetic evidence of BCR signaling in the pathogenesis of ABC DLBCL, genes in the BCR pathway in DLBCL cell lines and biopsies were sequenced.[50] Missense mutations in the CD79B protein of the BCR were identified in two cell lines and in 21% of ABC DLBCL and 3% of GCB DLBCL tumor biopsies.[50] These results suggest that a significant percentage of ABC DLBCL may have a heightened BCR antigenic response, leading to abnormal activation of NF-kB. BCR signaling also activates the PI3K/Akt/mTOR signaling pathway with effects on apoptosis, proliferation and metabolism. A recent study also shows ABC DLBCL has a dependence on MYD88, an adaptor protein that mediates TOLL and interleukin (IL)-1 receptor signaling, and oncogenic mutations in MYD88 (Figure 2A).[51] RNAi screening showed that MYD88 and the interleukin-1 receptor-associated kinases (IRAK), IRAK1 and IRAK4, are indispensable for ABC DLBCL survival. Analysis of ABC DLBCL tumors revealed 29% harbored mutations in MYD88, which were shown to be gain-of-function driver mutations. These results indicate that the MYD88 signaling pathway is important for the pathogenesis of some ABC DLBCL, and supports the development of inhibitors. It is important to note that the molecular distinctions between the GCB and ABC DLBCL subtypes have yet to have clinical application. However, they are critically important to advance the targeted treatment of DLBCL. In this regard, the best practical method(s) for identifying these subtypes remains a matter of controversy. While GEP remains the gold standard, it has obvious practical limitations for clinical practice. In its place, investigators have developed immunohistochemical models that have had variable reproducibility, but have successfully distinguished GCB from non-GCB DLBCL in a number of clinical trials.[52-54] Recent advances in paraffin-based gene expression profiling will likely emerge as the new standard due to its ability to replicate the validated GEP expression signatures for GCB and ABC DLBCL.

Leveraging biology: activated B-cell DLBCL

The constitutive activation of NF-kB activates genes associated with survival and proliferation. To help assess the clinical utility of this target, Staudt *et al.* treated showed that ABC DLBCL cell lines were differentially sensitive to an IkB kinase inhibitor, which is necessary for NF-κB activation (Figure 3A).[55] Dunleavy *et al.* undertook a 'proof of principal' clinical study to test whether inhibition of NF-κB might sensitize ABC but not GCB DLBCL to chemotherapy.[56,57] Based on *in vitro* evidence that bortezomib, a proteasome inhibitor, blocked degradation of phosphorylated IkBa and consequently inhibited NF-kB activity in ABC DLBCL cell lines (*data not shown*), bortezomib was combined with DA-EPOCH in patients with relapsed/refractory DLBCL.[58-60] Tumor tissue was analyzed to identify molecular DLBCL subtypes. Patients with ABC DLBCL had a significantly higher response (83% *vs.* 13%; P=0.0004) and median overall survival (10.8 *vs.* 3.4 months; P=0.0026) compared to GCB DLBCL (Figure 3B). These results provide a rational therapeutic approach based on genetically distinct DLBCL subtypes.[61] Based on these studies, several randomized studies of R-CHOP with or without bortezomib in untreated DLBCL patients have been instituted.

Lenalidomide, an immune modulatory agent, may also have activity in ABC DLBCL.[62,63] As a single agent, lenalidomide demonstrated a response rate of 55% in patients with ABC DLBCL compared with only 9% in patients with GCB DLBCL, suggesting differential activity.[62] *In vitro*, lenalidomide selectively kills ABC DLBCL cells by augmenting interferon b (IFNb) production through its effects on IRF4.[63] In ABC DLBCL cell lines, lenalidomide leads to the reduction of IRF4 which requires the expression of the E3 ubiquitin ligase complex co-receptor protein, cereblon.[64] It is also important to understand and target upstream targets involved in NF-κB activation (Figure 2A). Chronic BCR signaling, and activating mutations of CARD11 and MYD88 promote NF-κB activation, suggesting a number of targets. One potential target is Bruton's tyrosine kinase (Btk), where the selective inhibitor, ibrutinib, is selectively toxic to cell lines with chronic active BCR signaling (Figure 2B).[50] Importantly, the position of molecular lesions in the BCR and MYD88 signaling pathways could help guide therapy of ABC DLBCL.

Based on these studies, a phase II multicenter study of the Btk inhibitor, ibrutinib, was performed in patients with relapsed/refractory DLBCL. Objectives were to assess if ibrutinib had differential activity in ABC *versus* GCB DLBCL, and the role of MYD88, CARD11 and CD79 mutations on overall response rate. Seventy patients were enrolled with a median age of 64 years and 3 (range 1-7) prior regimens. Based on GEP analysis, there were 29 ABC, 20 GCB and 21 unclassified/unknown patients. Overall, 23% responded (41% ABC and 5% GCB DLBCL; P=0.007) supporting the hypothesis for the role of BCR signaling in ABC but not GCB DLBCL.[50] Furthermore, there was a trend toward improved overall survival in patients with ABC compared to GCB DLBCL (9.76 *vs.* 3.35 months; P=0.099). The investigators also assessed the relationship between mutations and overall response rate in a pilot analysis (Figure 4). Notably,



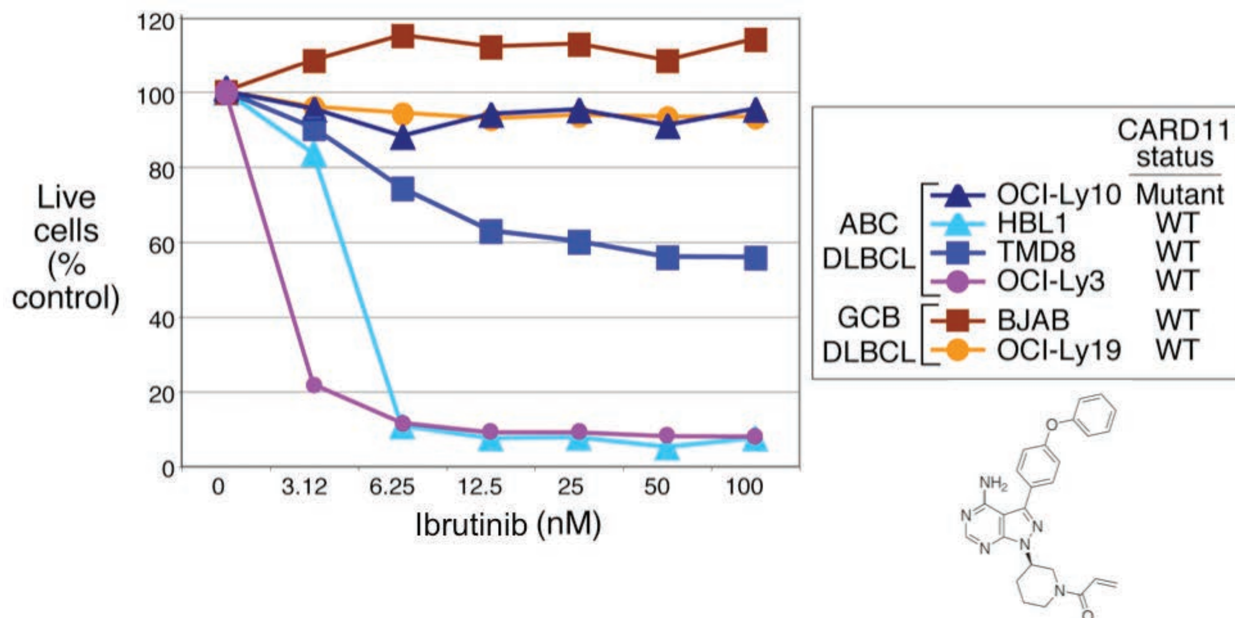
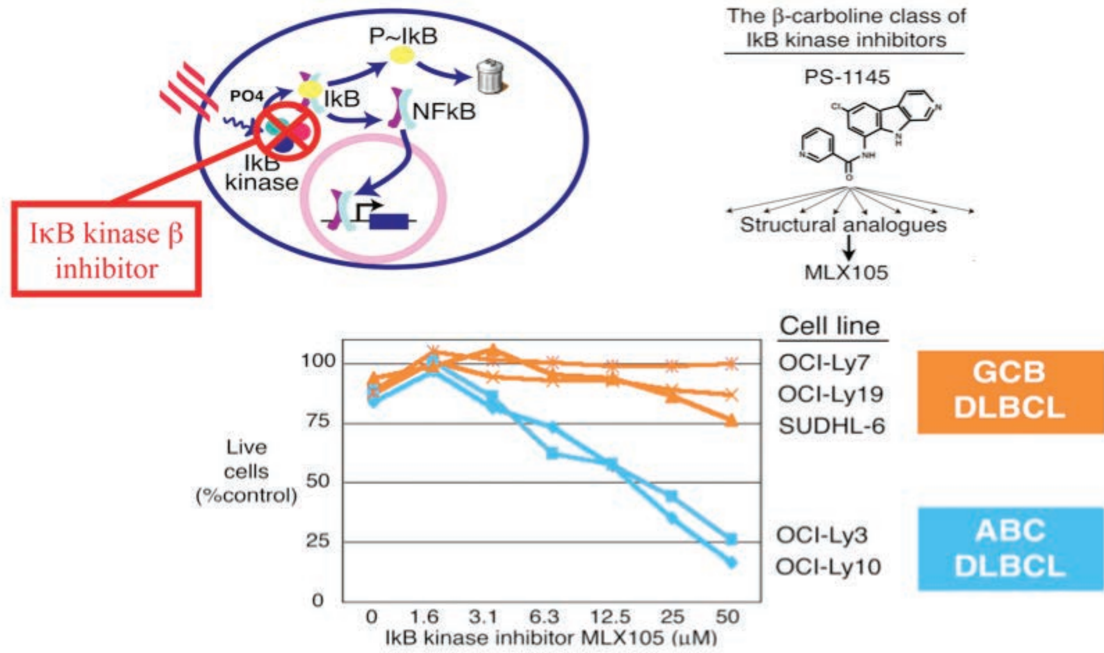
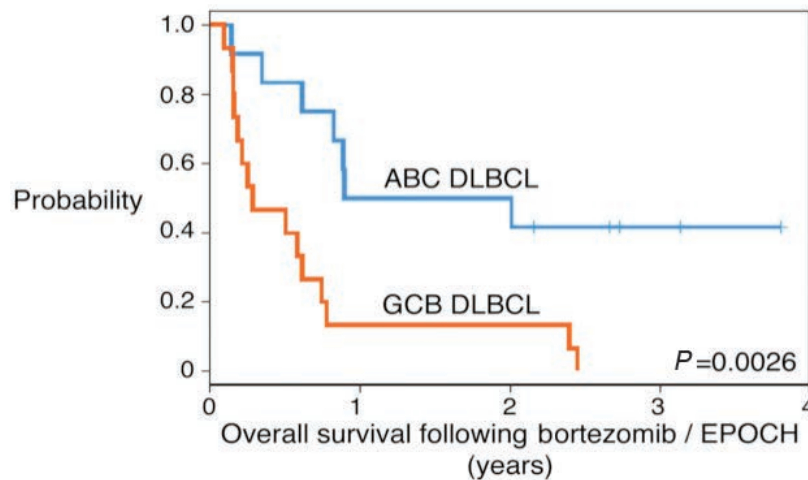


Figure 2. B-cell receptor (BCR) and MYD88 signaling pathways and potential targets. (A) Signaling through BCR leads to downstream activation of the NFκB transcription factor, which is a driver pathway in ABC DLBCL. Signaling also activates the AKT/MTOR and MAP kinase pathways. Constitutive MYD88 signaling is an alternative pathway leading to NFκB activation. (B) Inhibition of BTK by ibrutinib is toxic in ABC but not GCB DLBCL cell lines, providing evidence for the clinical relevance of the BCR signaling pathway.

A Validation of the NF-kB pathway as a therapeutic target in activated B cell-like diffuse large B-cell lymphoma[47]



B Response and survival is superior in ABC DLBCL than GCB DLBCL in the bortezomib / EPOCH phase II trial



Subtype	Total	Complete response	Partial response	No response	*P*-value
ABC DLBCL	12	5 (41.7%)	5 (41.7%)	2 (17%)	0.0004
GCB DLBCL	15	1 (6.5%)	1 (6.5%)	13 (87%)	

Figure 3. Inhibition of NFκB may promote ABC DLBCL cell death. (A) Inhibition of IκB kinase by MLX105 is lethal to ABC but not GCB DLBCL cell lines, supporting the importance of constitutive NFκB activation in ABC DLBCL and its clinical importance. (B) Outcome of a clinical trial of DA-EPOCH with bortezomib in 27 patients with relapsed/refractory ABC and GCB DLBCL. Overall survival of patients with ABC or GCB DLBCL showed a median survival of 10.8 and 3.4 months, respectively (*P*=0.0026). Patients with ABC DLBCL also had a significantly higher complete and overall response rate compared to patients with GCB DLBCL.

responses were documented in 71% (5 of 7) of patients with mutant CD79B and 34% (10 of 29) of patients with wild-type CD79B, suggesting chronic BCR signaling. Interestingly, 80% (4 of 5) of patients with both mutant CD79B and MYD88 responded whereas patients with wild-type CD79B and mutant MYD88 did not respond, suggesting an MYD88 independent pathway for NF-κB activation. Furthermore, patients with CARD11 mutations did not respond, suggesting the dominance of downstream signaling (W Wilson *et al*., personal communication, 2012).

PKCβ is a serine/threonine kinase amplified through the BCR signaling pathway that may also play an essential role in the activation of the NF-κB pathway in B cells (Figure 4A).[65] Gene expression profiling identified PKCβ as an unfavorable prognostic marker in DLBCL,[66] and *in vitro* evidence supported PKCβ as a rational therapeutic target in DLBCL.[67] Enzastaurin is a potent oral inhibitor of PKCβ that has been studied in relapsed/refractory DLBCL[68] as well as in combination with R-CHOP in patients with intermediate and high-risk DLBCL.[69] Unfortunately, it has shown little activity.

There are also studies that have targeted the PI3K/AKT/mTOR signaling pathway using mTOR inhibitors. Although the patients have been heterogeneous, mTOR inhibitors (temsirolimus and everolimus) have induced complete remissions across lymphoma subtypes.[70,71] These results suggested different types of lymphomas are dependent on an activated PI3K/AKT/mTOR pathway, including DLBCL. Although the ideal target for the PI3K/AKT/mTOR pathway is unknown, investigators are targeting upstream molecules such as AKT and PI3K. GS 1101 (formerly CAL 101) is a potent small molecule inhibitor of PI3K p110δ that blocks constitutive PI3K signaling *in vitro*.[72] GS 1101 was studied in 9 patients with DLBCL and was well tolerated, but did not result in clinical responses.[73]

Alternative activation of the classical NF-κB signaling pathway occurs through stimulation of MYD88 (Figure 2A). MYD88 mutations are present in 30% of ABC DLBCL cases and promote NF-κB activation through this pathway via the kinase activity of IRAK1 and IRAK4.[74] In ABC DLBCL cell lines, it is the activity of IRAK4 but not IRAK1 that is required for the oncogenic effect of MYD88.[74] Small molecule inhibitors of IRAK4 have demonstrated selective toxicity for ABC DLBCL cell lines and represent another potential therapeutic target in ABC DLBCL.[75]

Another important target activated by MYD88 is the janus activated kinase (JAK)/STAT signaling pathway.[74] STAT3 expression and activation are significantly higher in ABC cell lines than GCB cell lines and may play a role in the chemoresistance of this subset.[76-78] The STAT proteins are a family of transcription factors important for the regulation of cellular events such as proliferation and survival.[79] The secretion of IL-6 and IL-10 in ABC DLBCL promotes survival through the activation of the JAK family of kinases (JAK1, JAK2, JAK3, and TYK2).[80] A gene expression signature demonstrated that approximately half of ABC DLBCLs demonstrate elevated activity of STAT3 activity (STAT3-high) and have higher NF-κB activity than those with low STAT3 activity (STAT3-low) as well

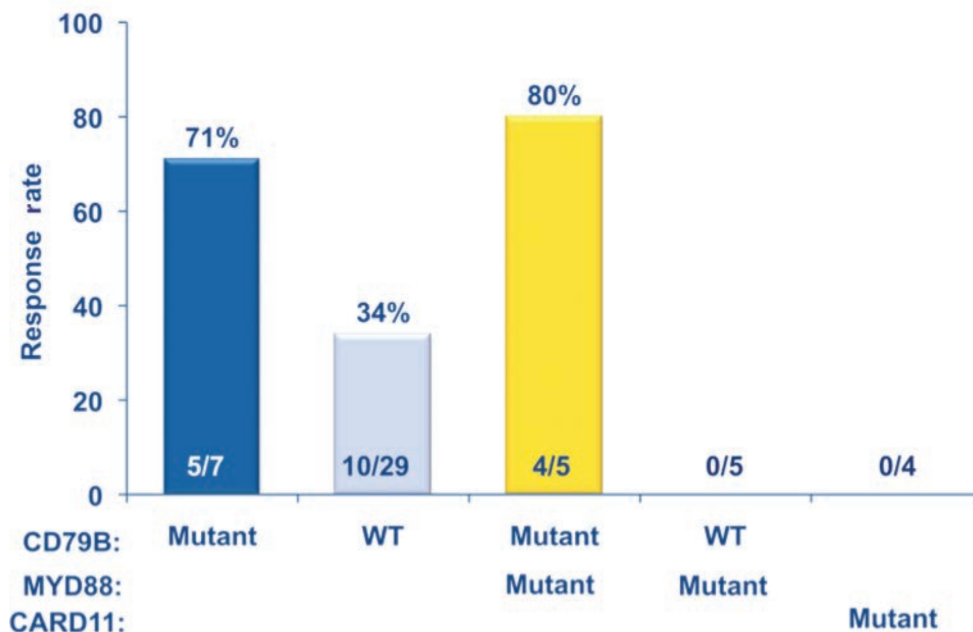


Figure 4. Blockade of BCR signaling in ABC DLBCL with ibrutinib, an irreversible inhibitor of BTK. Pilot analysis of ABC DLBCL gene mutations and response to ibrutinib.

as increased expression of proliferation genes.[76] Selective inhibition of STAT3 signaling resulted in dose-dependent cellular death in ABC but not GCB cell lines.[76] Direct therapeutic targeting of STAT3 signaling poses a challenge since it lacks its own enzymatic activity. Ruxolitinib is an oral selective inhibitor of JAK1 and JAK2 that has gained FDA approval in the United States for the treatment of primary myelofibrosis (PMF).[81,82]

Leveraging biology: germinal center B-cell DLBCL

While GCB DLBCL has a better prognosis than ABC DLBCL, over 30% are not cured with R-CHOP-based treatment (Figure 1A). Bcl-6 is a key transcription factor expressed by germinal center B cells, including GCB DLBCL, that regulates cell growth and apoptosis.[83,84] Bcl-6 suppresses genes involved in lymphocyte activation, differentiation, cell cycle arrest, and DNA damage response genes, p53 and ATR.[83] In GCB DLBCL, chromosomal translocations affecting the Bcl-6 locus juxtapose heterologous promoters from the partner chromosome with intact Bcl-6 coding sequences, leading to deregulated expression of Bcl-6; additionally, Bcl-6 can be altered by multiple somatic mutations. These mutations/translocations in Bcl-6 enhance its inhibitory effect on the apoptotic stress response and promote proliferation, both of which are associated with treatment failure.[83,85-88]

These results suggest that Bcl-6 is an important target for GCB DLBCL. BCL6 is difficult to target directly and is best affected by targeting protein-protein interactions. Recently, a small molecule known as 79-6 complex was identified that specifically disrupts the activity of BCL6 by blocking its co-repressors.[89] 79-6 was able to selectively induce apoptosis in DLBCL cell lines that were BCL6-dependent but not those that were BCL6-independent. In a xenograft model in mice, 79-6 induced tumor shrinkage validating BCL6 inhibition as a rational therapeutic strategy for GCB DLBCL tumors.[89] Targeting other Bcl-6 domains or using histone deacetylase inhibitors to overcome Bcl-6 repression of p53 and cell cycle inhibitory proteins may also be potentially useful, and are under investigation.[46]

A potentially important observation is the effect of topoisomerase II inhibition on Bcl-6 expression. Inhibition of topoisomerase II by etoposide leads to downregulation of Bcl-6 expression through ubiquitin-mediated protein degradation and possibly transcriptional inhibition.[90] This could account for the *in vitro* finding that sustained exposure of tumor cells to etoposide and low-dose doxorubicin promote the p53-p21 pathway and activates the check-point kinase (Chk2), effects that are inhibited in cells engineered to over-express Bcl-6.[91,92] This raises the possibility that inhibition of topoisomerase II may be important in GCB DLBCL. This may partially explain the finding by the German co-operative group (DSHNHL) that the addition of etoposide to CHOP (CHOEP) improved the event-free survival of younger patients, who have a higher incidence of GCB DLBCL, compared to older patients.[9,29,93] Although the benefit of etoposide in CHOEP was lost when rituximab was added (R-CHOPE), these results nonetheless suggest that topoisomerase inhibition is important.[30] The association between topoisomerase II inhibition and inhibition of Bcl-6 raises the hypothesis of whether regimens that more effectively inhibit topoisomerase II would be more effective in GCB DLBCL, even in the setting of rituximab. In this regard, the DA-EPOCH-R regimen inhibits topoisomerase II through several strategies: 1) incorporates two topoisomerase II inhibitors, etoposide and doxorubicin; 2) optimizes topoisomerase II inhibition through a prolonged 96-h infusion; 3) maximizes steady state concentrations through pharmacodynamic dose adjustment.[37] Interestingly, an analysis of outcome of GCB DLBCL in two DA-EPOCH-R trials showed a 95% EFS at five years in HIV+ GCB DLBCL, and a 100% EFS at 5-years in GCB DLBCL (CALGB study).[38,41] These studies suggest that DA-EPOCH-R may be particularly effective in GCB DLBCL, in part due to its effective inhibition of topoisomerase II and Bcl-6.

MYC is another potentially important target that is expressed in both GCB and ABC DLBCL and its expression level is associated with tumor proliferation.[9,94] Recent studies have shown that up to 10% of DLBCL cases harbor myc translocations, mostly in GCB DLBCL, which lead to high protein expression and are associated with a poor outcome with standard R-CHOP treatment.[95,96] The Myc oncoproteins (c-Myc, N-Myc, and L-Myc) have generally been considered 'undrugable' targets because the protein structures are not amenable to small molecule inhibition. However, recent epigenetic manipulation of the BET bromodomain protein BRD4 by the compound JQ1 has demonstrated exciting promise in inhibiting c-Myc in murine models of multiple myeloma.[97,98] Since bromodomain proteins serve as regulatory factors for c-Myc, this indirect approach may alter gene expression. Another mechanism by which Myc promotes lymphomagenesis is by suppressing the transcription of Tristetrapolin (TTP), which functions as a tumor suppressor.[99] Normal gene expression is tightly controlled by mRNA turnover that is, in turn, tightly regulated by AU-binding proteins (AUBP) that recognize AU-rich elements (ARE) within transcripts. TTP is an example of an AUBP that is suppressed in cancers with Myc involvement, and restoring TTP impairs Myc-induced lymphomagenesis and abolishes the malignant state.[99] Both of these strategies represent novel epigenetic targeting of MYC+ tumors that could potentially be combined with chemotherapy.

Bcl-2 is a drugable target expressed in both GCB and ABC DLBCL, albeit through different mechanisms. While some older studies found an association between bcl-2 expression and poor outcome in DLBCL, later studies have shown a more complex association.[86,100] The mechanism of bcl-2 overexpression has been related to its prognostic relevance in DLBCL. Gascoyne *et al.* showed that bcl-2 overexpression was only associated with a poor outcome in the absence of a t(14:18), which indicates that the mechanism of expression and not the protein itself is more relevant to prognosis.[100] This becomes more understandable when considering the relationship of bcl-2 expression to the molecular subtype of DLBCL. In GCB DLBCL, bcl-2 expression is associated with t(14:18), which is only found in GCB DLBCL, whereas in ABC DLBCL, bcl-2 overexpression is associated with gene amplification or NFkB transcriptional activation.[9,94] In this latter case, bcl-2 expression may primarily be a surrogate biomarker for ABC DLBCL, and may not in itself be an important ther-

apeutic target. More recently, Gascoyne *et al.* published a study showing that the concurrent protein expression of MYC and BCL-2 had an adverse outcome, whereas expression of either alone did not portend a worse outcome with R-CHOP.[101] While this study is only correlative, it provides additional evidence for testing inhibitors of BCL-2, such as navitoclax or ABT-199, and Myc.[102]

Primary mediastinal B-cell lymphoma: where are we therapeutically?

There is a near total absence of prospective studies in PMBL, which has led to conflicting findings and a lack of treatment standards.[103-106] Nonetheless, several observations have emerged from the literature. First, most patients with PMBL do not achieve adequate tumor control with standard immunochemotherapy, necessitating routine mediastinal radiotherapy.[105-107] Second, even with radiotherapy, which has serious late-term side effects, 20% of patients have disease progression.[103,105] Third, more aggressive chemotherapy is associated with an improved outcome.[104,105] Due to the widespread use of R-CHOP chemotherapy, it has become a *de facto* standard for PMBL.[103,104] Most strategies also incorporate consolidation radiotherapy to overcome the inadequacy of immunochemotherapy.[104,108] The most accurate assessment of R-CHOP and radiotherapy comes from a subset analysis of PMBL patients in the Mabthera International Trial Group study of R-CHOP-based treatment.[103] In 44 patients, 73% of whom received radiotherapy, the event-free survival was 78% at 34 months.[103] These results indicate that patients who receive R-CHOP-based treatment, the majority being young and female, will confront the potentially serious long-term consequences of radiotherapy.[109]

Retrospective studies have long suggested that PMBL has a better outcome with more dose intense regimens.[105] It is of interest that dose intensity appears to be important in nodular sclerosis Hodgkin's lymphoma, which is a closely related disease.[110] Based on evidence that dose intensity is important in PMBL, Dunleavy *et al*. assessed DA-EPOCH-R, a dose intense regimen, without radiotherapy in PMBL.[111,112] In a recent update of 40 patients with untreated PMBL, the EFS and OS were 95% and 100%, respectively, at the median follow up of four years. Importantly, only 2 patients required consolidation radiation treatment and no patients have progressed (K Dunleavy *et al.,* personal communication, 2011). These results suggest that DA-EPOCH-R obviates the need for radiation in most patients with PMBL, thus eliminating the risk of long-term toxicities such as secondary malignancies and heart disease. This is particularly important given that patients afflicted with PMBL are typically young and often women, and are at increased risk of breast and other cancers as well as late-term toxicities.

Although the outcome of PMBL is excellent with regimens such as DA-EPOCH-R, it would be important to further reduce the toxicity and length of treatment. Hence, targeted agents will be important to test. In this regard, JAK2 may be a potentially important target for PMBL. Trials are currently being planned to assess inhibitors of the JAK pathway in DLBCL including PMBL but no clinical data are available at this time.

References

1. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. The Non-Hodgkin's Lymphoma Pathologic Classification Project. Cancer. 1982;49:2112-35.
2. Stansfeld AG, Diebold J, Noel H, et al. Updated Kiel classification for lymphomas. Lancet. 1988;1:292-3.
3. Harris NL, Jaffe ES, Stein H, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood. 1994;84:1361-92.
4. Jaffe ES. The 2008 WHO classification of lymphomas: implications for clinical practice and translational research. Hematology Am Soc Hematol Educ Program. 2009:523-31.
5. Wilson WH, Hernandez-Ilizaliturri FJ, Dunleavy K, Little RF, O'Connor OA. Novel disease targets and management approaches for diffuse large B-cell lymphoma. Leuk Lymphoma. 2010;51(Suppl 1):1-10.
6. Tay K, Dunleavy K, Wilson WH. Novel agents for B-cell non-Hodgkin lymphoma: science and the promise. Blood Rev. 2010;24:69-82.
7. Jaffe ES, Harris NL, Stein H, Vardiman JW (eds.) Tumours of Haematopoietic and Lymphoid Tissues. In: World Health Organization Classification of Tumours (1st ed). Lyon: IARC Press; 2001.
8. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. A Predictive Model for Aggressive Non-Hodgkin's Lymphoma. N Engl J Med. 1993;329:987-94.
9. Rosenwald A, Wright G, Chan WC, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002;346:1937-47.
10. Lenz G, Wright GW, Emre NC, et al. Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. Proc Natl Acad Sci USA. 2008;105:13520-5.
11. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000;403:503-11.
12. Wilson WH, Teruya-Feldstein J, Fest T, et al. Relationship of p53, bcl-2, and tumor proliferation to clinical drug resistance in non-Hodgkin's lymphomas. Blood. 1997;89:601-9.
13. Wiestner A, Staudt LM. Towards a molecular diagnosis and targeted therapy of lymphoid malignancies. Semin Hematol. 2003;40:296-307.
14. Dalla-Favera R, Migliazza A, Chang CC, et al. Molecular pathogenesis of B cell malignancy: the role of BCL-6. Curr Top Microbiol Immunol. 1999;246:257-63.
15. Matsuyama T, Grossman A, Mittrucker HW, et al. Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). Nucleic Acids Res. 1995;23:2127-36.
16. Mittrucker HW, Matsuyama T, Grossman A, et al. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. Science. 1997;275:540-3.
17. Tschopp J, Irmler M, Thome M. Inhibition of fas death signals by FLIPs. Curr Opin Immunol. 1998;10:552-8.
18. Lenz G, Wright G, Dave SS, et al. Stromal gene signatures in large-B-cell lymphomas. N Engl J Med. 2008;359:2313-23.
19. Rosenwald A, Wright G, Leroy K, et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. J Exp Med. 2003;198:851-62.
20. Abou-Elella AA, Weisenburger DD, Vose JM, et al. Primary mediastinal large B-cell lymphoma: a clinicopathologic study of 43 patients from the Nebraska Lymphoma Study Group. J Clin Oncol. 1999;17:784-90.
21. Gonzalez CL, Medeiros LJ, Jaffe ES. Composite lymphoma. A clinicopathologic analysis of nine patients with Hodgkin's disease and B-cell non-Hodgkin's lymphoma. Am J Clin Pathol. 1991;96:81-9.
22. Savage KJ, Monti S, Kutok JL, et al. The molecular signature of mediastinal large B-cell lymphoma differs from that of other diffuse large B-cell lymphomas and shares features with classical Hodgkin's lymphoma. Blood. 2003:200306-1841.
23. Pileri SA, Zinzani PL, Gaidano G, et al. Pathobiology of primary mediastinal B-cell lymphoma. Leuk Lymphoma. 2003;44(Suppl 3):S21-26.
24. Pileri SA, Gaidano G, Zinzani PL, et al. Primary mediastinal B-cell lymphoma: high frequency of BCL-6 mutations and consistent expression of the transcription factors OCT-2, BOB.1, and PU.1 in the absence of immunoglobulins. Am J Pathol. 2003;162:243-53.
25. Joos S, Kupper M, Ohl S, et al. Genomic imbalances including

amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells. Cancer Res. 2000;60:549-52.
26. Joos S, Menz CK, Wrobel G, et al. Classical Hodgkin lymphoma is characterized by recurrent copy number gains of the short arm of chromosome 2. Blood. 2002;99:1381-7.
27. Fisher RI, Gaynor ER, Dahlberg S, et al. Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N Engl J Med. 1993;328:1002-6.
28. Pfreundschuh M. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of elderly patients with aggressive lymphomas: results of the NHL-B2 trial of the DSHNHL. Blood. 2004;104:634-41.
29. Pfreundschuh M, Trumper L, Kloess M, et al. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of young patients with good-prognosis (normal LDH) aggressive lymphomas: results of the NHL-B1 trial of the DSHNHL. Blood. 2004;104:626-33.
30. Pfreundschuh M, Trumper L, Osterborg A, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol. 2006;7:379-91.
31. Pfreundschuh M, Schubert J, Ziepert M, et al. Six versus eight cycles of bi-weekly CHOP-14 with or without rituximab in elderly patients with aggressive CD20+ B-cell lymphomas: a randomised controlled trial (RICOVER-60). Lancet Oncol. 2008;9:105-16.
32. Cunningham D, Smith P, Mouncey P, Qian W, Jack AS, Pocock C, et al. R-CHOP14 versus R-CHOP21: Result of a randomized phase III trial for the treatment of patients with newly diagnosed diffuse large B-cell non-Hodgkin lymphoma. ASCO 2011 Annual Meeting; 2011.
33. Récher C, Coiffier B, Haioun C, et al. Intensified chemotherapy with ACVBP plus rituximab versus standard CHOP plus rituximab for the treatment of diffuse large B-cell lymphoma (LNH03-2B): an open-label randomised phase 3 trial. The Lancet. 2011;378:1858-67.
34. Holte H, Leppa S, Bjorkholm M, et al. Dose-densified chemoimmunotherapy followed by systemic central nervous system prophylaxis for younger high-risk diffuse large B-cell/follicular grade 3 lymphoma patients: results of a phase II Nordic Lymphoma Group study. Ann Oncol. 2013;24(5):1385-92.
35. Metayer C, Curtis RE, Vose J, et al. Myelodysplastic syndrome and acute myeloid leukemia after autotransplantation for lymphoma: a multicenter case-control study. Blood. 2003;101:2015-23.
36. Wilson WH, Dunleavy K, Pittaluga S, et al. Phase II study of dose-adjusted EPOCH and rituximab in untreated diffuse large B-cell lymphoma with analysis of germinal center and post-germinal center biomarkers. J Clin Oncol. 2008;26:2717-24.
37. Wilson WH, Grossbard ML, Pittaluga S, et al. Dose-adjusted EPOCH chemotherapy for untreated large B-cell lymphomas: a pharmacodynamic approach with high efficacy. Blood. 2002;99:2685-93.
38. Wilson WH, Jung SH, Porcu P, et al. A Cancer and Leukemia Group B multi-center study of DA-EPOCH-rituximab in untreated diffuse large B-cell lymphoma with analysis of outcome by molecular subtype. Haematologica. 2012;97:758-65.
39. Wilson WH, Dunleavy K, Pittaluga S, et al. Phase II Study of Dose-Adjusted EPOCH and Rituximab in Untreated Diffuse Large B-Cell Lymphoma With Analysis of Germinal Center and Post-Germinal Center Biomarkers. Br J Hematol J Clin Oncol. 2008;26:2717-24.
40. Wilson WH, Jung SH, Porcu P, et al. A Cancer and Leukemia Group B multi-center study of DA-EPOCH-rituximab in untreated diffuse large B-cell lymphoma with analysis of outcome by molecular subtype. Haematologica. 2011;97:758-65.
41. Dunleavy K, Little RF, Pittaluga S, et al. The role of tumor histogenesis, FDG-PET, and short-course EPOCH with dose-dense rituximab (SC-EPOCH-RR) in HIV-associated diffuse large B-cell lymphoma. Blood. 2010;115:3017-24.
42. Garcia-Suarez J, Banas H, Arribas I, De Miguel D, Pascual T, Burgaleta C. Dose-adjusted EPOCH plus rituximab is an effective regimen in patients with poor-prognostic untreated diffuse large B-cell lymphoma: results from a prospective observational study. Br J Hematol J Clin Oncol. 2007; 136:276-85.
43. Garcia-Suarez J, Flores E, Callejas M, et al. Two-weekly dose-adjusted (DA)-EPOCH-like chemotherapy with high-dose dexamethasone plus rituximab (DA-EDOCH14-R) in poor-prognostic untreated diffuse large B-cell lymphoma. Br J Haematol. 2012 Dec 11. [Epub ahead of print]
44. Shaffer AL, Rosenwald A, Staudt LM. Lymphoid malignancies: the dark side of B-cell differentiation. Nat Rev Immunol. 2002;2:920-32.
45. Parekh S, Polo JM, Shaknovich R, et al. BCL6 programs lymphoma cells for survival and differentiation through distinct biochemical mechanisms. Blood. 2007;110(6):2067-74.
46. Parekh S, Prive G, Melnick A. Therapeutic targeting of the BCL6 oncogene for diffuse large B-cell lymphomas. Leuk Lymphoma. 2008;49:874-82.
47. Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med. 2001;194:1861-74.
48. Lenz G, Davis RE, Ngo VN, et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. Science. 2008;319:1676-9.
49. Ngo VN, Davis RE, Lamy L, et al. A loss-of-function RNA interference screen for molecular targets in cancer. Nature. 2006;441:106-10.
50. Davis RE, Ngo VN, Lenz G, et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature. 2010;463:88-92.
51. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. Nature. 2011;470(7332):115-9.
52. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004;103(1):275-82.
53. Dunleavy K, Janik J, Gea-Banacloche J, et al. Phase I/II study of Bortezomib Alone and Bortezomib with Dose-Adjusted EPOCH Chemotherapy in Relapsed or Refractory Aggressive B-Cell Lymphoma. ASH Annual Meeting Abstracts. 2004;104:1385.
54. Choi WW, Weisenburger DD, Greiner TC, et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. Clin Cancer Res. 2009;15:5494-502.
55. Lam LT, Davis RE, Pierce J, et al. Small molecule inhibitors of IkappaB kinase are selectively toxic for subgroups of diffuse large B-cell lymphoma defined by gene expression profiling. Clin Cancer Res. 2005;11:28-40.
56. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res. 2008;14:1649-57.
57. Orlowski RZ, Baldwin AS Jr. NF-kappaB as a therapeutic target in cancer. Trends Mol Med. 2002;8:385-9.
58. Allen C, Saigal K, Nottingham L, Arun P, Chen Z, Van Waes C. Bortezomib-induced apoptosis with limited clinical response is accompanied by inhibition of canonical but not alternative nuclear factor-{kappa}B subunits in head and neck cancer. Clin Cancer Res. 2008;14:4175-85.
59. Houldsworth J, Petlakh M, Olshen AB, Chaganti RS. Pathway activation in large B-cell non-Hodgkin lymphoma cell lines by doxorubicin reveals prognostic markers of in vivo response. Leuk Lymphoma. 2008;49:2170-80.
60. Strauss SJ, Higginbottom K, Juliger S, et al. The proteasome inhibitor bortezomib acts independently of p53 and induces cell death via apoptosis and mitotic catastrophe in B-cell lymphoma cell lines. Cancer Res. 2007;67:2783-90.
61. Dunleavy K, Pittaluga S, Czuczman MS, et al. Differential efficacy of bortezomib plus chemotherapy within molecular subtypes of diffuse large B-cell lymphoma. Blood. 2009;113:6069-76.
62. Hernandez-Ilizaliturri FJ, Deeb G, Zinzani PL, et al. Higher response to lenalidomide in relapsed/refractory diffuse large B-cell lymphoma in nongerminal center B-cell-like than in germinal center B-cell-like phenotype. Cancer. 2011;117:5058-66.
63. Yang Y, Shaffer AL 3rd, Emre NC, et al. Exploiting synthetic lethality for the therapy of ABC diffuse large B cell lymphoma. Cancer Cell. 2012;21:723-37.
64. Zhang LH, Kosek J, Wang M, Heise C, Schafer PH, Chopra R. Lenalidomide efficacy in activated B-cell-like subtype diffuse large B-cell lymphoma is dependent upon IRF4 and cereblon expression. Br J Haematol. 2013;160(4):487-502.
65. Saijo K, Mecklenbrauker I, Santana A, Leitger M, Schmedt C, Tarakhovsky A. Protein kinase C beta controls nuclear factor kappaB activation in B cells through selective regulation of the IkappaB kinase alpha. J Exp Med. 2002;195:1647-52.
66. Shipp MA, Ross KN, Tamayo P, et al. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med. 2002;8:68-74.
67. Rossi RM, Henn AD, Conkling R, et al. The PKC{beta} Selective Inhibitor, Enzastaurin (LY317615), Inhibits Growth of Human Lymphoma Cells. ASH Annual Meeting Abstracts. 2005;106:1483.
68. Robertson MJ, Kahl BS, Vose JM, et al. Phase II study of enzastaurin, a protein kinase C beta inhibitor, in patients with relapsed or refractory diffuse large B-cell lymphoma. J Clin Oncol.

2007;25:1741-6.
69. Hainsworth JD, Arrowsmith ER, Mccleod M, et al. Randomized Phase II Study of R-CHOP Plus Enzastaurin Versus R-CHOP in the First Line Treatment of Patients with Intermediate and High-Risk Diffuse Large B-cell Lymphoma (DLBCL) - Preliminary Analysis. Ann Oncol. 2011;22(Suppl 4):(Abstract 074).
70. Smith SM, van Besien K, Karrison T, et al. Temsirolimus has activity in non-mantle cell non-Hodgkin's lymphoma subtypes: The University of Chicago phase II consortium. J Clin Oncol. 2010;28:4740-6.
71. Witzig TE, Geyer SM, Ghobrial I, et al. Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma. J Clin Oncol. 2005;23:5347-56.
72. Lannutti BJ, Meadows SA, Herman SEM, et al. CAL-101, a p110 selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability. Blood. 2010;117:591-4.
73. Furman RR, Byrd JC, Brown JR, et al. CAL-101, An Isoform-Selective Inhibitor of Phosphatidylinositol 3-Kinase P110{delta}, Demonstrates Clinical Activity and Pharmacodynamic Effects In Patients with Relapsed or Refractory Chronic Lymphocytic Leukemia. ASH Annual Meeting Abstracts. 2010;116:55.
74. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. Nature. 2011;470:115-9.
75. Lim K-H, Romero DL, Chaudhary D, Robinson SD, Staudt LM. IRAK4 Kinase As A Novel Therapeutic Target in the ABC Subtype of Diffuse Large B Cell Lymphoma. ASH Annual Meeting Abstracts. 2012;120:62.
76. Lam LT, Wright G, Davis RE, et al. Cooperative signaling through the signal transducer and activator of transcription 3 and nuclear factor-{kappa}B pathways in subtypes of diffuse large B-cell lymphoma. Blood. 2008;111:3701-13.
77. Ding BB, Yu JJ, Yu RY, et al. Constitutively activated STAT3 promotes cell proliferation and survival in the activated B-cell subtype of diffuse large B-cell lymphomas. Blood. 2008;111:1515-23.
78. Scuto A, Kujawski M, Kowolik C, et al. STAT3 inhibition is a therapeutic strategy for ABC-like diffuse large B-cell lymphoma. Cancer Res. 2011;71:3182-8.
79. Calo V, Migliavacca M, Bazan V, et al. STAT proteins: from normal control of cellular events to tumorigenesis. J Cell Physiol. 2003;197:157-68.
80. Murray PJ. The JAK-STAT signaling pathway: input and output integration. J Immunol. 2007;178:2623-9.
81. Verstovsek S, Mesa RA, Gotlib J, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. N Engl J Med. 2012;366:799-807.
82. Verstovsek S, Kantarjian H, Mesa RA, et al. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. N Engl J Med. 2010;363:1117-27.
83. Phan RT, Dalla-Favera R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature. 2004;432:635-9.
84. Phan RT, Saito M, Basso K, Niu H, Dalla-Favera R. BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. Nat Immunol. 2005;6:1054-60.
85. Paik JH, Jeon YK, Park SS, et al. Expression and prognostic implications of cell cycle regulatory molecules, p16, p21, p27, p14 and p53 in germinal centre and non-germinal centre B-like diffuse large B-cell lymphomas. Histopathology. 2005;47:281-91.
86. Wilson WH, Teruya-Feldstein J, Fest T, et al. Relationship of p53, bcl-2, and tumor proliferation to clinical drug resistance in non-Hodgkin's lymphomas. Blood. 1997;89:601-9.
87. Ranuncolo SM, Polo JM, Dierov J, et al. Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. Nat Immunol. 2007;8:705-14.
88. Pasqualucci L, Migliazza A, Basso K, Houldsworth J, Chaganti RS, Dalla-Favera R. Mutations of the BCL6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma. Blood. 2003;101:2914-23.
89. Cerchietti LC, Ghetu AF, Zhu X, et al. A small-molecule inhibitor of BCL6 kills DLBCL cells in vitro and in vivo. Cancer Cell. 2010;17:400-11.
90. Kurosu T, Fukuda T, Miki T, Miura O. BCL6 overexpression prevents increase in reactive oxygen species and inhibits apoptosis induced by chemotherapeutic reagents in B-cell lymphoma cells. Oncogene. 2003;22:4459-68.
91. Theard D, Coisy M, Ducommun B, Concannon P, Darbon J-M. Etoposide and Adriamycin but Not Genistein Can Activate the Checkpoint Kinase Chk2 Independently of ATM/ATR. Biochem Bioph Res Co. 2001;289:1199-204.
92. Siu WY, Lau A, Arooz T, Chow JPH, Ho HTB, Poon RYC. Topoisomerase poisons differentially activate DNA damage checkpoints through ataxia-telangiectasia mutated-dependent and -independent mechanisms. Mol Cancer Ther. 2004;3:621-32.
93. Pfreundschuh M, Trumper L, Kloess M, et al. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of elderly patients with aggressive lymphomas: results of the NHL-B2 trial of the DSHNHL. Blood. 2004;104:634-41.
94. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000;403:503-11.
95. Savage KJ, Johnson NA, Ben-Neriah S, et al. MYC gene rearrangements are associated with a poor prognosis in diffuse large B-cell lymphoma patients treated with R-CHOP chemotherapy. Blood. 2009;114:3533-7.
96. Klapper W, Stoecklein H, Zeynalova S, et al. Structural aberrations affecting the MYC locus indicate a poor prognosis independent of clinical risk factors in diffuse large B-cell lymphomas treated within randomized trials of the German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL). Leukemia. 2008;22:2226-9.
97. Delmore JE, Issa GC, Lemieux ME, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell. 2011;146:904-17.
98. Chesi M, Matthews GM, Garbitt VM, et al. Drug response in a genetically engineered mouse model of multiple myeloma is predictive of clinical efficacy. Blood. 2012;120:376-85.
99. Rounbehler RJ, Fallahi M, Yang C, et al. Tristetraprolin Impairs Myc-Induced Lymphoma and Abolishes the Malignant State. Cell. 2012;150:563-74.
100. Gascoyne RD, Adomat SA, Krajewski S, et al. Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. Blood. 1997;90:244-51.
101. Johnson NA, Slack GW, Savage KJ, et al. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol. 2012;30:3452-9.
102. Wilson WH, O'Connor OA, Czuczman MS, et al. Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. Lancet Oncol. 2010;11:1149-59.
103. Rieger M, Osterborg A, Pettengell R, et al. Primary mediastinal B-cell lymphoma treated with CHOP-like chemotherapy with or without rituximab: results of the Mabthera International Trial Group study. Ann Oncol. 2011;22:664-70.
104. Savage KJ, Al-Rajhi N, Voss N, et al. Favorable outcome of primary mediastinal large B-cell lymphoma in a single institution: the British Columbia experience. Ann Oncol. 2006;17:123-30.
105. Zinzani PL, Martelli M, Bertini M, et al. Induction chemotherapy strategies for primary mediastinal large B-cell lymphoma with sclerosis: a retrospective multinational study on 426 previously untreated patients. Haematologica. 2002;87:1258-64.
106. Zinzani PL, Martelli M, Bendandi M, et al. Primary mediastinal large B-cell lymphoma with sclerosis: a clinical study of 89 patients treated with MACOP-B chemotherapy and radiation therapy. Haematologica. 2001;86:187-91.
107. Zinzani PL, Martelli M, Magagnoli M, et al. Treatment and clinical management of primary mediastinal large B-cell lymphoma with sclerosis: MACOP-B regimen and mediastinal radiotherapy monitored by (67)Gallium scan in 50 patients. Blood. 1999;94:3289-93.
108. Vassilakopoulos TP, Pangalis GA, Katsigiannis A, et al. Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone with or without radiotherapy in primary mediastinal large B-cell lymphoma: the emerging standard of care. Oncologist. 2012;17:239-49.
109. Hodgson DC. Late effects in the era of modern therapy for Hodgkin lymphoma. Hematology Am Soc Hematol Educ Program. 2011;2011:323-9.
110. Diehl V, Franklin J, Pfreundschuh M, et al. Standard and increased-dose BEACOPP chemotherapy compared with COPP-ABVD for advanced Hodgkin's disease. N Engl J Med. 2003; 348:2386-95.
111. Dunleavy K, Pittaluga S, Janik J, et al. Primary mediastinal large b-cell lymphoma (PMBL) may be significantly improved by the addition of rituximab to dose-adjusted EPOCH and obviates the need for radiation: results from a prospective study of 44 patients. Blood (ASH Annual Meeting Abstracts). 2006;108.
112. Wilson WH, Dunleavy K, Pittaluga S, et al. Dose-Adjusted EPOCH-Rituximab Is Highly Effective in the GCB and ABC Subtypes of Untreated Diffuse Large B-Cell Lymphoma. ASH Annual Meeting Abstracts. 2004;104:159.

Biology and diagnosis of Hodgkin's lymphoma

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A B S T R A C T

Modern diagnostic approaches now allow the establishment of a firm diagnosis of Hodgkin's lymphoma (HL) including the different subtypes. Entities to be considered in the differential diagnosis include T-cell lymphomas (follicular variant and angioimmunoblastic T cell lymphoma), T-cell/histiocyte rich B-cell lymphoma and progressively transformed germinal centers. Molecular techniques such as single cell investigations, gene expression and sequencing provide new insights into the biology and development of HL. In recent years, it has become more and more evident that not only T cells, but several other cell types, especially macrophages are key players in HL biology. Macrophages seem to be of prognostic relevance and show different morphologies depending on the immunological status of the patients (e.g. HIV status).

Learning goals

At the completion of this activity, participants should know that:
- new technologies allowing a precise knowledge of molecular mechanisms in HRS cells give a better understanding of the disease and diagnostic delineation;
- analysis of the microenvironment can give hints as to the immune status of the patient as well as to the predictive value of clinical behavior.

Hodgkin's lymphoma morphology

The infiltrate in Hodgkin's lymphoma (HL) is composed of only few, mostly scattered tumor cells and an abundant reactive background. The tumor cells in classic HL, the Hodgkin- and Reed-Sternberg (HRS) cells, can show single or several nuclei and usually show prominent eosinophilic nucleoli. Immunohistochemically, HRS cells strongly express CD30 (Figure 1), MUM1 and weakly PAX5. They show variable expression of markers usually found in other cell lineages including CD15,[1] fascin,[2] NOTCH1,[3] GATA3[4,5] and occasionally Granzyme B.[6] HRS cells can variably be Epstein-Barr virus (EBV)-infected. B-cell markers such as CD20, CD79a and CD19 are usually not expressed or strongly down-regulated. Weak, heterogeneous expression of CD20 in some of the HRS cells can sometimes be observed (Figure 1). HRS cells are frequently arranged around remnants of B-cell follicles or are found in the interfollicular areas. The reactive microenvironment includes mainly CD4-positive ($CD4^+$) T cells, epithelioid cells and eosinophils. T cells isolated from primary HL tissue are anergic to stimulation with mitogen and contain enriched populations of T-regulatory 1 and $CD4^+CD25^+$ regulatory T cells.[7] Reactive B-cell compartments are usually only partly preserved. Necrosis can be found at a variable frequency. Recently, a high content of macrophages in the tissue has been found to be associated with an adverse clinical behavior.[8-11] However, other studies could not confirm this finding.[12,13]

Hodgkin's lymphoma subtypes

Hodgkin's lymphoma is divided into four classical subtypes (90%-95%) and the nodular lymphocyte predominant HL (approx. 5%).

Nodular sclerosis (NSCHL)

Nodular sclerosis (NSCHL) is the most frequent subtype in the Western countries and often presents in the mediastinum. The histological picture is characterized by sclerotic bands forming macronodular compartments in the lymph node or involved tissue. In these compartments a mixed infiltrate containing HRS cells and usually abundant amounts of reactive T cells, eosinophils and some epithelioid cells and macrophages can be found. A high content of eosinophils was found to be a negative prognostic predictor.[14] HRS cells in nodular sclerosis often show a cytoplasmic retraction artifact when the tissue is formalin fixed and paraffin embedded; therefore, these cells are also called lacunar cells. In nodular sclerosis, HRS cells are more often EBV-negative, but this is largely dependent on geographical location. A subset of cases shows a syncytial growth pattern of HRS cells, usually around residual regressive follicles.

Mixed cellularity

In mixed cellularity (MCCHL) subtype, the neoplastic infiltrate is usually found in the interfollicular areas. Germinal centers can be preserved when there is early involvement of

lymph nodes. The neoplastic infiltrate expands from the interfollicular areas and destroys the B-cell areas. The reactive microenvironment usually consists of high numbers of T cells, eosinophils and epithelioid cells. Other cases show high amounts of macrophages. The HRS cells are more often EBV-infected. The mixed cellularity type is most frequently found either in young children or elderly and immunocompromised individuals.

Lymphocyte depleted subtype

Lymphocyte depleted subtype (LDCHL) is a very rare subtype. Like mixed cellularity subtype it predominantly occurs in immunocompromised patients. The HRS cells are often EBV-infected. Some of the cases previously diagnosed as lymphocyte depleted subtype may nowadays be better placed in other categories like nodular sclerosis with a high tumor cells content or gray zone lymphoma between Hodgkin's lymphoma and diffuse large B-cell lymphoma.[15]

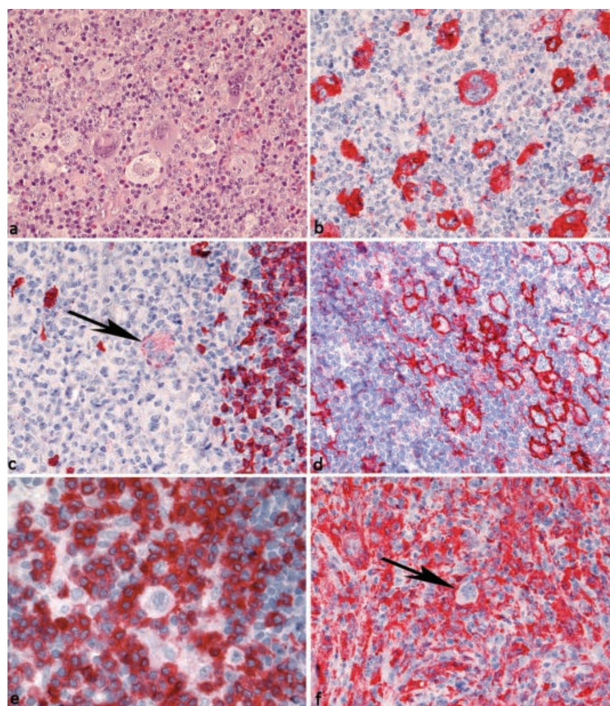

Figure 1. Classic Hodgkin's lymphoma. (A) Typical multinuclear Reed-Sternberg cells with a background of T cells, epithelioid cells and eosinophils in a nodular sclerosing HL (HE, 200x). (B) HRS cells are strongly positive for CD30 (CD30-immunostaining, 200x). (C) Some HRS cells can weakly express CD20 (arrow). Interestingly, the staining intensity is much weaker than in the reactive small B cells (right, CD20-immunostaining, 200x). (D) HRS cells in a nodular sclerosing HL strongly express the glucose transporter GLUT1 (GLUT1-immunostaining, 200x). (E) HRS cells show rosetting by T cells in a mixed cellularity HL (HIV-negative, CD3-immunostaining, 200x). (F) HIV-associated mixed cellularity HL with a high amount of macrophages, rosetting around an HRS cell (arrow, CD163-immunostaining, 200x).

Lymphocyte rich subtype

The lymphocyte rich subtype (LRCHL) is another rare variant of HL. It occurs relatively frequently in the Waldeyer´s ring. Patients are most often diagnosed in stage I.[16] In this particular subtype, HRS cells are located in the B-cell nodules or in enlarged mantle zones surrounding reactive germinal centers, which may be preserved. Eosinophils can occasionally be observed. The content of macrophages is relatively low in this subtype. HRS cells can be positive for EBV.

Nodular lymphocyte predominant Hodgkin's lymphoma

Nodular lymphocyte predominant Hodgkin's lymphoma (NLPHL) is the only non-classic HL subtype and it differs from classical HL in terms of the immunophenotype of tumor cells, molecular findings and clinical behavior. The tumor cells in NLPHL, the lymphocyte predominant cells or LP cells, have a preserved B-cell phenotype, although it can be partially down-regulated (Figure 2).[17,18] Like in LRCHL, the LP cells show prominent rosetting of follicular T-helper cells (PD-1$^+$) and, therefore, keep their germinal center derived microenvironment in contrast to the other classic HL subtypes.[19-21] On clinical grounds, patients with NLPHL often show slowly growing, massively enlarged lymph nodes. Axillary lymph nodes are frequently involved. Middle-aged male patients are often affected.[22] Like LRCHL, patients are often diagnosed in stage I.[16] Whereas the overall survival of patients is excellent, relapses are more common than in LRCHL.[23-25] Transformation into diffuse large B-cell lymphoma occurs in up to 30% in 20 years.[26,27] Variants of NLPHL with a diffuse growth pattern are associated with a higher relapse risk.[28,29]

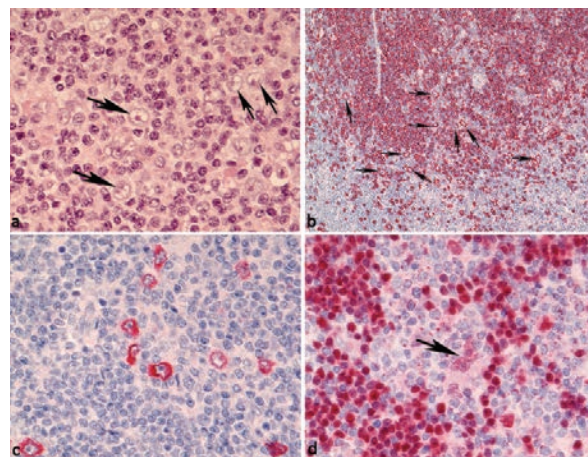

Figure 2. Nodular lymphocyte predominant Hodgkin's lymphoma. (A) LP cells (arrows) show popcorn-like nuclei. They are surrounded by stimulated T lymphocytes with slightly enlarged nuclei and open chromatin (HE, 200x). (B) CD20-positive LP cells (arrows) are found in a nodule of CD20-positive small B cells (CD20-immunostaining, 100x). (C) LP cells strongly express J-chain (J-chain immunostaining, 200x). (D) LP cells (arrow) show a slightly less intense PAX5-expression than the small surrounding B cells, indicating a partially down-regulated B cell phenotype (PAX5-immunostaining 200x).

Single cell polymerase chain reaction

Since HRS cells have usually lost their B-cell immunophenotype and express various markers of other cell lineages, the nature of HL remained unclear for many years. In 1994, Küppers *et al.*[30] could show for the first time in 3 cases of classic HL and one case of NLPHL that HRS and LP cells are clonally related B cells. In further studies, HRS cells were shown to be derived from crippled pre-apoptotic germinal center B cells which do not express functional antigen receptors.[31] In contrast, LP cells in NLPHL showed ongoing somatic hypermutation of the *IGH* locus producing functional immunoglobulin heavy and light chains, indicating that they are derived from, and still closely related to, germinal center B cells.[32] Interestingly, these molecular findings correlate very well with the localization of the LP cells in the follicular compartment and the expression of J-chain (Figure 2) as well as kappa light chain restriction.[33] HRS cells in classic HL neither express light chains nor J-chain and are found in the extrafollicular region in the affected lymph node.

Gene expression in Hodgkin's lymphoma

Gene expression analysis of primary HRS cells was for a long time hampered by the low tumor cell content in the tissue. In early gene expression studies,[34] HL cell lines displayed an activated B-cell signature similar to EBV-infected lymphoblastoid cell lines and activated B-cell (ABC)-type DLBCL. Recently, gene expression studies of primary microdissected HRS cells could be performed.[17,35,36] In one study, a group of HL cases showing upregulation of MYC-, NOTCH1- and IRF4-target genes could be identified.[35] Interestingly, in the same study, no major differences were found in the gene expression of EBV-positive and EBV–negative HL cases. One explanation may be that NF-κB activity, which is LMP1-driven in EBV-infected HRS cells, can be acquired by mutations of NF-κB inhibitors like TNFAIP3/A20[37] more common in the HRS cells of EBV-negative HL cases and consequently the gene expression profiles of EBV-positive and EBV-negative cases are similar. Another study showed that HL patients in which HRS cells show a macrophage-like signature including the lineage-inappropriate expression of CSF1R, had significantly inferior progression-free and overall survival.[38] CSF1R expression was correlated with mixed cellularity subtype and a high macrophage content in the tissue. The first gene expression study on microdissected LP cells showed that they show a partially down-regulated B-cell phenotype and constitutive NF-κB activity.[17] These cells were most closely related to the tumor cells of T-cell/histiocyte rich large B-cell lymphoma.

Mutations and deregulated pathways in Hodgkin's lymphoma

Two major deregulated pathways in HL are NF-κB and the JAK-STAT-signaling pathway. To activate these pathways, HRS cells use different mechanisms including NF-κB activation by the EBV-encoded latent membrane protein 1. EBV-negative HL cases frequently show mutations of the NF-κB inhibitor TNFAIP3/A20.[37] In different cases, mutations of other NF-κB inhibiting factors like IκBα, IκBε, CYLD and TRAF3 were found.[39-41] Loci covering REL as well as other important NF-κB factors frequently show genomic gains in HRS cells.[42-45] Although NF-κB activity has been observed in the LP cells of NLPHL[17], mutations of TNFAIP3/A20 and IκBα were only rarely found.[46] Similar findings of genomic aberrations and mutations were made concerning the JAK-STAT-signaling pathway, as HRS cells frequently present genomic gains of 9p24 including JAK2 as well as JMJD2C, PD-L1 and PD-L2.[44,47,48] A negative regulator of JAK2, SOCS1, is commonly mutated in HRS cells of classic HL and LP cells of NLPHL.[49,50] Inhibition of the JAK-STAT-pathway may, therefore, be one therapeutic option in future.[51]

HRS cells, particularly in EBV-negative cases, were shown to express several receptor tyrosine kinases,[52,53] that might in future have therapeutic implications. In many HL cases, HRS cells show a strong membrane bound expression of the glucose transporter GLUT1 (Figure 2), indicating a high glycolytic activity in the HRS cells.[54] HRS cells have many more peculiar features, such as strong expression of the Vitamin D_3 receptor,[55] which may explain hypercalcemia that is sometimes observed in HL patients.[56,57] Other deregulated genes in HL include the repression of the transcription factor FOXO1[58] as well as the expression of the c-Met oncogene, which was correlated with a favorable prognostic outcome.[59]

Hodgkin's lymphoma in HIV patients

Treatment of HIV-infection has much improved in the past years and the occurrence of aggressive B-cell lymphomas has consequently decreased with the application of combination antiretroviral therapy. However, HIV-patients still have a 10-fold increased risk for developing classic HL.[60,61] Due to the immunosuppression of patients, HRS cells are almost always EBV-infected in HIV-patients.[62] In the year before HL manifestation, HIV-patients show decreasing $CD4^+$ blood counts, indicating that $CD4^+$ T-helper cells are recruited to the lymph nodes affected by HL.[61] In several studies, a shift of the CD4/CD8 ratio in the reactive microenvironment of HIV-associated HL was described.[63-66] Since both $CD4^+$ T-helper cells and macrophages co-express the CD4-receptor and CCR5, both cell types can not only be infected by R5-HIV strains, but they are also both recruited to lymph nodes involved by HL via CCL5/RANTES, which is secreted by the HRS cells.[67] In HIV-patients with low $CD4^+$ blood counts, high numbers of macrophages are found in the tissue affected by HL[68] evoking a spindle shape appearance of the infiltrate (Figure 1) which may lead to confusion with a reactive process such as tuberculosis or other mycobacterial infection. However, under current treatment strategies, HIV-positive HL patients have almost the same prognosis as HIV-negative HL patients.[61,69]

Differential diagnosis of Hodgkin's lymphoma

In recent years, many neoplastic conditions mimicking HL have been identified. HRS cells are the hallmark of

HL, but they are not specific for the disease and thus can also occur in other benign and neoplastic entities. Therefore, a careful examination of the HRS cell morphology, their distribution in the tissue and their immunophenotype as well as the composition and the immunophenotype of the reactive bystander cells is necessary. Improved immunohistochemical techniques, as well as the application of new antibodies,[70] have led to a better definition of HL over the past years. HRS-like cells can occasionally be found in infectious mononucleosis.[71] One important neoplastic mimicker of HL are peripheral T-cell lymphomas.[72] It has been known for several years that HRS-like cells can occur in T-cell lymphomas.[73] Recently, by applying follicular T-helper cell markers, the follicular variant of peripheral T-cell lymphoma could be better defined,[74-76] which had previously often been misdiagnosed as HL.[77] Another HL look-alike is the nodal involvement of primary cutaneous CD30+ lymphoproliferative diseases or mycosis fungoides with CD30+ blasts.[78] B-CLL can also harbor HRS-like blasts, which are usually not clonally related to the B-CLL clone if they are EBV-positive.[79,80] Transformation into true HL can occur in approximately 1% of B-CLL cases,[81] but the diagnosis should only be made if in addition to the HRS cells, also the typical microenvironment with T cells, epithelioid cells and eosinophils is present. In the differential diagnosis of early involvement by NLPHL is the progressive transformation of germinal centers (PTGC). PTGCs are composed of small mantle zone B cells, but typical LP cells and epithelioid cells are characteristically absent. Sometimes it can be very challenging to distinguish residual centroblasts in PTGC from LP cells. The differential diagnosis of the diffuse variants of NLPHL includes T-cell/histiocyte rich large B-cell lymphoma (THRLBCL). This is characterized by a histiocyte-rich infiltrate including less than 10% CD20-positive blasts and a virtual absence of small B cells. Its clinical behavior is usually aggressive with a presentation in advanced stages.

References

1. Fellbaum C, Hansmann ML, Parwaresch MR, Lennert K. Monoclonal antibodies Ki-B3 and Leu-M1 discriminate giant cells of infectious mononucleosis and of Hodgkin's disease. Hum Pathol. 1988;19:1168-73.
2. Pinkus GS, Pinkus JL, Langhoff E, Matsumura F, Yamashiro S, Mosialos G, et al. Fascin, a sensitive new marker for Reed-Sternberg cells of hodgkin's disease. Evidence for a dendritic or B cell derivation? Am J Pathol. 1997;150:543-62.
3. Jundt F, Anagnostopoulos I, Forster R, Mathas S, Stein H, Dorken B. Activated Notch1 signaling promotes tumour cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. Blood. 2002;99:3398-403.
4. Küppers R, Klein U, Schwering I, Distler V, Bräuninger A, Cattoretti G, et al. Identification of Hodgkin and Reed-Sternberg cell-specific genes by gene expression profiling. J Clin Invest. 2003;111:529-37.
5. Stanelle J, Doring C, Hansmann ML, Kuppers R. Mechanisms of aberrant GATA3 expression in classical Hodgkin lymphoma and its consequences for the cytokine profile of Hodgkin and Reed/Sternberg cells. Blood. 2010;116:4202-11.
6. Asano N, Kinoshita T, Tamaru J, Ohshima K, Yoshino T, Niitsu N, et al. Cytotoxic molecule-positive classical Hodgkin's lymphoma: a clinicopathological comparison with cytotoxic molecule-positive peripheral T-cell lymphoma of not otherwise specified type. Haematologica. 2011;96:1636-43.
7. Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN, et al. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. Blood. 2004;103:1755-62.
8. Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, et al. Tumour-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med. 2010;362:875-85.
9. Tzankov A, Matter MS, Dirnhofer S. Refined prognostic role of CD68-positive tumour macrophages in the context of the cellular micromilieu of classical Hodgkin lymphoma. Pathobiology. 2010;77:301-8.
10. Kamper P, Bendix K, Hamilton-Dutoit S, Honore B, Nyengaard JR, d'Amore F. Tumour-infiltrating macrophages correlate with adverse prognosis and Epstein-Barr virus status in classical Hodgkin's lymphoma. Haematologica. 2011;96:269-76.
11. Tan KL, Scott DW, Hong F, Kahl BS, Fisher RI, Bartlett NL, et al. Tumour-associated macrophages predict inferior outcomes in classic Hodgkin lymphoma: a correlative study from the E2496 Intergroup trial. Blood. 2012;120:3280-7.
12. Azambuja D, Natkunam Y, Biasoli I, Lossos IS, Anderson MW, Morais JC, et al. Lack of association of tumour-associated macrophages with clinical outcome in patients with classical Hodgkin's lymphoma. Ann Oncol. 2012;23:736-42.
13. Harris JA, Jain S, Ren Q, Zarineh A, Liu C, Ibrahim S. CD163 versus CD68 in tumour associated macrophages of classical Hodgkin lymphoma. Diagn Pathol. 2012;7:12.
14. von Wasielewski R, Seth S, Franklin J, Fischer R, Hubner K, Hansmann ML, et al. Tissue eosinophilia correlates strongly with poor prognosis in nodular sclerosing Hodgkin's disease, allowing for known prognostic factors. Blood. 2000;95:1207-13.
15. Slack GW, Ferry JA, Hasserjian RP, Sohani AR, Longtine JA, Harris NL, et al. Lymphocyte depleted Hodgkin lymphoma: an evaluation with immunophenotyping and genetic analysis. Leuk Lymphoma. 2009;50:937-43.
16. Anagnostopoulos I, Hansmann ML, Franssila K, Harris M, Harris NL, Jaffe ES, et al. European Task Force on Lymphoma project on lymphocyte predominance Hodgkin disease: histologic and immunohistologic analysis of submitted cases reveals 2 types of Hodgkin disease with a nodular growth pattern and abundant lymphocytes. Blood. 2000;96:1889-99.
17. Brune V, Tiacci E, Pfeil I, Doring C, Eckerle S, van Noesel CJ, et al. Origin and pathogenesis of nodular lymphocyte-predominant Hodgkin lymphoma as revealed by global gene expression analysis. J Exp Med. 2008;205:2251-68.
18. Tedoldi S, Mottok A, Ying J, Paterson JC, Cui Y, Facchetti F, et al. Selective loss of B-cell phenotype in lymphocyte predominant Hodgkin lymphoma. J Pathol. 2007;213:429-40.
19. Nam-Cha SH, Roncador G, Sanchez-Verde L, Montes-Moreno S, Acevedo A, Dominguez-Franjo P, et al. PD-1, a follicular T-cell marker useful for recognizing nodular lymphocyte-predominant Hodgkin lymphoma. Am J Surg Pathol. 2008;32:1252-7.
20. Nam-Cha SH, Montes-Moreno S, Salcedo MT, Sanjuan J, Garcia JF, Piris MA. Lymphocyte-rich classical Hodgkin's lymphoma: distinctive tumour and microenvironment markers. Mod Pathol. 2009;22:1006-15.
21. Churchill HR, Roncador G, Warnke RA, Natkunam Y. Programmed death 1 expression in variant immunoarchitectural patterns of nodular lymphocyte predominant Hodgkin lymphoma: comparison with CD57 and lymphomas in the differential diagnosis. Hum Pathol. 2010;41:1726-34.
22. Diehl V, Sextro M, Franklin J, Hansmann ML, Harris N, Jaffe E, et al. Clinical presentation, course, and prognostic factors in lymphocyte-predominant Hodgkin's disease and lymphocyte-rich classical Hodgkin's disease: report from the European Task Force on Lymphoma Project on Lymphocyte-Predominant Hodgkin's Disease. J Clin Oncol. 1999;17:776-83.
23. Sextro M, Diehl V, Franklin J, Hansmann ML, Anagnostopoulos I, Marafioti T, et al. Lymphocyte predominant Hodgkin's disease—a workshop report. European Task Force on Lymphoma. Ann Oncol. 1996;7(4 Suppl):61-5.
24. Eichenauer DA, Fuchs M, Pluetschow A, Klimm B, Halbsguth T, Boll B, et al. Phase 2 study of rituximab in newly diagnosed stage IA nodular lymphocyte-predominant Hodgkin lymphoma: a report from the German Hodgkin Study Group. Blood. 2011;118:4363-5.
25. Savage KJ, Skinnider B, Al-Mansour M, Sehn LH, Gascoyne RD, Connors JM. Treating limited-stage nodular lymphocyte predominant Hodgkin lymphoma similarly to classical Hodgkin lymphoma with ABVD may improve outcome. Blood. 2011;118:4585-90.
26. Al-Mansour M, Connors JM, Gascoyne RD, Skinnider B, Savage KJ. Transformation to aggressive lymphoma in nodular lymphocyte-predominant Hodgkin's lymphoma. J Clin

Oncol. 2010;28:793-9.
27. Biasoli I, Stamatoullas A, Meignin V, Delmer A, Reman O, Morschhauser F, et al. Nodular, lymphocyte-predominant Hodgkin lymphoma: a long-term study and analysis of transformation to diffuse large B-cell lymphoma in a cohort of 164 patients from the Adult Lymphoma Study Group. Cancer. 2010;116:631-9.
28. Boudova L, Torlakovic E, Delabie J, Reimer P, Pfistner B, Wiedenmann S, et al. Nodular lymphocyte-predominant Hodgkin lymphoma with nodules resembling T-cell/histiocyte-rich B-cell lymphoma: differential diagnosis between nodular lymphocyte-predominant Hodgkin lymphoma and T-cell/histiocyte-rich B-cell lymphoma. Blood. 2003;102:3753-8.
29. Fan Z, Natkunam Y, Bair E, Tibshirani R, Warnke RA. Characterization of variant patterns of nodular lymphocyte predominant hodgkin lymphoma with immunohistologic and clinical correlation. Am J Surg Pathol. 2003;27:1346-56.
30. Küppers R, Rajewsky K, Zhao M, Simons G, Laumann R, Fischer R, et al. Hodgkin disease: Hodgkin and Reed-Sternberg cells picked from histological sections show clonal immunoglobulin gene rearrangements and appear to be derived from B cells at various stages of development. Proc Natl Acad Sci USA. 1994;91:10962-6.
31. Kanzler H, Küppers R, Hansmann ML, Rajewsky K. Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumour clone derived from (crippled) germinal center B cells. J Exp Med. 1996;184:1495-505.
32. Braeuninger A, Küppers R, Strickler JG, Wacker HH, Rajewsky K, Hansmann ML. Hodgkin and Reed-Sternberg cells in lymphocyte predominant Hodgkin disease represent clonal populations of germinal center-derived tumour B cells. Proc Natl Acad Sci USA. 1997;94:9337-42.
33. Schmid C, Sargent C, Isaacson PG. L and H cells of nodular lymphocyte predominant Hodgkin's disease show immunoglobulin light-chain restriction. Am J Pathol. 1991;139:1281-9.
34. Küppers R, Klein U, Schwering I, Distler V, Bräuninger A, Cattoretti G, et al. Identification of Hodgkin and Reed-Sternberg cell-specific genes by gene expression profiling. J Clin Invest. 2003;111:529-37.
35. Tiacci E, Doring C, Brune V, van Noesel CJ, Klapper W, Mechtersheimer G, et al. Analyzing primary Hodgkin and Reed-Sternberg cells to capture the molecular and cellular pathogenesis of classical Hodgkin lymphoma. Blood. 2012; 29;120(23):4609-20.
36. Steidl C, Diepstra A, Lee T, Chan FC, Farinha P, Tan K, et al. Gene expression profiling of microdissected Hodgkin Reed Sternberg cells correlates with treatment outcome in classical Hodgkin lymphoma. Blood. 2012; 120(17):3530-40.
37. Schmitz R, Hansmann ML, Bohle V, Martin-Subero JI, Hartmann S, Mechtersheimer G, et al. TNFAIP3 (A20) is a tumour suppressor gene in Hodgkin lymphoma and primary mediastinal B cell lymphoma. J Exp Med. 2009;206:981-9.
38. Steidl C, Diepstra A, Lee T, Chan FC, Farinha P, Tan K, et al. Gene expression profiling of microdissected Hodgkin Reed-Sternberg cells correlates with treatment outcome in classical Hodgkin lymphoma. Blood. 2012;120:3530-40.
39. Lake A, Shield LA, Cordano P, Chui DT, Osborne J, Crae S, et al. Mutations of NFKBIA, encoding IkappaB alpha, are a recurrent finding in classical Hodgkin lymphoma but are not a unifying feature of non-EBV-associated cases. Int J Cancer. 2009;125:1334-42.
40. Schmidt A, Schmitz R, Giefing M, Martin-Subero JI, Gesk S, Vater I, et al. Rare occurrence of biallelic CYLD gene mutations in classical Hodgkin lymphoma. Genes Chromosomes Cancer. 2010;49:803-9.
41. Otto C, Giefing M, Massow A, Vater I, Gesk S, Schlesner M, et al. Genetic lesions of the TRAF3 and MAP3K14 genes in classical Hodgkin lymphoma. Br J Haematol. 2012;157:702-8.
42. Joos S, Menz CK, Wrobel G, Siebert R, Gesk S, Ohl S, et al. Classical Hodgkin lymphoma is characterized by recurrent copy number gains of the short arm of chromosome 2. Blood. 2002;99:1381-7.
43. Barth TF, Martin-Subero JI, Joos S, Menz CK, Hasel C, Mechtersheimer G, et al. Gains of 2p involving the REL locus correlate with nuclear c-Rel protein accumulation in neoplastic cells of classical Hodgkin lymphoma. Blood. 2003;101:3681-6.
44. Hartmann S, Martin-Subero JI, Gesk S, Husken J, Giefing M, Nagel I, et al. Detection of genomic imbalances in microdissected Hodgkin and Reed-Sternberg cells of classical Hodgkin's lymphoma by array-based comparative genomic hybridization. Haematologica. 2008;93:1318-26.
45. Steidl C, Telenius A, Shah SP, Farinha P, Barclay L, Boyle M, et al. Genome-wide copy number analysis of Hodgkin Reed-Sternberg cells identifies recurrent imbalances with correlations to treatment outcome. Blood. 2010;116:418-27.
46. Schumacher MA, Schmitz R, Brune V, Tiacci E, Doring C, Hansmann ML, et al. Mutations in the genes coding for the NF-{kappa}B regulating factors I{kappa}B{alpha} and A20 are uncommon in nodular lymphocyte-predominant Hodgkin lymphoma. Haematologica. 2010;95:153-7.
47. Joos S, Küpper M, Ohl S, von Bonin F, Mechtersheimer G, Bentz M, et al. Genomic imbalances including amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells. Cancer Res. 2000;60:549-52.
48. Green MR, Monti S, Rodig SJ, Juszczynski P, Currie T, O'Donnell E, et al. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. Blood. 2010;116:3268-77.
49. Weniger MA, Melzner I, Menz CK, Wegener S, Bucur AJ, Dorsch K, et al. Mutations of the tumour suppressor gene SOCS-1 in classical Hodgkin lymphoma are frequent and associated with nuclear phospho-STAT5 accumulation. Oncogene. 2006;25:2679-84.
50. Mottok A, Renne C, Willenbrock K, Hansmann ML, Brauninger A. Somatic hypermutation of SOCS1 in lymphocyte-predominant Hodgkin lymphoma is accompanied by high JAK2 expression and activation of STAT6. Blood. 2007;110:3387-90.
51. Derenzini E, Lemoine M, Buglio D, Katayama H, Ji Y, Davis RE, et al. The JAK inhibitor AZD1480 regulates proliferation and immunity in Hodgkin lymphoma. Blood Cancer J. 2011;1:e46.
52. Renné C, Willenbrock K, Küppers R, Hansmann ML, Bräuninger A. Autocrine- and paracrine-activated receptor tyrosine kinases in classic Hodgkin lymphoma. Blood. 2005;105:4051-9.
53. Renne C, Hinsch N, Willenbrock K, Fuchs M, Klapper W, Engert A, et al. The aberrant coexpression of several receptor tyrosine kinases is largely restricted to EBV-negative cases of classical Hodgkin's lymphoma. Int J Cancer. 2007;120:2504-9.
54. Hartmann S, Agostinelli C, Diener J, Doring C, Fanti S, Zinzani PL, et al. GLUT1 expression patterns in different Hodgkin lymphoma subtypes and progressively transformed germinal centers. BMC Cancer. 2012;12:586.
55. Renne C, Benz AH, Hansmann ML. Vitamin D3 receptor is highly expressed in Hodgkin's lymphoma. BMC Cancer. 2012;12:215.
56. Rieke JW, Donaldson SS, Horning SJ. Hypercalcemia and vitamin D metabolism in Hodgkin's disease. Is there an underlying immunoregulatory relationship? Cancer. 1989;63:1700-7.
57. Jacobson JO, Bringhurst FR, Harris NL, Weitzman SA, Aisenberg AC. Humoral hypercalcemia in Hodgkin's disease. Clinical and laboratory evaluation. Cancer. 1989;63:917-23.
58. Xie L, Ushmorov A, Leithauser F, Guan H, Steidl C, Farbinger J, et al. FOXO1 is a tumour suppressor in classical Hodgkin lymphoma. Blood. 2012;119:3503-11.
59. Xu C, Plattel W, van den Berg A, Ruther N, Huang X, Wang M, et al. Expression of the c-Met oncogene by tumour cells predicts a favorable outcome in classical Hodgkin's lymphoma. Haematologica. 2012;97:572-8.
60. Biggar RJ, Jaffe ES, Goedert JJ, Chaturvedi A, Pfeiffer R, Engels EA. Hodgkin lymphoma and immunodeficiency in persons with HIV/AIDS. Blood. 2006;108:3786-91.
61. Bohlius J, Schmidlin K, Boue F, Fatkenheuer G, May M, Caro-Murillo AM, et al. HIV-1-related Hodgkin lymphoma in the era of combination antiretroviral therapy: incidence and evolution of CD4(+) T-cell lymphocytes. Blood. 2011;117:6100-8.
62. Audouin J, Diebold J, Pallesen G. Frequent expression of Epstein-Barr virus latent membrane protein-1 in tumour cells of Hodgkin's disease in HIV-positive patients. J Pathol. 1992;167:381-4.
63. Thompson LD, Fisher SI, Chu WS, Nelson A, Abbondanzo SL. HIV-associated Hodgkin lymphoma: a clinicopathologic and immunophenotypic study of 45 cases. Am J Clin Pathol. 2004;121:727-38.
64. Bosch Princep R, Lejeune M, Salvado Usach MT, Jaen Martinez J, Pons Ferre LE, Alvaro Naranjo T. Decreased number of granzyme B+ activated CD8+ cytotoxic T lymphocytes in the inflammatory background of HIV-associated Hodgkin's

lymphoma. Ann Hematol. 2005;84:661-6.
65. Pelstring RJ, Zellmer RB, Sulak LE, Banks PM, Clare N. Hodgkin's disease in association with human immunodeficiency virus infection. Pathologic and immunologic features. Cancer. 1991;67:1865-73.
66. Kiyasu J, Aoki R, Tanaka PY, Pracchia LF, Calore EE, Perez NM, et al. FOXP3(+) regulatory and TIA-1(+) cytotoxic T lymphocytes in HIV-associated Hodgkin lymphoma. Pathol Int. 2012;62:77-83.
67. Ma Y, Visser L, Roelofsen H, de Vries M, Diepstra A, van Imhoff G, et al. Proteomics analysis of Hodgkin lymphoma: identification of new players involved in the cross-talk between HRS cells and infiltrating lymphocytes. Blood. 2008;111:2339-46.
68. Hartmann S, Jakobus C, Rengstl B, Doring C, Newrzela S, Brodt HR, et al. Spindle-shaped CD163+ rosetting macrophages replace CD4+ T-cells in HIV-related classical Hodgkin lymphoma. Mod Pathol. 2013 Jan 11. [Epub ahead of print]
69. Hentrich M, Berger M, Wyen C, Siehl J, Rockstroh JK, Muller M, et al. Stage-adapted treatment of HIV-associated Hodgkin lymphoma: results of a prospective multicenter study. J Clin Oncol. 2012;30:4117-23.
70. Agostinelli C, Sabattini E, Gjorret JO, Righi S, Rossi M, Mancini M, et al. Characterization of a New Monoclonal Antibody Against PAX5/BASP in 1525 Paraffin-embedded Human and Animal Tissue Samples. Appl Immunohistochem Mol Morphol. 2010;18(6):561-72.
71. Kurth J, Hansmann ML, Rajewsky K, Küppers R. Epstein-Barr virus-infected B cells expanding in germinal centers of infectious mononucleosis patients do not participate in the germinal center reaction. Proc Natl Acad Sci USA. 2003;100:4730-5.
72. Hartmann S, Agostinelli C, Klapper W, Korkolopoulou P, Koch K, Marafioti T, et al. Revising the historical collection of epithelioid cell-rich lymphomas of the Kiel Lymph Node Registry: what is Lennert's lymphoma nowadays? Histopathology. 2011;59:1173-82.
73. Quintanilla-Martinez L, Fend F, Moguel LR, Spilove L, Beaty MW, Kingma DW, et al. Peripheral T-cell lymphoma with Reed-Sternberg-like cells of B-cell phenotype and genotype associated with Epstein-Barr virus infection. Am J Surg Pathol. 1999;23:1233-40.
74. Agostinelli C, Hartmann S, Klapper W, Korkolopoulou P, Righi S, Marafioti T, et al. Peripheral T cell lymphomas with follicular T helper phenotype: a new basket or a distinct entity? Revising Karl Lennert's personal archive. Histopathology. 2011;59:679-91.
75. Miyoshi H, Sato K, Niino D, Arakawa F, Kimura Y, Kiyasu J, et al. Clinicopathologic analysis of peripheral T-cell lymphoma, follicular variant, and comparison with angioimmunoblastic T-cell lymphoma: Bcl-6 expression might affect progression between these disorders. Am J Clin Pathol. 2012;137:879-89.
76. Hu S, Young KH, Konoplev SN, Medeiros LJ. Follicular T-cell lymphoma: a member of an emerging family of follicular helper T-cell derived T-cell lymphomas. Hum Pathol. 2012;43:1789-98.
77. Moroch J, Copie-Bergman C, de Leval L, Plonquet A, Martin-Garcia N, Delfau-Larue MH, et al. Follicular Peripheral T-cell Lymphoma Expands the Spectrum of Classical Hodgkin Lymphoma Mimics. Am J Surg Pathol. 2012;36:1636-46.
78. Eberle FC, Song JY, Xi L, Raffeld M, Harris NL, Wilson WH, et al. Nodal Involvement by Cutaneous CD30-positive T-cell Lymphoma Mimicking Classical Hodgkin Lymphoma. Am J Surg Pathol. 2012; 36(5):716-25.
79. Kanzler H, Küppers R, Helmes S, Wacker HH, Chott A, Hansmann ML, et al. Hodgkin and Reed-Sternberg-like cells in B-cell chronic lymphocytic leukemia represent the outgrowth of single germinal-center B-cell-derived clones: potential precursors of Hodgkin and Reed-Sternberg cells in Hodgkin's disease. Blood. 2000;95:1023-31.
80. Mao Z, Quintanilla-Martinez L, Raffeld M, Richter M, Krugmann J, Burek C, et al. IgVH mutational status and clonality analysis of Richter's transformation: diffuse large B-cell lymphoma and Hodgkin lymphoma in association with B-cell chronic lymphocytic leukemia (B-CLL) represent 2 different pathways of disease evolution. Am J Surg Pathol. 2007;31:1605-14.
81. Swerdlow SH, International Agency for Research on Cancer, World Health Organization. WHO classification of tumours of haematopoietic and lymphoid tissues 4th ed. Lyon, France: International Agency for Research on Cancer, 2008.

The integration of FDG PET/CT imaging in the management of Hodgkin's lymphoma

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A B S T R A C T

The majority pf patients affected by Hodgkin's Lymphoma (HL) become long-term survivors, wth cure and survival rates after first-line treatment exceeding 80% and 90%, respectively. These favorable results do not depend only on high tumor chemo- and radiosensitivity, but also on the continued improvements in the staging, formulation of effective prognostic factors, optimization of radiation treatment (RT) planning and adoption of intensified therapy strategies for high-risk groups. Currently, there are no conclusive data from prospective, randomized trials proving a benefit for overall disease control in patients undergoing tumor staging and restaging by functional imaging with [18F]-fluorodeoxyglucose (FDG) positron emission tomography-computed tomography (FDG-PET/CT) over those assessed with other conventional methods. However, there is indirect evidence that the survival of the HL patients has increased in the last decade for all age subgroup seven for patients over 60 in whom no major therapeutic improvement has been recorded. These results suggest that even FDG-PET/CT could have a place in the panoply of tools now available for an effective HL treatment. Early chemosensitivity assessment definitely proved to be the most important predictor of treatment outcome in ABVD-treated patients, but this premise is yet to be proven by the mature results of the ongoing prospective clinical trials.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe the role of FDG-PET at staging in Hodgkin's lymphoma;
- discuss the utility of bone marrow biopsy in the PET era for baseline staging workup;
- describe the rationale for interim therapy chemosensitivity assessment using PET/CT;
- describe the current interim PET/CT interpretation rules and the rationale for their proposal;
- describe the rationale behind the PET-adapted therapeutic strategy;
- describe the role of end of therapy PET scan for the management of a residual mass at the end of chemotherapy.

Introduction

The majority pf patients affected by Hodgkin's lymphoma (HL) become long-term survivors, wth cure and survival rates after first-line treatment exceeding 80% and 90%, respectively.[1] These favorable results do not depend only on high tumor chemo- and radiosensitivity but also on the continued improvements in the staging, formulation of effective prognostic factors, optimization of radiation treatment (RT) planning and adoption of intensified therapy strategies for high-risk groups. The combination of doxorubicin, bleomycin, vinblastine and dacarbazine (ABVD) as first-line treatment induces a durable remission in 75%-80% of patients.[2] However, for those with primary refractory disease or relapsing after the first complete remission (CR), efforts to develop valid predictors of treatment response and more effective therapy strategies are ongoing to counteract poor prognosis by achieving a better survival and safety outcome. In fact, 10%-15% of early stage and 20%-25% of advanced-stage patients fail to achieve durable remissions, ultimately succumbing to resistant or recurrent HL.[3] In the early stage group, the main challenge is: 1) to develop an effective regimen to reduce toxicity effects while maintaining excellent outcomes; and 2) in the late stage or poor-risk group, to increase the efficacy of treatment while controlling the toxicity within an acceptable range. Identification of the patient subgroup who would not benefit from continuation of standard therapy would be most desirable at the outset prior to initiation of therapy, or at least early during treatment, to make a timely shift to a more aggressive treatment. Positron emission tomography (PET) using F-18-fluorodeoxyglucose (FDG), integrated with computed tomography (PET/CT) is now a widely used imaging modality in the staging, re-staging and evaluation of therapy response in lymphoma, and may provide the means for such an individualized approach.[4-8]

FDG PET/CT for lymphoma staging

Despite major improvements in prognostic models and surrogate predictors for treatment

outcome and survival, staging remains the most important prognostic parameter for treatment planning.[9] PET/CT proved to be a more sensitive and specific imaging technique than other conventional modalities including Gallium and CT for determination of extent of nodal and extra-nodal disease.[10] Stage migration occurs in nearly 25% of patients, mostly upstaging disease, leading to a change in treatment strategy in nearly 10%-15% of them.[5] The role of a contrast-enhanced CT (CECT) performed simultaneously in the same diagnostic session as the PET scan is still a subject of debate. Direct comparison of unenhanced low-dose PET-CT and PET performed with CECT has shown no statistically significant differences in the number of detected nodal and extranodal sites, but lymphoma was occasionally upstaged with the help of CECT or additional clinically relevant findings were identified.[11-12] PET/CECT may be useful in patients with abdominal and pelvic involvement for delineating lymph nodes from adjacent bowel loops and vasculature.[13]

To determine bone marrow involvement (BMI), BM biopsy has a high false-negative rate because of the small sample size[14] and the limited area of evaluation in the pelvis. The sensitivity of FDG-PET in detecting BMI in HL ranges between 55% and 85%.[15-18] FDG-PET proved more accurate than trephine bone marrow biopsy (BMB) in one study.[16] In another recent study, the role of routine BMB was assessed in a cohort of 454 HL patients staged with PET/CT: BMB up-staged only 5 patients from stage III to IV and no BMB allocated patients in another treatment or risk group.[17] Although BMB remains essential for the diagnostic workup, BMI is a rare presentation for HL patients, thus BMB should no longer be a routine procedure for staging HL patients in the PET era. In selected patients, however, FDG PET should be the first test preceding BMB and biopsy should be directed to PET positive sites if indicated. Tumor bulk assessment at baseline by functional imaging proved an independent prognostic factor for disease control by chemotherapy, and several methods using semi-automated contouring methods have been proposed to measure tumor metabolic volume (MTV).[19-20] The MTV can be calculated by selecting tumor with uptake above an arbitrary cut-off value for maximum standardized uptake value (SUVmax) or using a threshold-based method.[20] Combined with interim-PET results, MTV has shown promising results in the prediction of treatment outcome in advanced-stage HL.[21] However, the optimal methodology still has to be found with further controlled studies.

The role of base-line FDG PET/CT for RT planning of early-stage HL

As a consequence of radical innovations in modern radiotherapy, the RT fields for single modality therapy in lymphoma have been dramatically reduced to include only the non-bulky and bulky nodal disease regions involved by disease to avoid unnecessary radiation to the healthy surrounding tissue.[22] Accordingly, the risk of serious early and late consequences has sharply decreased.[23,24] The reduction of the irradiated area while maintaining similar doses delivered to the tumor, as well as a more precise calculation of tumor shrinkage following first-line chemotherapy, both require the precision provided by PET/CT to define more accurate tumor contours before and after chemotherapy. In radiotherapy planning for early-stage HL, the initial lymphoma volume determined on the staging PET/CT scan, as well as the reduced volume after chemotherapy, should be contoured on a planning scan to be performed after chemotherapy. Since tumor shrinkage is assessed both in the longitudinal and in transverse axes, fused images obtained by anatomical and functional imaging pre- and post-chemotherapy should be used to delineate the areas of irradiation. This approach forms the basis of the current conformal RT for involved field (IFRT) and involved-nodal radiotherapy (INRT), in which the irradiated volume delineated by PET/CT differs significantly from that that would be defined by traditional CT imaging.[25,26]

FDG-PET/CT for the assessment of prognosis and chemosensitivity

Various prognostic models have been proposed to tailor treatment in HL patients. These models, however, are of limited clinical value, and their predictive power for treatment outcome has been seriously questioned.[27,28] In recent years, there has been growing concern about toxicity of HL treatment for both early[29] and advanced[30] stage disease because the long-term consequences of treatment, in particular for combined modality of chemotherapy and radiotherapy, have become manifest.[31] To reduce treatment-associated morbidity and mortality, therapeutic strategies tailored to the individual patient's risk of chemoresistance have been proposed, with the aim of maintaining and even improving on the high cure rates. Recently, a novel class of prognostic factor has been proposed in lymphoma, based on early assessment of the individual risk of chemoresistance during treatment. This was attained either by the evaluation of minimal residual disease (MRD) using molecular biology techniques[32] or evaluating the chemosensitivity during the first few cycles of chemotherapy. Traditional radiological assessment of tumor bulk shrinkage is not an accurate predictor of outcome since any reduction in tumor volume can lag behind metabolic slowdown of the neoplastic tissue. Consequently, up to two-thirds of the HL patients show a residual mass at the end of treatment.[33,34] Moreover, the inherent non-neoplastic inflammatory cells accounting for up to 90% of HL burden, are not necessarily affected by cytostatic treatment.[35] The use of functional imaging with FDG-PET enables an early evaluation of the metabolic changes that occur during the induction treatment as early as after the first,[8] the second[6,7,36,37] and third[38] cycle of chemotherapy. In this regard, PET/CT yielded highly promising results as a surrogate for predicting tumor chemosensitivity and progression-free survival (PFS)[6-8,36] with a sensitivity and specificity of 43%-100% and 67%-100%, respectively,[39] even performing better than the International Prognostic Score (IPS).[40] Because the success of an imaging test predicting outcome is closely related to interpretation criteria, the recent developments in the efforts to improve the reading accuracy have been significant. In April 2009, at a workshop on interim PET in lymphoma held in Deauville, France, simple and reproducible rules were proposed for interim visual PET interpretation,[41,42] and these criteria have recently been retrospectively validated (A Gallamini, submitted manuscript, 2013). Briefly, the adopted rules include

the following statements: i) visual assessment is preferred, but SUV determination can be used in some cases; ii) interim-PET interpretation should always be made by comparing the single foci of FDG uptake to those recorded in the baseline study; iii) the intensity of FDG uptake should be graded according to a 5-point scale in which two reference organs, the mediastinal blood pool structures (MBPS) and liver, are used to define different grades of FDG uptake. Accordingly, the so-called Deauville 5-point scale (5-PS) has been proposed (Figure 1). 5-PS is now considered to be an effective method for interim PET interpretation in HL. Secondly, the best time interval between interim PET and cytotoxic therapy should not be before 7-10 days after the start of chemotherapy infusion to avoid the critical window of inflammatory response peaking during this period.[44,45] Evaluation at an earlier period could coincide with stunning of cellular glucose metabolism by the immediate effects of chemotherapy, compromising the test sensitivity.[46] Most of the published literature supports post-2 cycle PET imaging as the best time-point during chemotherapy to assess chemosensitivity. However, recently a better negative predictive value (NPV) has also been stressed after one chemotherapy cycle.[9] Therefore, the recommended timing for interim PET scan is Day 11-13 of the second chemotherapy administration, considering that two 14-day treatments constitute one cycle in the HL treatment schedule. The importance of adherence to international guidelines for PET scanning and image acquisition cannot be emphasized enough, in order to generate consistently reliable data and for intra- as well as inter-institutional cross comparative studies.[47,48] Interestingly, in the aforementioned retrospective validation study for 5-PS interpretation, only 39% of patients were found to have undergone a PET scan performed in accordance with the above guidelines. The positive predictive value (PPV) of interim PET in predicting treatment outcome in the entire patient cohort and in the patient subset, scanned according international guidelines, was 0.73 and 0.86, respectively (P<0.01).

Deauville score (5-point scale)

- Score 1: no uptake
- Score 2: uptake ≤ mediastinum
- Score 3: uptake > mediastinum but ≤ liver
- Score 4: moderately ↑uptake > liver
- Score 5: markedly ↑uptake > liver and/or new sites of disease

Figure 1. The 5-point Deauville scale.[41,43]

FDG PET/CT-based response adapted therapy

In advanced-stage HL the primary treatment objective differs significantly from that of limited stage HL. In light of the outstanding event-free and overall survivals in the limited stage group (10-year overall survival, 84% to 97%) the late toxicities, such as secondary tumors and cardiac events, become the most concerning issues.[49] In advanced stage HL, however, the less favorable EFS and OS compared to limited stage disease (10-year overall survival 75%-85%)[30] are the main reason for escalated therapy trials with the goal of improving treatment efficacy at the cost of increase in unwanted treatment effects. Multiple clinical trials are now underway to assess the survival benefit and safety of risk-adapted strategies based on interim-PET results[50] (Table 1). The current management of early stage HL warrants one of the most crucial questions: whether radiotherapy could be safely avoided in patients showing a rapid response as evidenced by a negative interim PET scan. In this setting, avoidance of RT and allowing for a slightly reduced treatment efficacy may be justifiable on the basis of effective second-line treatments at the time of a possible relapse, because the reduction in radiation related long-term effects would outweigh the attendant disadvantages. In line with this concept, the results of a GHLSG study of early-stage favorable HL reported that a reduced-intensity regimen of two cycles of ABVD chemotherapy followed by 20 Gy of IFRT could achieve comparable treatment success with a freedom from treatment failure rate of 91% that is similar to that of the standard four cycles of ABVD with 30 Gy of IFRT.[66] Again using this strategy, the results of the English RAPID trial have been recently reported.[67] By contrast in the large multicenter European study H 10 on behalf of the EORTC, GELA and FIL exploring the role of a PET-adapted therapy in early stage HL, omitting radiotherapy in the experimental arm in patients with a negatve interim PET was associated with an increasing number of events both in favorable and unfavorable subsets. As a result, the PET-negative arm of the study was prematurely closed.[68] In advanced stage lymphoma, two opposite risk-adapted strategies are currently being tested: 1) starting with BEACOPP escalated regimen and de-intensifying treatment to ABVD in interim PET negative patients; 2) starting with ABVD and intensifying treatment only in interim PET-positive patients. The results of the interim analysis of the latter trial, the GITIL/FIL HD 0607, have recently been presented.[69] Several other trials are underway, starting first-line therapy with an intensified regimen (BEACOPP escalated) to overcome chemoresistance early during treatment, and subsequently de-escalating treatment in patients with a negative interim PET, as shown in Table 1. A preliminary report from one of these trials, the GHSG HD-18, was reported in 240 patients.[70] Using modified Deauville criteria with a highly sensitive threshold for interim PET positivity, 98 patients (41%) were PET-2 positive and 142 (59%) were PET-2 negative. Other ongoing trials are pursued by the GELA and the national Israeli lymphoma study group, both of which employ a strategy starting with BEACOPP escalated regimen and randomizing PET-2 negative patients to either continuation on BEACOPP escalated or de-escalate treatment to ABVD in the experimental arm. Despite preliminary evidence of the advantages of a risk-adapted strategy over conventional

treatment, although using a retrospective and observational design,[71,72] for the moment interim PET should be considered an investigational procedure, and planned therapy schemes should not be changed according to interim PET results outside a clinical trial setting.[50] Only the results of ongoing clinical trials will provide guidance concerning the outcome benefits of a PET response-adapted strategy.

FDG PET/CT to guide post-chemotherapy consolidation RT in advanced-stage HL

As previously reported, 60%-80% of HL patients show a residual mass during end-of-treatment re-staging mostly in sites of previous bulky disease,[33,34] but only less than half of these masses will harbor residual disease.[35] In pre-PET era, involved field RT had been proposed for bulky nodal lesions or residual masses in advanced stage HL patients as an integral part of ABVD treatment.[3] More recently, end-therapy PET/CT proved effective in the discrimination between residual active disease and fibrotic masses with a sensitivity of 43%-100% and a specificity of 67%-100%.[73] Not surprisingly, the NPV of the end-treatment PET depends on the efficacy of the administered chemotherapy, being as high as 94% after the high-intensity regimen, BEACOPP,[74] or as low as 75% after the low-intensity regimen VEBEP.[75] In the large HD15 trial of the GHSG, consolidation radiotherapy was selectively administered to advanced-stage HL patients with a PET-positive residual mass of more than 2.5 cm at the end of three different BEACOPP escalated regimens. The 4-year PFS of irradiated *versus* non-irradiated patients were 86.2% *versus* 92.6%, (P=0.022). The NPV of end-therapy PET was 94%.[74] A similar approach for advanced-stage, ABVD-treated patients was reported by the British Columbia Cancer Agency in a retrospective analysis of 163 patients with a residual mass of 2 cm or more at the end of chemotherapy. Consolidation RT was given in patients with an FDG-avid mass while PET-negative patient had no further treatment. Patients with a PET-negative scan (n=130, 80%) had a far superior 3-year time to progression compared to those with a PET-positive scan (89% *vs.* 55%, P=0.00001) with no difference between those with bulky *versus* non-bulky disease. The NPV for end-treatment PET was 92%.[76] These results strongly support a strategy for omission of RT in advanced stage HL patients who achieve a PET-negative remission at the end of chemotherapy. However, the decision to irradiate a PET-positive lesion should be made with the awareness of false positive results as well as the possibility of a radioresistant disease.

Table 1. Ongoing clinical trials based on a PET response-adapted therapeutic strategy.

Group/title/ NCT n.	Stage/risk	S.ple	Pre-PET-2 Tx	PET-2-negative Tx	PET-2 positive Tx	End point
RHC/na[76]	i-IIA F I-II A UF	660	ABVD x 2 ABVD x 2	INT ABVD x 2 + INT	ABVDx2 ± INT ($_{PET4-}$) ABVDx2 ± ABVD 2 ±INT $_{(PET-4-)}$	5-year EFS
EORTC/LYSA/ FILH10[77]	I-II A F I-IIA UF	1797	ABVD x 2 ABVD x 2	ABVD x 1 + INT ABVD x 2 + INT	BEACOPP esc x 2 BEACOPP esc x 2	3-year PFS
CALGB 50801[78]	IA-IIB Bulky	123	ABVD x 2	ABVD x 4	BEACOPP esc x 4 + IFRT	3-year PFS
ECOG 2410[79]	IA-IIB Bulky	200	ABVD x 2	ABVD x 4 + INRT	BEACOPP esc x 4 + INRT	3-year PFS
CALGB 50604[80]	IA-IIB Non Bulky	149	ABVD x 2	ABVD x 2 + IFRT	BEACOPP esc x 2	3-year PFS
NCRI/RAPID[81]	I-IIA Non Bulky	575	ABVD x 3	IFRT or NFT	ABVD x 1 + IFRT	3-year PFS
GHSG/HD 16[82]	I-IIA F	1100	ABVD x 2 + IFRT	NFT	ABVD x 2	5-year PFS
GHSG HD 17[83]	I-II UF	1100	BEACOPP esc x 2 + ABVD x 2+ INT	NFT	BEACOPP esc x 2 + ABVD x 2	5-year PFS
GITIL/FIL HD0607[84]	IIB-IVB	750	ABVD x 2	ABVD x 4 ± IFRT	BEACOPP esc x 4 + BEACOPP bas x 4 ± Rit.	3-year PFS
SWOG S0816[85]	IIIA-IVB	230	ABVD x 2	ABVD x 2	BEACOPP esc x 6	2-year PFS
LYSA AHL 2011[86]	IIB-IVB	798	BEACOPP esc x 2	ABVD x 2 ± ABVD x 2	BEACOPP esc x 2 ± BEACOPP esc x 2	5-year PFS
NCRI/RATHL[87]	IIB-IVB	1200	ABVD x 2	ABVD x 4 or AVD x 4	BEACOPP esc x 4	3-year PFS
GHSG HD 18[88]	IIB-IVB	1500	BEACOPP esc x 2	BEACOPP esc x 4 BEACOPP esc x 2	BEACOPP esc x 4 BEACOPP-R x 4	5-year PFS
FIL HD0801[89]	IIB-IVB	400	ABVD x 2	ABVD x 4 ± IFRT	IGEV x 4 + ASCT	2-year PFS
RHC/na[90]	III-IVB $I_{PS\ 0-2}$ III-IVB $_{IPS\ 3-7}$	660	ABVD x 2 BEACOPP esc x 2	ABVD x 4 ABVD x 4	BEACOPP esc x 2 ± BEACOPP esc x 2	3-year PFS

FDG PET/CT for follow up of HL

Despite an improved disease control with the evolutions in first-line treatment strategies, relapses still occur in 20%-30% of HL patients.[77] The approach to monitoring for early detection of recurrence is heavily dependent on the probability of relapse in the population being tested, as well as the sensitivity, specificity and the frequency of the test.[78] Most treatment failures are usually observed within three years of treatment completion, the majority of relapses occurring in the first 12 months.[79,80] In advanced-stage HL, the most important factor predicting relapse in ABVD-treated patients is interim-PET positivity.[38,39,79] The prevalence of relapse in HL is rare, being reported in the pre-PET era with only one relapse per 68 visits based on routine CT scans irrespective of residual masses seen on CT.[81] According to the results from a meta-analysis on the role of surveillance FDG-PET, disease relapse is detected with a sensitivity and specificity of 50%-100% and 67%-100%, respectively.[73] Recently, El-Galaly *et al.* reported on the value of surveillance PET/CT in a retrospective cohort of 161 HL patients who achieved a CR or PR after first-line treatment.[82] Fourteen percent of patients experienced a relapse after a mean follow up of 34 months. With an average of 1.9 PET/CT scans per patient, the PPV of routine PET/CT and clinically indicated PET/CT was 22% and 37%, respectively (P=0.02). However, in a subset of high-risk patients (with extranodal disease or a positive interim PET) the PPV increased to 36%, while in those without risk factors, the PPV was only 5%. Consequently, the routine use of surveillance PET in HL patients achieving complete remission after first-line treatment should be reserved for high-risk patients. In summary, the design limitations of the prior studies, scarcity of prospective data, the overall cost of surveillance PET schedule, high rate of false-positive results (30%-80%)[79,82] and the lack of evidence for a survival benefit provided by early detection of relapse do not support the routine use of serial follow-up PET scans after first-line therapy in HL patients. However, a strong statement cannot be made for an optimized follow-up algorithm for high-risk patients.

Interim PET during second-line treatment for relapsed/refractory HL

The current standard approach to relapsed or refractory HL involves high-dose chemotherapy and autologous hematopoietic stem cell transplantation (HDT/ASCT), producing a long-term PFS in up to 65% of patients. Successful outcome is a function of remission duration after first-line chemotherapy and chemosensitivity to second-line or salvage therapy prior to ASCT regardless of the chemotherapy that induced the response.[83-86] Furthermore recent meta-analysis data confirmed the prognostic value of pre-ASCT FDG-PET imaging in lymphoma demonstrating a poor long-term PFS in PET-positive patients after induction chemotherapy (31%-41%) compared with a PFS of 73%-82% in those who achieved a PET-negative remission before undergoing HDT/ASCT.[87] However, there are few published data with respect to the predictive value for the treatment outcome of an interim-PET scan during salvage therapy. In a small cohort of 24 relapsing or refractory HL patients treated with salvage chemotherapy consisting of iphosphamide, gemcytabine and vinorelbine (IGEV) followed by ASCT, PET scan was predictive of treatment outcome when performed after the second cycle. The 2-year PFS was 93% *versus* 10% for patients with PET-negative and PET-positive results, respectively (P<0.001).[88] In another small cohort of relapsing HL patients (n=26) interim PET scan after two courses of chemotherapy with DHAP (dexamethasone, cytarabine, and cisplatin) and VIM (etoposide, iphosphamide, and methotrexate) recurring HL score (rHPS)[89] was predictive of a 2-year failure-free survival after transplantation.[90] Consequently, more studies are warranted to obtain meaningful results for clinical applications of interim PET scans in this setting.

Conclusions

Currently, there are no conclusive data from prospective, randomized trials proving a benefit for overall disease control in patients undergoing tumor staging and restaging by functional imaging with FDG-PET/CT over those assessed with other conventional methods. However, there is indirect evidence that the survival of the HL patients has increased in the last decade for all age subgroups, even for patients over 60 years of age in whom no major improvement has been recorded.[91] These results suggest that even the availability of such a powerful imaging modality could have a place in the panoply of tools now available for an effective HL treatment. Early chemosensitivity assessment definitely proved to be the most important predictor of treatment outcome in ABVD-treated patients, but this premise still has to be confirmed by the mature results of the ongoing prospective clinical trials.

References

1. Diehl V, Stein H, Hummel M, Zollinger R, Connors JM. Hodgkin's lymphoma: biology and treatment strategies for primary, refractory, and relapsed disease. Am Soc Hematol Educ Program. 2003;225-47.
2. Canellos GP, Niedzwiecki D, Johnson JL. Long-Term Follow-up of Survival in Hodgkin's Lymphoma N Engl J Med. 2009;361;2390-1.
3. Bonadonna G, Viviani S, Bonfante V, Gianni AM, Valagussa P. Survival in Hodgkin's disease patients – Report of 25 years of experience at the Milan cancer Institute. Eur J Cancer. 2005;41:998-1006.
4. Wiedmann E, Baican B, Hertel A, Baum RP, Chow KU, Knupp B, et al. Positron emission tomography (PET) for staging and evaluation of response to treatment in patients with Hodgkin's disease. Leuk Lymphoma. 1999;34(5-6):545-51.
5. Cheson BD. Role of Functional Imaging in the Management of Lymphoma. J Clin Oncol. 2011;29:1-11.
6. Hutchings M, Loft A, Hansen M, Pedersen LM, Buhl T, Jurlander J, et al. FDG-PET after two cycles of chemotherapy predicts treatment failure and progression-free survival in Hodgkin lymphoma. Blood 2006; 107,52–9.
7. Gallamini A, Rigacci L, Merli F, Nassi L, Bosi A, Capodanno I, et al. The predictive value of positronemission tomography scanning performed after two courses of standard therapy on treatment outcome in advanced stage Hodgkin's disease. Haematologica. 2006;91:475-81.
8. Kostakoglu L, Goldsmith SJ, Leonard JP, Christos P, Furman RR, Atasever T, et al. FDG-PET after 1cycle of therapy predicts outcome in diffuse large cell lymphoma and classic Hodgkin disease. Cancer. 2006;107:2678-87.
9. Hutchings M. How does PET/CT help in selecting therapy for patients with Hodgkin lymphoma? Hematology Am Soc

Hematol Educ Program. 2013;322-7.
10. Hutchings M, Loft A, Hansen M, et al. Position emission tomography with or without computed tomography in the primary staging of Hodgkin's lymphoma. Haematologica. 2006;91(4):482-9.
11. Rodriguez-Vigil B, Gomez-Leon N, Pinilla I, et al. PET/CT in lymphoma: Prospective study of enhanced full-dose PET/CT versus unenhanced low-dose PET/CT. J Nuc Med. 2006;47:1643-8.
12. Chalaye J, Luciani A, Meignan M, Enache C, Amraoui K, Beaussart P, et al. Clinical impact of contrast-enhanced computed tomography (CECT) combined with low-dose FDG PET/CT on lymphoma patient management. J Nuc Med. 2012;53:1369-75.
13. Pinilla I, Gomez-Leon N, Del Campo-Del Val L, et al. Diagnostic value of CT, PET and combined PET/CT performed with low-dose unenhanced CT and full-dose enhanced CT in the initial staging of lymphoma. The quarterly journal of nuclear medicine and molecular imaging: official publication of the Italian Association of Nuclear Medicine (AIMN) [and] the International Association of Radiopharmacology (IAR), Section of the Society of Radiopharmaceutical Chemistry and Biology. 2011;55:567-75.
14. Wang J, Weiss LM, Chang KL, et al. Diagnostic utility of bilateral bone marrow examination: significance of morphologic and ancillary technique study in malignancy. Cancer. 2002:94:1522-31.
15. Pakos EE, Fotopoulos AD, Ioannidis JPA. 18F-FDG PET for Evaluation of Bone Marrow Infiltration in Staging of Lymphoma:□A Meta-Analysis. J Nucl Med. 2005;46:958-63.
16. Moulin-Romsee G, Hindie E, Cuenca X, et al. (18)F-FDG PET/CT bone/bone marrow findings in Hodgkin's lymphoma may circumvent the use of bone marrow trephine biopsy at diagnosis staging. Eur J Nucl Med Mol Imaging. 2010;37:1095-105.
17. El-Galaly TC, d'Amore F, Mylam KJ, de Nully Brown P, Bøgsted M, Bukh A, et al. Routine bone marrow biopsy has little or no therapeutic consequence for positron emission tomography/computed tomography-staged treatment-naive patients with hodgkin lymphoma. J Clin Oncol. 2012;30(36):4508-14.
18. Wu LM, Chen FY, Jiang XX, Gu HY, Yin Y, Xu JR. (18)F-FDG PET, combined FDG-PET/CT and MRI for evaluation of bone marrow infiltration in staging of lymphoma: A systematic review and meta-analysis Eur J Radiol. 2012;8:303-11.
19. Yan J, Zhao B, Wang L, et al. Marker-controlled watershed for lymphoma segmentation in sequential CT images. Medical Physics. 2006;33:2452-60.
20. Yan J, Zhuang TG, Zhao B, et al. Lymph node segmentation from CT images using fast marching method. Computerized medical imaging and graphics: the official journal of the Computerized Medical Imaging Society. 2004;28:33-8.
21. Meignan M, Barrington S, Gallamini A et al.: Report on the 4th international workshop on Positron Emission Tomography in Lymphoma held in Menton, France, 3-5 October 2012. Leukemia and lymphoma 2013, in press.
22. Girinsky T, Ghalibafian M. Radiotherapy of Hodgkin lymphoma: indications, new fields, and techniques. Semin Radiat Oncol. 2007;17(3):206-22.
23. Maraldo MV, Brodin NP, Vogelius IR, et al. Risk of developing cardiovascular disease after involved node radiotherapy versus mantle field for Hodgkin lymphoma. Int J RadiatOncolBiol Phys. 2012;83(4):1232-7.
24. Hodgson DC. Late effects in the era of modern therapy for Hodgkin lymphoma. Hematology Am Soc Hematol Educ Program. 2011;2011:323-9.
25. Hutchings M, Loft A, Hansen M, Berthelsen AK, Specht L. Clinical impact of FDG-PET/CT in the planning of radiotherapy for early-stage Hodgkin lymphoma. Eur J Haematol. 2007;78(3):206-12.
26. Girinsky T, Ghalibafian M, Bonniaud G, et al. Is FDG-PET scan in patients with early stage Hodgkin lymphoma of any value in the implementation of the involved-node radiotherapy concept and dose painting? Radiother Oncol. 2007;85(2):178-86.
27. Gobbi PG, Zinzani PL, Broglia C, Comelli M, Magagnoli M, Federico M, et al. Comparison of prognostic models in patients with advanced Hodgkin disease. Promising results from integration of the best three systems. Cancer. 2001;91;1467-78.
28. Hasenclever D. The disappearance of prognostic factors in Hodgkin's disease. Ann Oncol. 2002;13(1 Suppl):75-8.
29. Armitage JO. Early-Stage Hodgkin's Lymphoma. N Engl J Med. 2010;363:653-62.
30. Engert A, Diehl V, Franklin J, Lohri A, Dörken B, Wolf-Dieter L. Escalated-Dose BEACOPP in the Treatment of Patients With Advanced-Stage Hodgkin's Lymphoma: 10 Years of Follow-Up of the GHSG HD9 Study. J Clin Oncol. 2009; 27:4548-4554.
31. Meyer RM, Gospodarowicz MK, Connors JM, Pearcey RG, Wells AW, Winter JN. ABVD Alone versus Radiation-Based Therapy in Limited-Stage Hodgkin's Lymphoma. N Engl J Med. 2012;366:399-408.
32. Rambaldi A, Carlotti E, Oldani E, Della Starza I, Baccarani M, Cortelazzo S, et al. Quantitative PCR of bone marrow BCL2/IgH+ cells at diagnosis predicts lymphoma treatment response and long-term outcome in follicular non-Hodgkin lymphoma. Blood. 2005;105:3428-33.
33. Naumann R, Vaic A, Beuthien-Baumann B, Bredow J, Kropp J, Kittner T, et al. Prognostic value of positron emission tomography in the evaluation of post-treatment residual mass in patients with Hodgkin's disease and non-Hodgkin's lymphoma. Br J Haematol. 2001;115(4):793-800.
34. Radford JA, Cowan RA, Flanagan M, Durn G, Crowther D, Johnson RJ, et al. The significance of residual mediastinal abnormality on the chest radiograph following treatment for Hodgkin's disease. J Clin Oncol. 1988;6(6):940-6.
35. Canellos GP. Residual mass in lymphoma may not be residual disease. J Clin Oncol. 1988;6(6):931-3.
36. Zinzani PL, Rigacci L, Stefoni V, Broccoli A, Puccini B, Castagnoli A, et al. Early interim 18F-FDG PET in Hodgkin's lymphoma: evaluation on 304 patients. Eur J Nucl Med Mol Imaging. 2012;39:4-12.
37. Cerci JJ, Pracchia LF, Linardi CC, et al. 18F-FDG PET after 2 cycles of ABVD predicts event-free survival in early and advanced Hodgkin lymphoma. J Nucl Med. 2010;51(9):1337-43.
38. Hutchings M, Mikhaeel NG, Fields PA, Nunan T, Timothy AR. Prognostic value of interim FDG-PET after two or three cycles of chemotherapy in Hodgkin lymphoma. Ann Oncol. 2005;16(7):1160-8.
39. Terasawa T, Lau J, Bardet S, Couturier O, Hotta T, Hutchings M, et al. Fluorine-18-fluorodeoxyglucose positron emission tomography for interim response assessment of advanced stage Hodgkin's lymphoma and diffuse large B-cell lymphoma: a systematic review. J Clin Oncol. 2009;27:1906-14.
40. Gallamini A, Hutchings M, Rigacci L, Specht L, Merli F, Hansen M, et al. Early interim 2-[18F]fluoro-2-deoxy-D glucose positron emission tomography is prognostically superior to international prognostic score in advanced-stage Hodgkin's lymphoma: a report from a joint Italian-Danish study. J Clin Oncol. 2007;25:3746-52.
41. Meignan M, Gallamini A, Haioun C. Report on the first international workshop on interim-PET scan in Lymphoma. Leuk Lymphoma. 2009;50(8):1257-60.
42. Gallamini A, Fiore F, Sorasio P, Meignan M. Interim positron emission tomography scan in Hodgkin lymphoma: definitions, interpretation rules, and clinical validation Leuk Lymphoma. 2009;50(11):1761-4.
43. Barrington SF, Qian W, Somer EJ, Franceschetto A, Bagni B, Brun E. Concordance between four European centres of PET reporting criteria designed for use in multicenter trials in Hodgkin lymphoma. . Eur J Nucl Med Mol Imaging. 2010;37 (10):1824-33.
44. Juweid ME, Stroobants S, Hoekstra OS, Mottaghy FM, Dietlein M, Guermazi A, et al. Use of Positron Emission Tomography for Response Assessment of Lymphoma: Consensus of the Imaging Subcommittee of International Harmonization Project in Lymphoma. J Clin Oncol. 2007;25 (5):571-8.
45. Spaepen K, Stroobants S, Dupont P, Bormans G, Balzarini J, Verhoef G, et al. [(18)F]FDG PET monitoring of tumour response to chemotherapy: does [(18)F]FDG uptake correlate with the viable tumour cell fraction? Eur J Nucl Med Mol Imaging. 2003;30(5):682-8.
46. Engles JM, Quarless SA, Mambo E, Ishimori T, Cho SY, Wahl RL. Stunning and its effect on 3HFDG uptake and key gene expression in breast cancer cells undergoing chemotherapy. J Nucl Med. 2006;47(4):603-8.
47. Boellaard R, O'Doherty MJ, Weber WA, Mottaghy FM, Lonsdale MN, Stroobants SG, et al. FDG PETand PET/CT: EANM procedure guidelines for tumour PET imaging: version 1.0. Eur J Nucl Med Mol Imaging. 2010;37(1):181-200.
48. Podoloff DA, Ball DW, Ben-Josef E, Benson AB 3rd, Cohen SJ, Coleman RE, et al. NCCN task force: clinical utility of PET in a variety of tumor types. J Natl Compr Canc Netw. 2009;7(2 Suppl):S1-26.
49. Favier O, Heutte N, Stamatoullas-Bastard A, et al. Survival

after Hodgkin lymphoma: causes of death and excess mortality in patients treated in 8 consecutive trials. Cancer. 2009;115:1680-91.
50. Gallamini A, Kostakoglu L. Interim FDG-PET in Hodgkin Lymphoma: a compass for a safe navigation in clinical trials? Blood. 2012;120(25):4913-20.
51. US National Institutes of Health. Tailored Therapy for Hodgkin Lymphoma Using Early Interim Therapy PET for Therapy Decision. [Internet] Available from: http://www.clinicaltrials.gov/ct2/show/NCT00392314
52. US National Institutes of Health. Fludeoxyglucose F 18 PET Scan-Guided Therapy or Standard Therapy in Treating Patients With Previously Untreated Stage I or II Hodgkin's Lymphoma (EORTC/GELA). [Internet]. Available from: http://clinicaltrials.gov/ct2/show/NCT00433433
53. US National Institutes of Health. Response-Based Therapy Assessed By PET Scan in Treating Patients With Bulky Stage I and Stage II Classical Hodgkin Lymphoma (CALGB 50801). [Internet]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT01118026
54. US National Institutes of Health. Chemotherapy Based on PET Scan in Treating Patients With Stage I or Stage II Hodgkin Lymphoma (ECOG). [Internet]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT01390584
55. US National Institutes of Health. Chemotherapy Based on Positron Emission Tomography Scan in Treating Patients With Stage I or Stage II Hodgkin Lymphoma (CALGB 50604). [Internet]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT01132807
56. US National Institutes of Health. PET Scan in Planning Treatment in Patients Undergoing Combination Chemotherapy For Stage IA or Stage IIA Hodgkin Lymphoma. [Internet]. Available from: http://clinicaltrials.gov/ct2/show/
57. US National Institutes of Health. HD16 for Early Stage Hodgkin Lymphoma. [Internet]. Available from: http://clinicaltrials.gov/ct2/show/NCT00736320
58. US National Institutes of Health. HD17 for Intermediate Stage Hodgkin Lymphoma. [Internet]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT01356680
59. US National Institutes of Health. Positron Emission Tomography (PET)-Adapted Chemotherapy In Advanced Hodgkin Lymphoma (HL) (HD0607). [Internet]. Available from:
http://www.clinicaltrials.gov/ct2/show/NCT00795613
60. US National Institutes of Health. Fludeoxyglucose F 18-PET/CT Imaging and Combination Chemotherapy With or Without Additional Chemotherapy and G-CSF in Treating Patients With Stage III or Stage IV Hodgkin Lymphoma (SWOG-CALG-B). [Internet]. Available from: http://clinicaltrials.gov/ct2/show/NCT00822120
61. US National Institutes of Health. Study of a Treatment Driven by Early PET Response to a Treatment Not Monitored by Early PET in Patients With AA Stage 3-4 or 2B HL (AHL 2011). [Internet]. Available from: http://clinicaltrials.gov/ct2/show/NCT01358747
62. US National Institutes of Health. Fludeoxyglucose F 18-PET/CT Imaging in Assessing Response to Chemotherapy in Patients With Newly Diagnosed Stage II, Stage III, or Stage IV Hodgkin Lymphoma (RATHL). [Internet]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT00678327
63. US National Institutes of Health. HD18 for Advanced Stages in Hodgkin Lymphoma. [Internet]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT00515554
64. US National Institutes of Health. High-Dose Chemotherapy and Stem Cell Transplantation, in Patients PET-2 Positive, After 2 Courses of ABVD (HD0801) and Comparison of RT Versus no RT in PET-2 Negative Patients (HD0802). [Internet]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT00784537
65. US National Institutes of Health. Tailored Therapy for Hodgkin Lymphoma Using Early Interim Therapy PET for Therapy Decision. [Internet]. Available from: http://www.clinicaltrials.gov/ct2/show/NCT00392314
66. Engert A, Plütschow A, Eich HT, Lohri A, Dörken B, Borchmann P, et al. Reduced Treatment Intensity in Patientswith Early-Stage Hodgkin’s Lymphoma. N Engl J Med. 2010;363:640-52.
67. Radford J, Barrington S, Counsell N, Pettengell R, Johnson P, Wimperis J, et al. Involved field Radiotherapy versus no further treatment in patients with clinical stages IA and IIA Hodgkin Lymphoma and a ‘negative’ PET Scan after 3 cycles ABVD. Results of the UK NCRI RAPID trial. Blood. 2012;120:547.
68. André MPE, Reman O, Federico M, Girinski T, Brice P, Brusamolino E, et al. Interim Analysis of the Randomized Eortc/Lysa/Fil Intergroup H10 Trial On Early PET-Scan Driven Treatment Adaptation in Stage I/II Hodgkin Lymphoma. Blood. 2012 120:549.
69. Gallamini A, Rossi A, Patti C, Picardi M, Di Raimondo FCantonetti M, et al. Early Treatment Intensification in Advanced-Stage High-Risk Hodgkin Lymphoma (HL) Patients, with a Positive FDG-PET Scan After Two ABVD Courses – First Interim Analysis of the GITIL/FIL HD0607 Clinical Trial. Blood. 2012;120:550.
70. Gallamini A, O’Doherthy M. Report of satellite workshop on interim-PET in Hodgkin lymphoma: 8th International Symposium on Hodgkin Lymphoma, Cologne, 2010. Leuk Lymphoma. 2011;52(4):583-6.
71. Gallamini A, Patti C, Viviani S, Rossi A, Fiore F, Di Raimondo F, et al. Gruppo Italiano Terapie Innovative ne nei Linfomi (GITIL) Early chemotherapy intensification with BEACOPP in advanced stage Hodgkin lymphoma patients with a interim-PET positive after two ABVD courses. Br J Haematol. 2011;152(5):551-60.
72. Dann EJ, Bar-Shalom R, Tamir A, Haim N, Ben-Shachar M, Avivi I, et al. Risk-adapted BEACOPP regimen can reduce the cumulative dose of chemotherapy for standard and high-risk Hodgkin lymphoma with no impairment of outcome. Blood. 2007;109(3):905-9.
73. Terasawa T, Nihashi T, Hotta T, Nagai H. 18F-FDG PET for posttherapy assessment of Hodgkin's disease and aggressive Non-Hodgkin's lymphoma: a systematic review. J Nucl Med. 2008;49(1):13-21.
74. Engert A, Haverkamp H, Kobe C, Markova J, Renner C, Ho A, et al. Reduced-intensity chemotherapy and PET-guided radiotherapy in patients with advanced stage Hodgkin's lymphoma (HD15 trial): a randomised, open-label, phase 3 non-inferiority trial Lancet. 2012;379(9828):1791-9.
75. Magagnoli M, Marzo K, Balzarotti M, Rodari M, Mazza R, Giordano L, et al. Dimension of Residual CT Scan Mass in Hodgkin's Lymphoma (HL) Is a Negative Prognostic Factor in Patients with PET Negative After Chemo+/– Radiotherapy. Blood. 2011;118:93.
76. Savage KJ, Connors JM, Klasa RJ, Hoskins P, Shenkier TN, Gascoyne RD, et al. The use of FDG-PET to guide consolidative radiotherapy in patients with advanced-stage Hodgkin lymphoma with residual abnormalities on CT scan following ABVD chemotherapy. J Clin Oncol. 2011;29:8034.
77. Quddus F, Armitage JO. Salvage therapy for Hodgkin's lymphoma. Cancer J. 2009;15(2):161-3.
78. Armitage JO, Loberiza FR. Is there a place for routine imaging for patients in complete remission from aggressive lymphoma? Ann Oncol. 2006;17(6):883-4
79. Zinzani PL, Stefoni V, Tani M, Fanti S, Musuraca G, Castellucci P, et al. Role of [18F]fluorodeoxyglucose positron emission tomography scan in the follow-up of lymphoma. J ClinOncol. 2009;27(11):1781-7.
80. Canellos GP, Horwich A. Management of recurrent Hodgkin’s disease. In: Hodgkin’s disease. Mauch P, Armitage J (eds.) Lippincott, William & Wilkins; 1999;505-19.
81. Radford JA, Eardley A, Woodman C, Crowther D. Follow up policy after treatment for Hodgkin's disease too many clinic visits and routine test? A review of hospital records. BMJ. 1997;314(7077):343-6.
82. El-Galaly TC, Mylam KJ, Brown P, Specht L, Christiansen I, Munksgaard L, et al. Positron emission tomography/computed tomography surveillance in patients with Hodgkin lymphoma in first remission has a low positive predictive value and high costs. Haematologica. 2012;97(6):931-6.
83. Brice P, Bouabdallah R, Moreau P, et al. Prognostic factors for survival after high-dose therapy and autologous stem cell transplantation for patients with relapsing Hodgkin's disease: analysis of 280 patients from the French registry. Société Française de Greffe de Moëlle. Bone Marrow Transplant. 1997;20:21-6.
84. Josting A, Muller H, Borchmann P, Baars JW, Metzner B, Dohner H, et al. Dose intensity of chemotherapy in patients with relapsedHodgkin’s Lymphoma J Clin Oncol. 2010;28:5074-80
85. Moskowitz AJ, Yahalom J, Kewalramani T, et al. Pre-transplantation functional imaging predicts outcome following autologous stem cell transplantation for relapsed and refractory Hodgkin lymphoma. Blood. 2010;116:4934-7.
86. Moskowitz CH, Matasar MJ, Zelenetz AD, et al. Normalization of pre-ASCT, FDG-PET imaging with second-line, non-cross-resistant, chemotherapy programs improves event-free survival in patients with Hodgkin lymphoma.

Blood. 2012;119:1665-70.
87. Poulou LS, Thanos L, Ziakas PD. Unifying the predictive value of pretransplant FDG PET in patients with lymphoma: a review and meta-analysis of published trials. Eur J Nucl Med Mol Imaging. 2010;37:156-62.
88. Castagna L, Bramanti S, Balzarotti M, Sarina B, Todisco E, Anastasia A, et al. Predictive value of early 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) during salvage chemotherapy in relapsing/refractory Hodgkin lymphoma (HL) treated with high-dose chemotherapy. Brit J Haematol. 2009;145:369-72.
89. Schot BW, Zijlstra JM, Sluiter WJ, van Imhoff GW, Pruim J, Vaalburg W, et al. Early FDG-PET assessment in combination with clinical risk scores determines prognosis in recurring lymphoma. Blood. 2007;109:486-91
90. Josting A, Franklin J, May M, et al. New prognostic score based on treatment outcome of patients with relapsed Hodgkin's lymphoma registered in the database of the German Hodgkin's Lymphoma Study Group. J Clin Oncol. 2002;20: 221-30.
91. Brenner H, Gondos A, Pulte D. Ongoing improvement in long-term survival of patients with Hodgkin disease at all ages and recent catch-up of older patients. Blood. 2008;111:2977-83.

Late effects of Hodgkin's lymphoma treatment: implications for management of contemporary patients

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A B S T R A C T

Over the last several years, the management of Hodgkin's lymphoma (HL) has been influenced by the recognition that primary treatment can lead to significant delayed morbidity among long-term survivors. But despite substantial literature regarding late effects among historically treated patients, applying the resulting knowledge to modify modern HL treatment remains challenging. This review will outline: 1) how recent and anticipated changes in radiotherapy and chemotherapy are expected to reduce the risks of late toxicity among current HL patients; 2) individual patients' characteristics that could reasonably affect treatment choices among patients in the same HL 'risk group' in order to limit late effects; 3) recommendations for follow up of HL survivors to reduce the risk of late morbidity. It is apparent that while studies on late effects have produced extremely valuable information, the exact magnitude and nature of late effects described in many of them cannot be directly applied to contemporary patients, and consideration of individual differences in patients' and disease characteristics is needed to deliver optimal treatment.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe the potential late effects of contemporary chemotherapy and radiation therapy regimens;
- describe the relative radiation dose to normal tissues received by patients in late effects studies *versus* contemporary Hodgkin's lymphoma patients;
- describe patient and disease factors that can affect the potential risk of developing late effects following treatment for Hodgkin's lymphoma;
- describe screening tests that are recommended for Hodgkin's lymphoma survivors to reduce the morbidity of late treatment toxicity.

Introduction

Late effects associated with historic treatment

Observational studies that describe late morbidity among Hodgkin's lymphoma (HL) survivors are often limited to evaluating outdated treatments. These studies typically include patients whose primary treatment was extended-field radiation therapy (RT), prescribed to higher doses and substantially larger volumes of normal tissue that would be the case today.[1-5] In addition, patients were exposed to cumulative doses of alkylating agents no longer relevant to most contemporary treatment regimens.

As a result of these exposures, studies of patients treated before 1995 have reported 45-80 excess malignancies per 10^4 person-years of follow up, most of which are solid tumors (Figure 1).[2,4,6] The risk of breast cancer following mantle RT has been of particular concern. Mantle RT (35-45 Gy to bilateral axillary, mediastinal and neck nodes) is associated with a 2- to 20-fold increased relative risk of breast cancer, with younger patients (i.e. treated at ages <20 years) found to have higher relative risks and expected excess lifetime risks than older patients.[1,3,6-9] Ng *et al.*, for example, reported a 20-year cumulative risk of second cancer of 23% following mantle RT + para-aortic and spleen RT (median age at RT 25 years, median prescribed dose 40Gy), with breast cancer accounting for almost 40% of SC among female survivors.[8] Other studies of survivors treated with similar RT fields and doses have reported 30-year cumulative incidence of second cancer of approximately 30%.[7,10-12] Mantle RT is also associated with an increased risk of lung cancer, although the absolute excess risk is small in the first 20 years after exposure, particularly among those treated at young ages (i.e. 20-year cumulative risk <2% among those treated before 20 years of age).[1,4] The risks of other solid cancers have also been shown to be elevated after RT.[6,7]Unlike leukemia risk following alkyator exposure, which declines to almost baseline after 5-10 years, risks for solid tumor remain elevated for as long as follow up has been conducted, presumably for life, with some evidence of a small decline in relative risk in survivors aged 60-70 years.[7]

Alkylating agents are well known to increase the risk of treatment-related myeloid leukemia. Less well recognized is that these agents are also associated with a significant increase in the risk of lung cancer and gastrointestinal solid tumors.[2,13,14] Travis *et al*

reported a 4.2-fold increased risk of lung cancer among HL survivors treated with alkylating agents,[2,14] and increased risks of gastrointestinal second cancers have also been found.[4,15]

Cardiac disease

Among survivors treated with 35-45 Gy mantle RT the cumulative risks of significant heart disease among survivors of adult HL are approximately 5%-10% at 15 years, 15% at 20 years, and 35% at 30 years.[16-18] Coronary artery disease accounts for approximately half of the cardiac morbidity occurring among HL survivors. Valvular disease is less common and typically has a late onset (>10 years after RT) and is related to higher doses (>30 Gy) or young age at treatment. Treatment of a large cardiac volume to high dose can produce acute or late onset pericarditis, though this is uncommon. The duration of increased cardiac risk associated with RT is not well defined, but extrapolating 15-30 year risks suggests a persistent increase in elevated risk beyond 30 years after exposure. The combined use of doxorubicin with mantle RT has generally been associated with a greater risk of late cardiac toxicity than either treatment given alone.[18 16] Given the widespread adoption of ABVD in the 1990s, frequently followed by 35-45 Gy mantle RT, it is possible that cardiac toxicity will emerge as the dominant late toxicity among those entering into more than ten years of HL-free survival.

Fertility

Treatment with mechlorethamine, vincristine, procarbazine, prednisone (MOPP) either alone or with doxorubicine, bleomycine, vinblastine (MOPP/ABV) has been shown to significantly increase the risk of azoospermia or premature ovarian failure.[19,20] MOPP/ABV hybrid produces significant dose-related reduction in sperm counts in approximately half of patients, and in one study COPP chemotherapy was found to produce azoospermia in all of 19 males and premature ovarian failure in 8 of 14 (57%) of females.[21] Procarbazine, in particular, appears to be gonadotoxic and, to a lesser extent, cyclophosphamide.[22,23]

In addition to the late effects described above, survivors of HL treated with extended field RT and alkyator-based chemotherapy have been found to have increased risks of stroke,[24] thyroid dysfunction,[25] lung function abnormalities,[26] and fatigue.[27] And the historic use of splenectomy for staging increases survivors' risk of potentially fatal infection.

Late effects: contemporary therapy

In contrast to mantle RT/extended-field RT, contemporary IFRT treats only initially involved lymph node regions (+/- immediately adjacent regions in some cases), and prescribed RT doses are typically 30 Gy for adults and around 20 Gy for children. Very favorable risk adult patients can receive 20 Gy IFRT following two cycles of ABVD. In the majority of patients with mediastinal disease, IFRT produces significant dose reductions to the breasts (approximately 65% dose reduction), lungs, and heart (approximately 30% dose reduction) compared to with mantle or extended-field RT.[28,29] Females treated with 20 Gy mediastinal IFRT (as per GHSG HD10) are typically receiving average breast doses 80% lower than those received by patients treated with mantle RT in historic

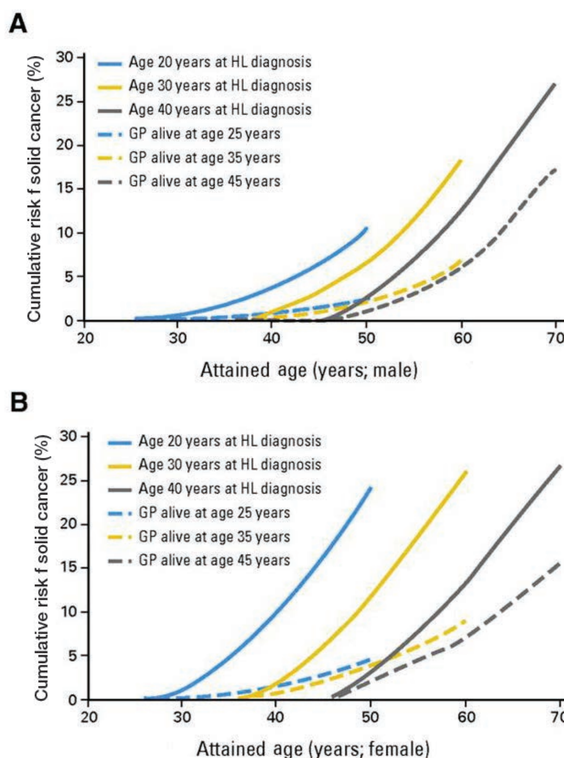

Figure 1. Cumulative incidence of solid cancers among 5-years survivors of Hodgkin's lymphoma (HL) compared with controls of the same age in the general population (GP). (A) Males (n=10,619 survivors). (B) Females (n=8,243 survivors). (Reprinted with permission from the American Society of Clinical Oncology. Hodgson DC, Gilbert ES, Dores GM, *et al*. Long-term solid cancer risk among 5-year survivors of Hodgkin's lymphoma. J Clin Oncol. 2007;25:1489-97. © 2007 American Society of Clinical Oncology. All rights reserved.[7])

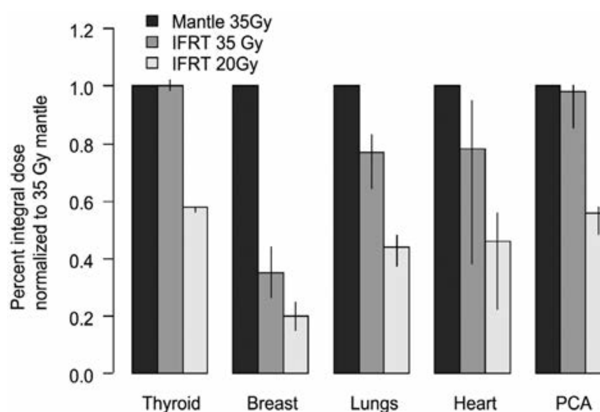

Figure 2. Reduction in integral dose to normal tissues with mantle RT *versus* IFRT. (Reprinted with permission from BioMed Central Ltd. Koh ES, Tran TH, Heydarian M, *et al.* A comparison of mantle versus involved-field radiotherapy for Hodgkin's lymphoma: reduction in normal tissue dose and second cancer risk. Radiat Oncol 2:13, 2007. © 2007 BioMed Central Ltd. All rights reserved.[28])

series, and cardiac doses approximately 60% lower (Figure 2).[28]

Emerging clinical evidence is showing that the transition to IFRT should translate into reduced risks of SC. Studies examining the dose-risk relationship for solid tumors suggest a decrease in risk of most solid cancers with decreasing dose to normal tissues below 40 Gy.[9,30-32] A Dutch study of 1122 female 5-year survivors of HL examined the effect of radiation field size on the risk of breast cancer after treatment of HL.[10] The cumulative incidence of breast cancer at 30 years was approximately 8% after IFRT, compared to approximately 27% after mantle RT (Figure 3). Similarly, Serdlow *et al.* reported a higher 40-year cumulative incidence of breast cancer with greater mantle RT doses, increasing from 22.8% among patients prescribed less than 34 Gy to 46.3% among those receiving 41 Gy or over.[9] These findings suggest that the decrease in the volume of irradiated tissue with the transition from mantle to IFRT, and the lower prescribed doses used in contemporary protocols (typically 30 Gy for adults and 20 Gy for children), should translate into a roughly proportional reduction in the risk of most forms of second

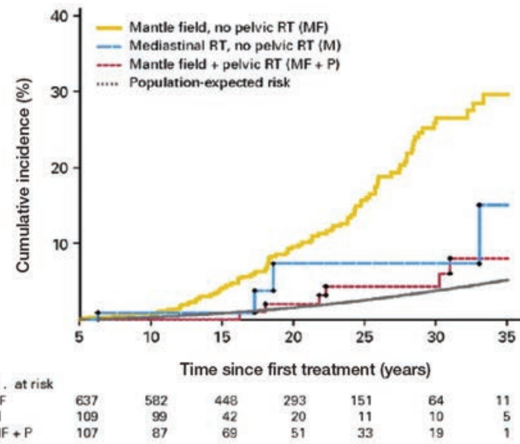

Figure 3. (Reprinted with permission from BioMed Central Ltd. Koh ES, Tran TH, Heydarian M, *et al.* A comparison of mantle versus involved-field radiotherapy for Hodgkin's lymphoma: reduction in normal tissue dose and second cancer risk. Radiat Oncol 2:13, 2007. © 2007 BioMed Central Ltd. All rights reserved.[28])

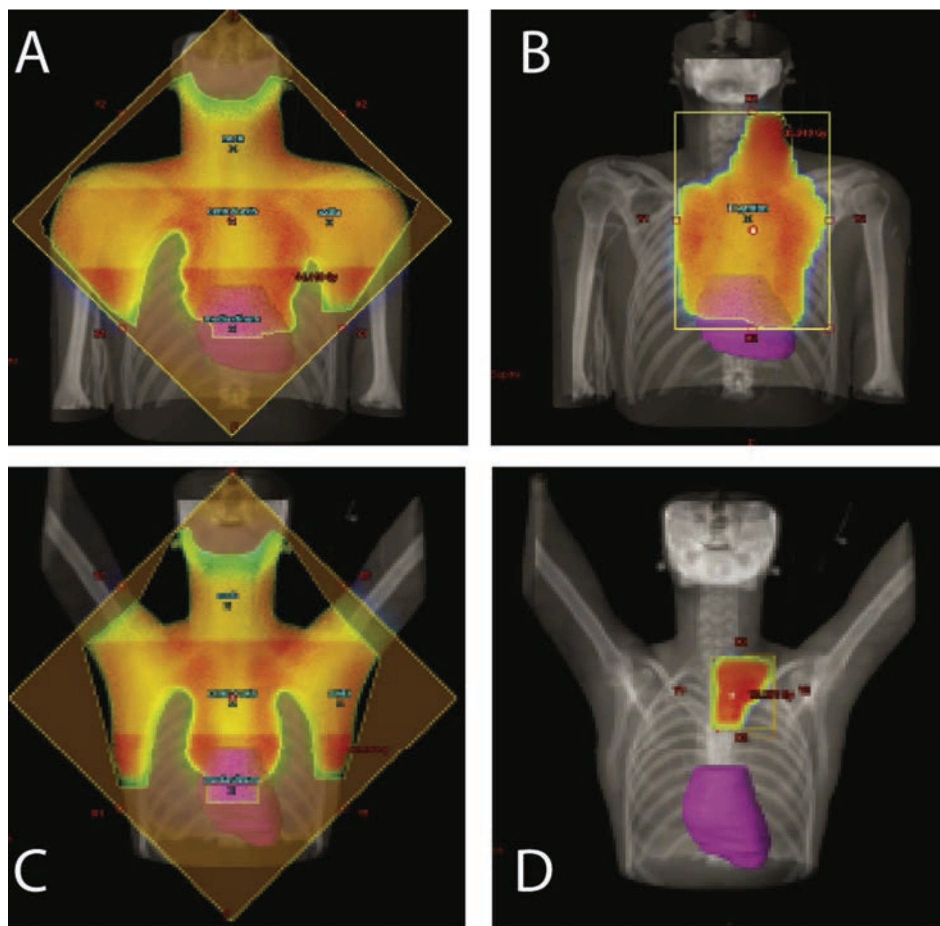

Figure 4. The evolution of RT fields for patients with mediastinal HL. (A) and (B) Mantle field and INRT for a patient with large mediastinal bulk. (C) and (D) Mantle fields and INRT for the same patient with a small mediastinal tumor. (Reprinted with permission from Elsevier, 2012. Maraldo MV, Brodin NP, Vogelius IR, *et al.* Risk of developing cardiovascular disease after involved node radiotherapy versus mantle field for Hodgkin lymphoma. Int J Radiat Oncol Biol Phys. 2012;83:1232-7. © 2012 Elsevier. All rights reserved.[29])

solid cancer. However, not all studies have demonstrated lower second cancer risks with lower prescribed doses,[33] and additional clinical data will be needed to fully characterize the dose-risk relationships.

Intensity-modulated RT, volumetric-modulated arc therapy, involved node RT (INRT)

IFRT fields encompass the lymph node *regions* (as described by Kaplan) that initially contained enlarged nodes at the time of diagnosis plus immediately adjacent nodal regions in some cases. Involved-node RT fields (INRT, also called involved site RT, ISRT) encompass the post-chemotherapy volumes of the initially involved *nodes,* not the entire nodal regions. For patients with mediastinal disease, this often allows further reduction in normal tissue dose compared to IFRT due to the exclusion of uninvolved hila and subcarinal nodes (Figure 4). Studies comparing normal tissue doses reported that for patients with mediastinal disease, INRT reduces the mean heart dose by approximately 70% compared to mantle fields and 50% compared to IFRT.[29,34] In one study, INRT reduced breast dose by 42% compared to IFRT, in addition to reducing dose to lung, thyroid and total body dose.[34] A study of 29 patients with mediastinal disease reported that the mean heart dose associated with 30 Gy INRT was 7.7 Gy, in contrast to 27.5 Gy among patients receiving historic 36 Gy mantle fields (Figure 5).[29]

It should be noted that the normal tissue sparing of INRT will be highly individualized depending on the distribution of disease: patients with disease that does not include the axillae, hila or subcarinal nodes will benefit more from the omission of these sites from the RT fields.

Early clinical results suggest INRT will be effective. In one study, INRT had an equivalent risk of relapse compared to IFRT,[35] and Maraldo *et al.* reported a 4-year freedom from disease progression rate of 96.4% in a cohort of 97 patients with stage 1-2 HL treated with combined ABVD + 30Gy INRT.[36] An interim analysis of the European Intergroup H10 trial, which randomized PET-2 negative patients to ABVD alone or ABVD + INRT, led to the closure of the chemotherapy alone arm when the hazard ratio of relapse was found to be 2.42 (80.4%; CI: 1.35-4.36) with the omission of INRT (one-year progression-free rates 97.3% and 94.7%, respectively). These results suggest that INRT is effective in reducing the risk of relapse among patients with early stage HL. It will be many years before an associated reduction in late toxicity will be clinically demonstrable.

Intensity modulated radiation therapy (IMRT) facilitates the more conformal shaping of the high-dose RT volume around irregularly shaped targets. Volumetric modulated arc therapy (VMAT) also delivers radiation with varying beam intensity; the major distinction from IMRT being that the beam source rotates through one or more arcs with the treatment unit continuously on, thereby reducing the treatment time. Although these methods can reduce the volume of tissue receiving higher doses (i.e. > 10 Gy), their major limitation is that the multiple beam angles used typically cause larger volumes of normal tissue to be exposed to low doses (i.e. 2-10 Gy). This is of greatest concern among young females with mediastinal disease for whom increasing the volume of irradiated breast tissue is not desirable. IMRT and VMAT are likely to be of greatest clinical advantage to male patients with mediastinal

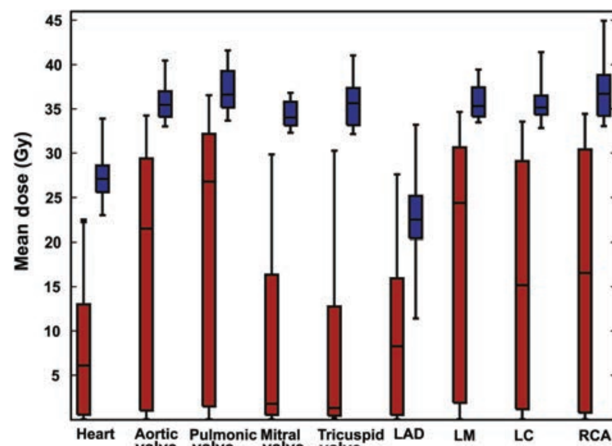

Figure 5. Comparison of dose to cardiac substructures with mantle RT versus INRT for patients with mediastinal HL. (Reprinted with permission from Elsevier, 2012. Maraldo MV, Brodin NP, Vogelius IR, *et al*. Risk of developing cardiovascular disease after involved node radiotherapy versus mantle field for Hodgkin lymphoma. Int J Radiat Oncol Biol Phys. 2012;83:1232-7. © 2012 Elsevier. All rights reserved.[29])

disease in whom they facilitate significant reductions in cardiac dose.[37,38] Early RT planning studies indicate that the smaller target volumes used for INRT could significantly enhance the relative merits of IMRT and VMAT for both males and females.[34,39]

Modern chemotherapy

Few studies have evaluated the long-term cardiac outcomes of patients treated with ABVD. Anthracyclines are known to produce asymptomatic echocardiographic abnormalities among some pediatric patients, the clinical significance of which is not well understood. Swerdlow *et al*. reported that with a median follow up of 2.7 years, treatment with doxorubicin without supradiaphragmatic RT was associated with a significantly increased risk of fatal myocardial infarction (standardized mortality ratio 3.2; P<0.001) and ABVD was associated with a 7.7-fold increased risk (P=0.01).[40] Vincristine was also associated with a significant 2-fold increase in the risk of MI. In contrast, two other studies[16,18] did not find increased risks of late cardiac toxicity among young adults initially treated with chemotherapy alone, although the number of patients treated with ABVD in these series was relatively small. Young children appear to be at greater risk for the development of late onset anthracycline-related cardiac dysfunction. Consequently, pediatric protocols have generally aimed to keep the cumulative dose of doxorubicin for low- and intermediate-risk patients to below 250 mg/m^2.

ABVD chemotherapy is known to be less gonadotoxic than alkylator-based regimens, and is associated with preserved sperm counts among males[41] and one study demonstrated that fertility among females attempting pregnancy following ABVD was comparable to that of untreated peers.[42] BEACOPP includes 700 mg/m^2 procarbazine and 650 mg/m^2 (base-line regimen) or 1200 mg/m^2 (escalated

regimen) cyclophosphamide per cycle. Both regimens have been shown to produce significant risks of azoospermia in males. Among females, fertility can be preserved after six cycles of baseline BEACOPP, although the risk of premature ovarian failure is significant among women treated at 30 years of age or over, and biochemical measures suggest that reproductive lifespan may be shortened among younger females.[43-46]

Implications for selection of primary therapy for contemporary patients

Ideally, the lowest treatment intensity possible will be selected to cure the disease and also minimize the risk of late effects. This relatively simple principle is often challenging to implement for an individual patient, and has produced considerable debate regarding the relative merits of combined modality therapy *versus* chemotherapy alone, and the selection of chemotherapy regimens (e.g. BEACOPP *vs.* ABVD). Insofar as late effects are an important consideration in HL management, it is useful to individualize treatment decisions not only based on patients' HL risk strata *per se*, but also on clinical factors that influence the risks of late toxicity. Some of these factors include the following.

Patient age: young age at treatment has generally been associated with higher relative risks and greater expected excess lifetime risks of second malignancy related to RT exposure. For example, the absolute excess risk of breast cancer is highest among females treated before 20 years of age, and is minimally elevated among women treated after 40 years of age. Thyroid cancer risk is also higher among patients treated at younger ages.[2,4,6,8,10,42] Recognizing these age effects, most pediatric protocols use lower doses of RT than adult protocols, and avoidance of RT, particularly in circumstances in which the distribution of disease would require significant normal tissue exposure (female breast exposure in particular), is of much greater importance for children and adolescents than for patients aged over 40 years. In contrast, Aleman *et al.* reported higher absolute excess risks of cardiac toxicity among patients treated with mediastinal RT at an older age, compared to those treated before 20 years of age, although higher relative risks among younger patients may in future translate into higher lifetime excess risks among younger patients. [18]

Patient gender: the excess risk of second solid cancer following mantle RT is substantially greater among females than among males. For example, Constine *et al.* reported a 25-year cumulative incidence of SC of approximately 35% among female survivors treated with mantle or extended-field RT (mean dose 32.9Gy) *versus* approximately 15% for males. Among females, cancers of the breast and thyroid accounted for approximately 40% and 15% of second malignancies, respectively, and these two SCs accounted for the significant risk difference between females and males.[47]

Males, in contrast, have higher risks of heart disease than females and, RT that encompasses significant cardiac volumes appear to magnify this difference. Aleman *et al*, reported an absolute excess risk of coronary artery disease of 60.7 per 10^4 person-years for males, and 9.7 per10^4 person-years for females,[18] and Galper *et al.* reported the SIR of cardiac complications of 1.56 (*P*=0.003) compared to females.[17]

Distribution of disease: for patients with early stage HL for whom the merits of chemotherapy alone *versus* combined modality therapy are being considered, an important consideration is the distribution of disease and the associated normal tissues that would be exposed in a contemporary IFRT/INRT volume. It is important to recognize that modern RT is not a monolithic treatment with similar risks of late toxicity for all patients. In a study of 37 patients receiving IFRT for mediastinal disease, for example, breast and lung doses varied 11-fold and 3.6-fold, respectively, due to the anatomic variation in the distribution of disease.[48] A young female with small volume disease that involves both axillae in addition to the mediastinum will have an IFRT field similar to a traditional mantle field, and will have a subsequent risk of breast cancer comparable to that described in historic cohorts, while most patients with mediastinal disease will have a lower risk.

Likewise, the cardiac radiation dose associated with RT fields encompassing a mediastinal disease distribution above the T5 vertebral level has been shown to be very low, while RT to disease that extends along pre-cardiac lymph nodes can produce clinically significant cardiac dose.

Pre-existing cardiac risk factors: management of patients with a significant history of heart disease is challenging since impaired ventricular function may limit the delivery of full-dose doxorubicin. In addition, Myrehaug *et al.* found that mediastinal RT given to older patients with pre-existing heart disease was associated with a significant increase in the risk of cardiac-related hospitalization, occurring with a shorter latency after treatment than typically described among younger patients.[49] Other studies have also found that a family history of heart disease or the presence of conventional cardiac risk factors confers a high risk of cardiac morbidity among HL survivors (i.e. to a greater degree than among the general population).[50]

Desire to preserve fertility: as noted above, the cumulative dose of alkylating agents used in different protocols varies to the point that the desire of younger patients to preserve their fertility may reasonably influence treatment selection. A female with high-risk HL who has completed childbearing may opt for BEACOPP-based therapy, whereas a comparable risk male who wishes to preserve fertility may prefer ABVD. With regard to adjuvant RT, a young female with non-bulky pelvic disease and a rapid complete response to chemotherapy will have a worse risk-benefit profile with respect to fertility preservation than an older patient with mediastinal disease. These examples illustrate that, even among patients within the same HL risk stratum, variation in clinical features may make different treatment approaches preferable to optimize the balance between curing the HL and limiting the risks of late toxicity. While some may view one of these options as medically preferable, defining the optimal trade off between relapse and delayed risks can be a value judgement that requires substantial patient input.

Implications for management of survivors

Many patients in long-term follow up will have been exposed to treatments that are currently no longer in use. Moreover, oncologists will often wish to shift the follow up of long-term survivors to the primary care physician. To ameliorate the morbidity associated with the late effects described above, early initiation of cancer screen-

ing is recommended for selected survivors.[51] Selected screening recommendations among HL survivors are described below.

Prior mediastinal RT

Women who have received mediastinal, axillary, or whole lung RT should have breast cancer screening initiated 8-10 years after RT or by 25-30 years of age, whichever comes later.[51-53] Breast MRI should be considered for younger patients (aged 30-40 years) due to the mammographic density of the breast tissue among young women. Women receiving RT after 40 years of age should start breast cancer screening start at 50 years of age.

Patients should be counseled to avoid (or stop) smoking, and to have conventional cardiac risk factors (blood pressure, serum lipids) monitored periodically (i.e. every 1-2 years). As noted above, the risk of clinically significant heart disease associated with conventional risk factors appears to be elevated among HL survivors, so if these become abnormal they should be treated. Some investigators recommend stress echocardiography and radionuclide perfusion imaging be performed 5-10 years after more than 36 Gy mediastinal RT, finding that this will detect significant wall motion abnormalities or perfusion defects in approximately 10-20% of survivors.[54] Thyroid function should be evaluated annually following neck or mediastinal RT. A single elevation of thyroid stimulating hormone (TSH) should not be taken as an indication to initiate L-thyroxine, however, as TSH levels can fluctuate over repeated measurements. The practice among some clinicians is to obtain a thyroid ultrasound ten years following neck RT among pediatric HL survivors, and every 1-3 years thereafter depending on the initial result. There is limited evidence to support the use of low-dose computed tomography (CT) to screen for lung cancer among survivors treated with prior mediastinal RT. In fact, the absolute risk of lung cancer for most HL survivors is small. However, smoking, radiation exposure, and alkylating agent chemotherapy appear to have multiple effects on lung cancer risk, and in view of recent results from the National Cancer Institute's National Lung Screening Trial, it is reasonable to consider low-dose CT lung cancer screening among HL survivors with prior chest RT and/or alkylating agent exposure and a history of smoking.[55]

Abdominal RT (i.e. para-aortic and splenic RT) and/or splenectomy

The absolute risk of colorectal cancer screening among HL survivors is increased, although the onset of this risk is delayed compared to breast cancer.[7] For a patient treated at 20 years of age, the absolute risk of colorectal cancer among HL survivors by 40-45 years of age is comparable to an average 50-year old, and some expert groups recommend that patients who received abdominal RT doses of 25 Gy or over (e.g. for para-aortic RT) should consider colorectal cancer screening ten years after treatment or by 35 years of age, whichever is later.[51]

Guidelines for patients who have had prior splenectomy or splenic dysfunction are available. Although there is little evidence on splenic function following splenic RT, it would be reasonable to apply these guidelines to patients receiving more than 25 Gy RT. Highlights of these guidelines are shown in Table 1. Identification of patients at 'high risk' for infection, and counseling regarding the potential merits of prophylactic antibiotics, may warrant referral to an infectious disease specialist.[56]

Alkylating agent exposure

Subfertility and premature ovarian failure (POF) are relatively neglected late effects of therapy. Alkylating agent chemotherapy and pelvic RT can be associated with dose- and age-dependent premature ovarian failure and subfertility. Emerging technologies for assisted reproduction now make it worthwhile to evaluate the ovarian function of female survivors exposed to alkylating agents, including those with normal menstrual cycles. Studies have shown significant subfertility can occur in the presence of normal cycles, so that a clinical history alone is not an adequate guide to fertility status among such patients. Moreover, young survivors who are found to be sub-fertile now have options for assisted reproduction, but these options become impracticable if delayed for too long.

Anti-Müllerian hormone (AMH) has been shown to be a good predictor of menopause in healthy females and in

Table 1. Guidelines for patients who have had prior splenectomy or splenic dysfunction.

Treatment received	Recommended follow up
Mediastinal radiation	Initiate breast cancer screening 8 years after treatment or at 25-30 years of age, whichever is later.
·	Cardiac stress test 5-10 years after RT if >36 Gy prescribed.
·	Monitor cardiac risk factors every 1-2 years.
·	Thyroid function tests annually.
Splenectomy/splenic RT	Pneumococcal vaccination (Haemophilus influenzae type b conjugate vaccine, meningococcal conjugate vaccine).
·	Annual influenza immunization.
·	Lifelong prophylactic antibiotics offered to patients considered at continued high risk of pneumococcal infection.
·	Patients should carry a supply of appropriate antibiotics for emergency use.
·	Patients developing infection, despite the above measures, to be given systemic antibiotics and admitted urgently to hospital.
Abdominal RT	Initiate colorectal cancer screening starting 10 years after RT or at 35 years of age, whichever is later.
Alkylating agent exposure	Ovarian function evaluation in females 25-40 years of age (even if reporting regular cycles).

comparison to other serum markers of ovarian reserve, AMH has the advantage of showing little or no fluctuation during menstrual cycle, making it logistically easy to test as part of routine follow up. AMH level also seems to be a good indicator of the longitudinal decline of oocyte reserve, and becomes abnormal before the onset of irregular cycles and before follicle stimulating hormone level begins to rise. Data are accumulating indicating that measurement of AMH level in young females exposed to alkylating agents may identify those who would benefit from early fertility counseling before clinically evident symptoms of ovarian failure arise.

There is little information on the relative risks and benefits of using hormone therapy to alleviate symptoms of POF in female survivors. Of particular concern is the possibility of increasing breast cancer risk among those with prior chest RT, as the loss of endogenous estrogens has been shown to reduce breast cancer risk among such patients. For a young woman with symptomatic POF, it is reasonable to prescribe oral contraception provided she is willing to undertake breast cancer screening as outlined above. Some clinical experts will discontinue hormonal therapy after 40-45 years of age.

Late effects: emerging developments

Emerging treatments

The rapid pace of drug development suggests that treatment (and the associated toxicities) of HL may change substantially over the next decade. For example, brentuximab vedotin, is an anti-CD30 antibody conjugated to the anti-microtubule agent monomethyl auristatin E. This agent has produced complete response in 34% of patients with relapse/refractory HL, and clinical trials are being planned to evaluate it as a part of upfront trials in both adult and pediatric HL.[57,58]

Proton therapy is qualitatively different from conventional photon therapy insofar as a beam of a given energy has a specific range of penetration in tissue with little dose delivered beyond that distance. Consequently, proton therapy can significantly reduce the volume of normal tissue receiving low and intermediate doses. In a study of patients with mediastinal HL, Hoppe *et al.* reported that compared to photon RT, proton therapy significantly reduced the cardiac dose (from mean 19.4 Gy to 8.9 Gy proton equivalent) among patients with subcarinal disease, and to a lesser extent the female breast dose.[59,60] The major limitation of protons is the complexity and uncertainty of the treatment planning and the achievement of homogeneous dose, particularly for large irregularly shaped targets.

These innovations promise to facilitate better disease control, particularly among high risk patients, while potentially reducing treatment toxicity, although it will be some years before their effectiveness will be demonstrated in clinical trials, and even longer for their impact on late morbidity to be well understood.

Genetic factors and risk for treatment-related toxicity

As the biological correlates of treatment-related toxicity are better understood, it will become increasingly possible to modify treatment or employ chemoprohpylaxis to prevent or reduce late effects. Genetic polymorphisms involved in the regulation of anthracycline metabolism and handling of reactive oxygen species have been shown to be associated with the development of anthracycline induced CHF.[61]

The genetic contributors to second cancer risk among HL survivors have been more challenging to clarify. Recognized genetic variants associated with radiation sensitivity, such as the *ATM* gene, have not been found to be associated with an increased risk of second cancer in HL survivors, and there is no clear association between heterozygous BRCA carrier status and the risk of RT-related breast cancer in female survivors.[62] Recently, variants at chromosome 6q21 were found to be associated with an increased risk of second cancers in survivors of Hodgkin's lymphoma treated with RT as children, but not as adults.[63]

While these findings are encouraging, considerably more work is required before it will be feasible to tailor treatment intensity or follow up based on molecular assays of late toxicity risk.

Conclusion

Significant changes in the chemotherapy and RT used to treat HL patients over the last 10-20 years will result in different risks of delayed toxicity than those described in many published studies of late effects. Familiarity with these changes and their expected impact on late toxicity is an important feature of accurate disclosure and discussion of treatment options with newly diagnosed HL patients, and also the appropriate management of survivors. As the risk of late effects are reduced, increasingly sophisticated judgments (and larger trials) will be required to find patients who can have further treatment reductions without compromising cure.

References

1. Bhatia S, Yasui Y, Robison LL, et al. High risk of subsequent neoplasms continues with extended follow-up of childhood Hodgkin's disease: report from the Late Effects Study Group. J Clin Oncol. 2003;21:4386-94.
2. Dores GM, Metayer C, Curtis RE, et al. Second malignant neoplasms among long-term survivors of Hodgkin's disease: a population-based evaluation over 25 years. J Clin Oncol. 2002;20:3484-94,.
3. Metayer C, Lynch CF, Clarke EA, et al. Second cancers among long-term survivors of Hodgkin's disease diagnosed in childhood and adolescence. J Clin Oncol. 2000;18:2435-43.
4. Swerdlow AJ, Barber JA, Hudson GV, et al. Risk of second malignancy after Hodgkin's disease in a collaborative British cohort: the relation to age at treatment. J Clin Oncol. 2000;18:498-509.
5. van Leeuwen FE, Klokman WJ, Hagenbeek A, et al. Second cancer risk following Hodgkin's disease: a 20-year follow-up study. J Clin Oncol. 1994;12:312-25.
6. van Leeuwen FE, Klokman WJ, Veer MB, et al. Long-term risk of second malignancy in survivors of Hodgkin's disease treated during adolescence or young adulthood. J Clin Oncol. 2000;18:487-97.
7. Hodgson DC, Gilbert ES, Dores GM, et al. Long-term solid cancer risk among 5-year survivors of Hodgkin's lymphoma. J Clin Oncol. 2007;25:1489-97.
8. Ng AK, Bernardo MV, Weller E, et al. Second malignancy after Hodgkin disease treated with radiation therapy with or without chemotherapy: long-term risks and risk factors. Blood. 2002;100:1989-96.
9. Swerdlow AJ, Cooke R, Bates A, et al. Breast cancer risk after supradiaphragmatic radiotherapy for Hodgkin's lymphoma in England and Wales: a National Cohort Study. J Clin Oncol. 2012;30:2745-52.
10. De Bruin ML, Sparidans J, Van't Veer MB, et al. Breast Cancer Risk in Female Survivors of Hodgkin's Lymphoma:

Lower Risk After Smaller Radiation Volumes. J Clin Oncol. 2009;27(26):4239-46.
11. Kenney L, Yasui Y, Inskip P, et al. Breast cancer after childhood cancer: a report from the Childhood Cancer Survivor Study. Ann Intern Med. 2004;141(8):590-7.
12. Travis LB, Hill D, Dores GM, et al. Cumulative absolute breast cancer risk for young women treated for Hodgkin lymphoma. J Natl Cancer Inst. 2005;97:1428-37.
13. Swerdlow AJ, Higgins CD, Smith P, et al. Second cancer risk after chemotherapy for Hodgkin's lymphoma: a collaborative British cohort study. J Clin Oncol. 2011;29:4096-104.
14. Travis LB, Gospodarowicz M, Curtis RE, et al. Lung cancer following chemotherapy and radiotherapy for Hodgkin's disease. J Natl Cancer Inst. 2002;94:182-92.
15. Birdwell SH, Hancock SL, Varghese A, et al. Gastrointestinal cancer after treatment of Hodgkin's disease. Int J Radiat Oncol Biol Phys. 1997;37:67-73.
16. Myrehaug S, Pintilie M, Tsang R, et al. Cardiac morbidity following modern treatment for Hodgkin lymphoma: supra-additive cardiotoxicity of doxorubicin and radiation therapy. Leuk Lymphoma. 2008;49:1486-93.
17. Galper SL, Yu JB, Mauch PM, et al. Clinically significant cardiac disease in patients with Hodgkin lymphoma treated with mediastinal irradiation. Blood. 2011;117:412-8.
18. Aleman BM, van den Belt-Dusebout AW, De Bruin ML, et al. Late cardiotoxicity after treatment for Hodgkin lymphoma. Blood. 2007;109:1878-86.
19. Anselmo AP, Cartoni C, Bellantuono P, et al. Risk of infertility in patients with Hodgkin's disease treated with ABVD vs MOPP vs ABVD/MOPP. Haematologica. 1990;75:155-8.
20. Heikens J, Behrendt H, Adriaanse R, et al. Irreversible gonadal damage in male survivors of pediatric Hodgkin's disease. Cancer. 1996;78:2020-4.
21. Kreuser ED, Xiros N, Hetzel WD, et al. Reproductive and endocrine gonadal capacity in patients treated with COPP chemotherapy for Hodgkin's disease. J Cancer Res Clin Oncol. 1987;113:260-6.
22. De Bruin ML, Huisbrink J, Hauptmann M, et al. Treatment-related risk factors for premature menopause following Hodgkin lymphoma. Blood. 2008;111:101-8.
23. Viviani S, Ragni G, Santoro A, et al. Testicular dysfunction in Hodgkin's disease before and after treatment. Eur J Cancer. 1991;27:1389-92.
24. Bowers DC, McNeil DE, Liu Y, et al. Stroke as a late treatment effect of Hodgkin's Disease: a report from the Childhood Cancer Survivor Study. J Clin Oncol. 2005;23:6508-15.
25. Jereczek-Fossa BA, Alterio D, Jassem J, et al. Radiotherapy-induced thyroid disorders. Cancer Treat Rev. 2004;30:369-84.
26. Ng AK, Li S, Neuberg D, et al. A prospective study of pulmonary function in Hodgkin's lymphoma patients. Ann Oncol. 2008;19:1754-8.
27. Hjermstad MJ, Oldervoll L, Fossa SD, et al. Quality of life in long-term Hodgkin's disease survivors with chronic fatigue. Eur J Cancer. 2006;42:327-33.
28. Koh ES, Tran TH, Heydarian M, et al. A comparison of mantle versus involved-field radiotherapy for Hodgkin's lymphoma: reduction in normal tissue dose and second cancer risk. Radiat Oncol. 2007;2:13.
29. Maraldo MV, Brodin NP, Vogelius IR, et al. Risk of developing cardiovascular disease after involved node radiotherapy versus mantle field for Hodgkin lymphoma. Int J Radiat Oncol Biol Phys. 2012;83:1232-7.
30. Inskip PD, Robison LL, Stovall M, et al. Radiation dose and breast cancer risk in the childhood cancer survivor study. J Clin Oncol. 2009;27:3901-7.
31. Travis LB, Hill DA, Dores GM, et al. Breast cancer following radiotherapy and chemotherapy among young women with Hodgkin disease. JAMA. 2003;290:465-75.
32. van Leeuwen FE, Klokman WJ, Stovall M, et al. Roles of radiation dose, chemotherapy, and hormonal factors in breast cancer following Hodgkin's disease. J Natl Cancer Inst. 2003; 95:971-80.
33. O'Brien MM, Donaldson SS, Balise RR, et al. Second malignant neoplasms in survivors of pediatric Hodgkin's lymphoma treated with low-dose radiation and chemotherapy. J Clin Oncol. 2010;28:1232-9
34. Weber DC, Peguret N, Dipasquale G, et al. Involved-node and involved-field volumetric modulated arc vs. fixed beam intensity-modulated radiotherapy for female patients with early-stage supra-diaphragmatic Hodgkin lymphoma: a comparative planning study. Int J Radiat Oncol Biol Phys. 2009;75:1578-86.
35. Campbell BA, Voss N, Pickles T, et al. Involved-nodal radiation therapy as a component of combination therapy for limited-stage Hodgkin's lymphoma: a question of field size. J Clin Oncol. 2008;26:5170-4.
36. Maraldo MV, Aznar MC, Vogelius IR, et al. Involved Node Radiation Therapy: An Effective Alternative in Early-Stage Hodgkin Lymphoma. Int J Radiat Oncol Biol Phys, 2013; 85(4):1057-65.
37. Chera BS, Rodriguez C, Morris CG, et al. Dosimetric comparison of three different involved nodal irradiation techniques for stage II Hodgkin's lymphoma patients: conventional radiotherapy, intensity-modulated radiotherapy, and three-dimensional proton radiotherapy. Int J Radiat Oncol Biol Phys. 2009;75:1173-80.
38. Girinsky T, van der Maazen R, Specht L, et al. Involved-node radiotherapy (INRT) in patients with early Hodgkin lymphoma: concepts and guidelines. Radiother Oncol. 2006;79: 270-7.
39. Weber DC, Johanson S, Peguret N, et al. Predicted risk of radiation-induced cancers after involved field and involved node radiotherapy with or without intensity modulation for early-stage hodgkin lymphoma in female patients. Int J Radiat Oncol Biol Phys. 2011;81:490-7.
40. Swerdlow AJ, Higgins CD, Smith P, et al. Myocardial infarction mortality risk after treatment for Hodgkin disease: a collaborative British cohort study. J Natl Cancer Inst. 2007;99:206-14.
41. Kulkarni SS, Sastry PS, Saikia TK, et al. Gonadal function following ABVD therapy for Hodgkin's disease. Am J Clin Oncol. 1997;20:354-7.
42. Hodgson DC, Pintilie M, Gitterman L, et al. Fertility among female hodgkin lymphoma survivors attempting pregnancy following ABVD chemotherapy. Hematol Oncol. 2007;25:11-5.
43. Behringer K, Breuer K, Reineke T, et al. Secondary amenorrhea after Hodgkin's lymphoma is influenced by age at treatment, stage of disease, chemotherapy regimen, and the use of oral contraceptives during therapy: a report from the German Hodgkin's Lymphoma Study Group. J Clin Oncol. 2005;23: 7555-64.
44. Behringer K, Wildt L, Mueller H, et al. No protection of the ovarian follicle pool with the use of GnRH-analogues or oral contraceptives in young women treated with escalated BEACOPP for advanced-stage Hodgkin lymphoma. Final results of a phase II trial from the German Hodgkin Study Group. Ann Oncol. 2010;21:2052-60.
45. Sieniawski M, Reineke T, Nogova L, et al. Fertility in male patients with advanced Hodgkin lymphoma treated with BEACOPP: a report of the German Hodgkin Study Group (GHSG). Blood. 2008;111:71-6.
46. Sieniawski M, Reineke T, Josting A, et al. Assessment of male fertility in patients with Hodgkin's lymphoma treated in the German Hodgkin Study Group (GHSG) clinical trials. Ann Oncol. 2008;19:1795-801.
47. Constine LS, Tarbell N, Hudson MM, et al. Subsequent malignancies in children treated for Hodgkin's disease: associations with gender and radiation dose. Int J Radiat Oncol Biol Phys. 2008;72:24-33.
48. Hodgson DC, Koh ES, Tran TH, et al. Individualized estimates of second cancer risks after contemporary radiation therapy for Hodgkin lymphoma. Cancer. 2007;110(11):2576-86.
49. Myrehaug S, Pintilie M, Yun L, et al. A population-based study of cardiac morbidity among Hodgkin lymphoma patients with preexisting heart disease. Blood. 2010;116:2237-40.
50. Andersson A, Naslund U, Tavelin B, et al. Long-term risk of cardiovascular disease in Hodgkin lymphoma survivors-Retrospective cohort analyses and a concept for prospective intervention. Int J Cancer. 2009;124(8):1914-7.
51. Group CsO. [Internet] Available from: www.survivorshipguidelines
52. Howell SJ, Searle C, Goode V, et al. The UK national breast cancer screening programme for survivors of Hodgkin lymphoma detects breast cancer at an early stage. Br J Cancer. 1009;101:582-8.
53. Smith RA, Saslow D, Sawyer KA, et al. American Cancer Society guidelines for breast cancer screening: update 2003. CA Cancer J Clin. 2003;53:141-69.
54. Heidenreich PA, Schnittger I, Strauss HW, et al. Screening for coronary artery disease after mediastinal irradiation for Hodgkin's disease. J Clin Oncol. 2007;25:43-9.
55. Wender R, Fontham ET, Barrera E Jr, et al. American Cancer Society lung cancer screening guidelines. CA Cancer J Clin. 2013; 63(2):107-17.
56. Davies JM, Lewis MP, Wimperis J, et al. Review of guidelines for the prevention and treatment of infection in patients with

an absent or dysfunctional spleen: prepared on behalf of the British Committee for Standards in Haematology by a working party of the Haemato-Oncology task force. Br J Haematol. 2011;155:308-17.
57. Deng C, Pan B, O'Connor OA. Brentuximab vedotin. Clin Cancer Res. 2013;19:22-7.
58. Kelly KM, Hodgson D, Appel B, et al. Children's Oncology Group's 2013 blueprint for research: Hodgkin lymphoma. Pediatr Blood Cancer. 2012 Dec 19. [Epub ahead of print]
59. Cella L, Liuzzi R, Magliulo M, et al. Radiotherapy of large target volumes in Hodgkin's lymphoma: normal tissue sparing capability of forward IMRT versus conventional techniques. Radiat Oncol. 2010;5:33.
60. Hoppe BS, Flampouri S, Lynch J, et al. Improving the therapeutic ratio in Hodgkin lymphoma through the use of proton therapy. Oncology. 2012;26:456-65.
61. Blanco JG, Sun CL, Landier W, et al. Anthracycline-related cardiomyopathy after childhood cancer: role of polymorphisms in carbonyl reductase genes—a report from the Children's Oncology Group. J Clin Oncol. 2012;30:1415-21.
62. Travis LB, Rabkin CS, Brown LM, et al. Cancer Survivorship - Genetic Susceptibility and Second Primary Cancers: Research Strategies and Recommendations. J Natl Cancer Inst. 2006;98:15-25.
63. Best T, Li D, Skol AD, et al. Variants at 6q21 implicate PRDM1 in the etiology of therapy-induced second malignancies after Hodgkin's lymphoma. Nat Med. 2011;17:941-3.

From monoclonal gammopathy of undetermined significance to symptomatic multiple myeloma: genetic heterogeneity on all levels

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A B S T R A C T

Multiple myeloma (MM) is preceded by pre-malignant disease phases of monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM). The genetic abnormalities found in MM comprise of intrachromosomal translocations, largely involving the *IGH* locus, copy number abnormalities, somatic mutations and changes in DNA and histone methylation. Many of these genetic lesions are also present in MGUS and SMM but do not result in the clinical symptoms associated with MM. Here we discuss the common abnormalities in these disease phases along with the impact of intraclonal heterogeneity on the future of myeloma biology and treatment.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe the common genetic abnormalities in multiple myeloma;
- know the common somatic mutations in myeloma and targeted therapy options;
- understand the complex subclonal genetic architecture of myeloma.

Introduction

Multiple myeloma is a genetically complex disease that is becoming more common in today's aging population. Myeloma belongs to a group of related paraproteinemias that are characterized by an abnormal clonal plasma cell infiltration in the bone marrow.[1,2] A number of distinct clinical phases of myeloma can be recognized, including monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic or smoldering multiple myeloma (SMM). Both these phases lack the clinical features of myeloma but share some of the genetic features of a myeloma clone.[3] By contrast, symptomatic multiple myeloma (MM) is defined by clinical symptoms and evidence of organ damage. A characteristic feature of myeloma cells is the requirement for an intimate relationship with the bone marrow microenvironment, where plasma cells are nurtured in specialized niches that maintain their survival long term.[4-8] However, during the progression of the disease, clonal cells develop the ability to proliferate at sites outside of the bone marrow, manifesting as extra-medullary myeloma (EMM) and plasma cell leukemia (PCL).[9] These cells constitute the end stages in the multistep transformation process from normal to malignant plasma cells. Here we will review the genetics and techniques used to study the events in the process of transformation from MGUS through SMM, MM and finally to PCL. These include the classically studied translocations and hyperdiploidy, copy number abnormalities and, finally, how genome sequencing strategies are identifying new potential targets in somatic mutations and how these can be used to determine the evolutionary course of disease progression.

Translocations

Chromosomal translocations arise when DNA double strand breaks at different sites in the genome are brought together and aberrantly rejoined.[10] They are common in tumors of the lymphoid lineage because of the 'off target' effects of the normal physiological mechanisms mediating DNA rearrangement at the immunoglobulin (*IGH*) locus. Translocations into the *IGH* locus predominantly occur either during recombination activation gene (RAG) complex-mediated V(D)J rearrangement, such as in mantle cell lymphoma (t(11;14)),[11] or during class switch recombination (CSR). In myeloma, the primary translocations are thought to be generated via abnormal CSR events mediated by activation-induced cytidine deaminase (AID).[12] This concept has been developed and is based on the location of the translocation breakpoints determined in myeloma cell lines and a few primary samples. Added to this, the myeloma clone is derived from a mature plasma cell that has undergone somatic hypermutation in the germinal center[13] and does not express the RAG complex.

In myeloma, primary aberrant rearrangements into the *IGH* locus are present in up to 40% of cases.[14,15] There are five main translocation partner chromosomes including the t(4;14) (11%), t(6;14) (2%), t(11;14) (15%), t(14;16) (3%) and t(14;20) (1.5%) which result in the overexpression of *MMSET* and *FGFR3*,

CCND3, *CCND1*, *MAF* and *MAFB*, respectively, and are thought to confer a selective advantage to the clone (Figure 1).[16] Although the translocations over-express very different genes, they have in common downstream deregulation of cyclin D genes, which have been grouped together under the Translocation/Cyclin D (TC) classification.[17] In its simplest form, this classification defines groups of myeloma samples based on their expression of *CCND1* (t(11;14)), *CCND2* (t(4;14),t(14;16) and t(14;20)), and CCND3 (t(6;14)). However, the translocations themselves are not sufficient to cause progression to myeloma. Evidence for this comes from analysis of MGUS, SMM and MM samples in which translocations are detected, but not at the same frequency.[18] For example, the t(14;20) is present in 5% of MGUS samples but only 1.5% of MM samples, and conversely, the t(4;14) is present in 3% of MGUS but rises to 11% in MM samples. The conclusions drawn from these data are that some translocations, such as the t(14;20), can be stable in MGUS patients for long periods of time resulting in higher frequencies present in MGUS, whereas the t(4;14) progresses to MM faster, resulting in a lower frequency in MGUS patients.

Copy number changes

In addition to translocations, copy number abnormalities are common in myeloma (Figure 1). These abnormalities have been studied by many techniques from karyotyping and fluorescence *in situ* hybridization (FISH) through to SNP-based mapping arrays, and more recently, exome sequencing. The most prevalent copy number abnormality is the presence of hyperdiploidy, through trisomy of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21, and like the translocations is considered a primary event. Hyperdiploidy is present in approximately 50% of myeloma samples and is almost mutually exclusive with *IGH* translocations, where both translocations and hyperdiploidy occur in only 9% of samples. The most commonly gained chromosomes are 9, 15 and 19 but the genetic mechanism of gain and pathogenic advantage still remain elusive. Hyperdiploid patients tend to have a better prognostic outcome than those with *IGH* translocations. The myeloma genome is rife with additional copy number abnormalities, with almost all chromosomes being affected across samples, indicating genomic instability in myeloma. Aside from the trisomies related to hyperdiploidy, the most common chromosomal abnormalities are del(1p) (30%), 1q+ (36%), del(6q) (33%), del(8p) (25%), 11q+ (24%), del(13q) (58%), del(16q) (35%) and del(17p) (7%).[19] In some of these chromosomes, the genes of interest have been identified but in others they remain elusive. For example, on 1p *FAM46C*, *CDKN2C* and *FAF1* have been identified as potential targets,[19-21] on 16q *CYLD* and *WWOX* are targets of interstitial deletions,[22,23] on 1q *CKS1B*, *ANP32E*, *BCL9* and *PDZK1* have all attracted interest,[19,24,25] and on 17p *TP53* is the clear gene of interest.[19,26,27] However, for many of the chromosomal abnormalities (6q, 8p) there is no clear target gene. These last two regions have not been so well studied, in part because they currently have no prognostic value.

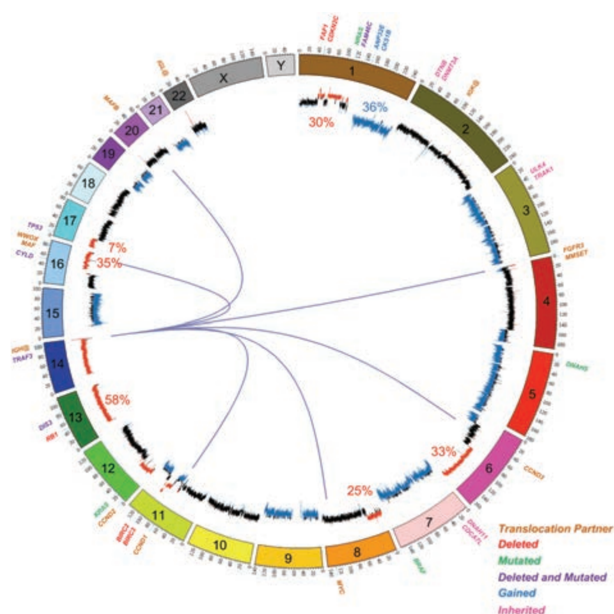

Figure 1. The common genetic abnormalities in myeloma. The circos plot shows chromosomes arranged around the outside in a clockwise direction. The internal track shows the common copy number changes with deletions (red) and gains (blue) shown with their frequencies in myeloma. Translocations are indicated by lines across the center between loci. The genes of interest are shown around the outside of the circle and are color-coded according to the legend.

Cytogenetic risk stratification

Cytogenetics has been used to determine which genetic lesions have an impact on overall and progression-free survival. Concerning the translocation groups, t(4;14), t(14;16) and t(14;20) are considered to be high risk genetic events resulting in a decreased overall survival.[28] However, much of the high risk nature of the t(4;14) can be overcome by treatment with bortezomib.[29] t(11;14) and t(6;14) are considered standard risk groups, as is hyperdiploidy. Many of the copy number abnormalities do have a prognostic value in several datasets. In the UK MRC Myeloma IX trial, we have shown del(1p), 1q+ and del(17p) all have an independent statistically significant impact in overall survival.[19,26] This has been confirmed in other datasets with several different treatment contexts.[30-33] Together with t(4;14), these cytogenetic markers have been used to identify patients with high-risk myeloma, which could be managed differently to standard risk patients. One analysis has also determined that the poor prognostic effect of high-risk genetics (t(4;14), t(14;16), t(14;20) or del(17p)) can be ameliorated by the presence of trisomies.[34] Bortezomib administration can also improve outcome in patients with del(17p) when administered before and after autologous stem cell transplantation.[35]

The accumulation of adverse markers has a profound effect on the overall survival of a patient. Many of the adverse lesions co-segregate, so the chance of a patient having more than one abnormality is increased, for example 72% of patients with an *IGH* translocation also have

1q+. By integrating these known adverse lesions it is possible to more accurately estimate the overall survival of a patient where those without any adverse markers (OS=60.6 months) do better than those with one (OS=41.9 months), two (OS=23.4 months) or three (OS=9.1 months) adverse markers. [36]

Somatic mutations

The most recent developments in myeloma genetics revolve around genome and exome sequencing of samples, allowing the identification of somatic mutations and structural variations. This has been exemplified by the initial publication of the landscape of mutations in myeloma through sequencing of 38 myeloma samples.[37] The number of non-synonymous (NS) somatic mutations found in myeloma is around 30-35.[37,38] This number is higher than some other hematologic malignancies such as hairy cell leukemia (NS-mutations = 5),[39] acute myeloid leukemia (NS-mutations = 8)[40] but much lower than solid tumors such as lung cancer (NS-mutations = 540).[41] This level of mutation indicates that myeloma is more complex than most hematologic malignancies.

The main finding of this initial screen is that there is no unifying mutation in myeloma. In some other hematologic malignancies, a common mutation in most or all samples has been discovered and is thought to be the primary driver mutation. For example, in hairy cell leukemia, the *BRAF* V600E mutation is found in all samples,[39] and in Waldenströms macroglobulinemia, the *MYD88* L265P mutation is found in 91% of samples.[42] In myeloma, the most frequent mutations were found in *NRAS* (23%) and *KRAS* (26%), followed by *FAM46C* (13%, previously identified as deleted and mutated)[19,20] and *TP53* (8%). The *NRAS* and *KRAS* mutations, with the addition of *BRAF* mutations (4%), indicates the ERK pathway is critical in at least 53% of myeloma patients and points to a treatment strategy that has so far not been harnessed. ERK pathway mutations are not new to myeloma, but the whole genome strategies have identified some novel mutations not previously identified by other means. These include *DIS3* (mutated in 10%) on chromosome 13, a highly conserved RNA nuclease, which is also deleted in 58% of samples. The function of this mutation is not understood, but may be involved in regulation of the available pool of mRNAs available for translation.[43] However, the number of myeloma samples sequenced to date is small and the true landscape of somatic mutations is yet to be realized. As the number of samples sequenced increases, it will be possible to identify groups of genes with related functions or pathways that can be used as therapeutic targets. For example, DNA and histone methylation are important biological processes in myeloma which is characterized by overexpression of MMSET, a histone methyltransferase, in t(4;14) myeloma, and mutations in other methyltransferases, such as *EZH2* and *MLL3*, can also be present. Additionally, histone lysine demethylases such as *KDM6A* (also known as *UTX*) can be deleted or mutated in myeloma,[44] making histone methylation a common and attractive target for drug therapy.

The discovery of *BRAF* mutations in 4% of myeloma patients has also brought the possibility of targeted therapy to the forefront of myeloma treatment in the clinic. BRAF is part of the MAP kinase pathway, which is activated by RAS through phosphorylation and results in the subsequent activation of the MEK/MAPK/ERK signaling cascade, resulting in proliferation and survival.[45] The *BRAF*^{V600E} mutation is present in 50%-60% of all melanomas and results in constitutive activation of BRAF, bypassing the requirement for RAS, activating the MEK/MAPK/ERK cascade, and culminating in cell proliferation and malignant conversion.[46] The drug vemurafenib is a competitive selective inhibitor of BRAF^{V600E} which is approved for use in melanoma and results in relative reduction of 63% in risk for death compared to other treatments.[47] Vemurafenib, therefore, represents a potential targeted therapy for patients harboring a *BRAF*^{V600E} mutation and clinical trials are underway in myeloma to determine its efficacy.

Intraclonal heterogeneity

Like many malignancies, myeloma cells are not uniform within a patient. A great deal of genetic variation exists within the population of tumor cells, and it is this variation that allows the cancer to persist and diversify. The genetic events within a cancer cell consist of 'driver' and 'passenger' lesions, where drivers confer a selective advantage to the progeny. The acquisition of these lesions allows for the rapid evolution of a clone in a Darwinian fashion. Selection pressures are exerted on the tumor cells allowing the outgrowth of any favorable trait. These selection pressures may give a growth advantage to a cell, confer a better interaction with the bone marrow microenvironment, or even allow independence from the bone marrow resulting in a plasma cell leukemia or an extramedullary tumor. Aside from this, mutations gained in subpopulations of cells may confer drug resistance, allowing the eventual repopulation of the tumor in a drug resistant state.

Although myeloma is considered to be a clonal disease, due to the presence of one V(D)J rearrangement and a monoclonal secreted immunoglobulin, at a genetic level the cells are far from clonal. *IGH* translocations and hyperdiploidy are accepted as being primary events in myeloma pathogenesis; however, the rate at which other abnormalities are accrued has been less well studied. Studies utilizing FISH were the first to investigate the relationship of abnormalities within a sample by using probes to a translocation and a copy number abnormality and comparing the frequencies. When comparing a translocation with del(13q) it was found that the majority of cells carry the translocation (as expected given it is a primary event) but the proportion of cells with del(13q) can vary dramatically from patient to patient, but is always lower than the frequency of the translocation.[48] It can be inferred from these data that the copy number abnormalities occur subsequent to the translocation. By analyzing the disease at different time points it becomes clear that the frequency of any given abnormality increases through MGUS and SMM towards MM in a population of individuals. This has been shown for del(13q), del(17p) and 1q+ where the proportion of myeloma patients with an abnormality increases as the disease progresses.[18,49] However, such an analysis can be even more informative if sequential samples from the same patient are used, particularly when they are taken at different stages of disease (for

example SMM and MM). Several papers have been published analyzing such patients by FISH and SNP-based mapping array.[49,50] The overarching theme of these papers is that the frequency of abnormalities increases within a tumor sample as the disease progresses, but they are generally always present at low levels in the preceding stage of disease. For example, in a patient there may be 29% of cells with del(17p) when the patient is diagnosed with high risk-SMM and this may increase to 86% when they present with symptomatic MM.[51] The genetic landscape of these tumors gets more interesting as the technologies used get more advanced. Using genome sequencing technologies it is possible to estimate the proportion of cells in a tumor mass with any somatic mutation found. This has been achieved in many cancers,[52,53] including myeloma.[38,54] Taking the RAS pathway mutations as an example, it has been shown that these activating oncogenic driver mutations are not necessarily present in the dominant clone. That is, they can be present only in a subset of the cells in the tumor.[38] This is true for *NRAS*, *KRAS* and *BRAF* mutations, indicating that although they are known oncogenic drivers they are not necessarily present early on in the disease and can be acquired as the tumor evolves. Using information on the subclonal nature of multiple mutations or copy number abnormalities it is possible to piece together the history of a tumor, determining which genetic events occurred first or occurred together.[52,55] This can also be done at the single cell level using FISH with multiple probes per cell, or at a nucleotide level using single cell sorting and genotyping assays.[38,55] These techniques clearly indicate a complex substructure of branched evolution in tumor development. Other studies have focused on the genetic evolution of myeloma following treatment.[54] Analysis of tumor DNA collected at multiple time points during a patient's treatment can illustrate the genetic diversity within a myeloma tumor and the effect that treatment has on the dynamics of the sub-clones present. By studying seven time points from diagnosis, remission, four relapse phases and progression to plasma cell leukemia the different subclones present can be seen using arrays, gaining and losing dominance in the myeloma population as the patient undergoes different treatment regimens. Ultimately, the clone that was dominant as the disease progresses to PCL was barely detectable at diagnosis.

Given that myeloma exists as multiple foci of lytic lesions throughout the bone marrow, it remains to be determined how these subpopulations of cells relate to one another, whether they evolve independently, and whether they can be treated as a whole.

Conclusions

Myeloma is a genetically complex malignancy in which translocations involving the *IGH* locus and hyperdiploidy are primary events. These events are followed by an accrual of additional lesions through MGUS and SMM before transforming to MM. These additional lesions include, but are not limited to, chromosomal gains and losses, somatic mutations and DNA methylation changes. It is clear that there is a subclonal genetic structure within the myeloma cell population where copy number and somatic mutations are gained or lost over time, resulting in a mixed population of cells capable of exploiting any selective advantages laid upon them. This intraclonal heterogeneity may prove to be an extra obstacle in the fight towards curing myeloma, but through using therapies towards key genetic mechanisms it should prove possible to selectively target mutated clones.

References

1. Greipp PR, San MJ, Durie BG, et al. International staging system for multiple myeloma. J Clin Oncol. 2005;23(15):3412-20.
2. Kyle RA, Gertz MA, Witzig TE, et al. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc. 2003;78(1):21-33.
3. Kyle RA, Remstein ED, Therneau TM, et al. Clinical course and prognosis of smoldering (asymptomatic) multiple myeloma. New Eng J Med. 2007;356(25):2582-90.
4. Akiyama M, Hideshima T, Hayashi T, et al. Cytokines modulate telomerase activity in a human multiple myeloma cell line. Cancer Res. 2002;62(13):3876-82.
5. Chauhan D, Li G, Hideshima T, et al. Blockade of ubiquitin-conjugating enzyme CDC34 enhances anti-myeloma activity of Bortezomib/Proteasome inhibitor PS-341. Oncogene. 2004;23(20):3597-602.
6. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood. 1999;93(5):1658-67.
7. Hideshima T, Catley L, Yasui H, et al. Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces in vitro and in vivo cytotoxicity in human multiple myeloma cells. Blood. 2006;107(10):4053-62.
8. Mitsiades CS, Mitsiades NS, Munshi NC, Richardson PG, Anderson KC. The role of the bone microenvironment in the pathophysiology and therapeutic management of multiple myeloma: interplay of growth factors, their receptors and stromal interactions. Eur J Cancer. 2006;42(11):1564-73.
9. Palumbo A, Anderson K. Multiple myeloma. N Engl J Med. 2011;364(11):1046-60.
10. Richardson C, Jasin M. Frequent chromosomal translocations induced by DNA double-strand breaks. Nature. 2000;405 (6787):697-700.
11. Jares P, Colomer D, Campo E. Molecular pathogenesis of mantle cell lymphoma. J Clin Invest. 2012;122(10):3416-23.
12. Gonzalez D, van der Burg M, Garcia-Sanz R, et al. Immunoglobulin gene rearrangements and the pathogenesis of multiple myeloma. Blood. 2007;110(9):3112-21.
13. Bakkus MH, Heirman C, van Riet I, van Camp B, Thielemans K. Evidence that multiple myeloma Ig heavy chain VDJ genes contain somatic mutations but show no intraclonal variation. Blood. 1992;80(9):2326-35.
14. Bergsagel PL, Chesi M, Nardini E, Brents LA, Kirby SL, Kuehl WM. Promiscuous translocations into immunoglobulin heavy chain switch regions in multiple myeloma. Proc Natl Acad Sci USA. 1996;93(24):13931-6.
15. Pratt G, Fenton JA, Proffitt JA, et al. True spectrum of 14q32 translocations in multiple myeloma. Br J Haematol. 1998;103(4):1209-10.
16. Morgan GJ, Walker BA, Davies FE. The genetic architecture of multiple myeloma. Nat Rev Cancer. 2012;12(5):335-48.
17. Bergsagel PL, Kuehl WM. Molecular pathogenesis and a consequent classification of multiple myeloma. J Clin Oncol. 2005;23(26):6333-8.
18. Ross FM, Chiecchio L, Dagrada G, et al. The t(14;20) is a poor prognostic factor in myeloma but is associated with long-term stable disease in monoclonal gammopathies of undetermined significance. Haematologica. 2010;95(7):1221-5.
19. Walker BA, Leone PE, Chiecchio L, et al. A compendium of myeloma-associated chromosomal copy number abnormalities and their prognostic value. Blood. 2010;116(15):e56-65.
20. Boyd KD, Ross FM, Walker BA, et al. Mapping of chromosome 1p deletions in myeloma identifies FAM46C at 1p12 and CDKN2C at 1p32.3 as being genes in regions associated with adverse survival. Clin Cancer Res. 2011;17(24):7776-84.
21. Leone PE, Walker BA, Jenner MW, et al. Deletions of CDKN2C in multiple myeloma: biological and clinical implications. Clin Cancer Res. 2008;14(19):6033-41.
22. Jenner MW, Leone PE, Walker BA, et al. Gene mapping and expression analysis of 16q loss of heterozygosity identifies WWOX and CYLD as being important in determining clinical outcome in multiple myeloma. Blood. 2007;110(9):3291-300.

23. Keats JJ, Fonseca R, Chesi M, et al. Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell. 2007;12(2):131-44.
24. Shaughnessy J. Amplification and overexpression of CKS1B at chromosome band 1q21 is associated with reduced levels of p27Kip1 and an aggressive clinical course in multiple myeloma. Hematology. 2005;10(1 Suppl):117-26.
25. Le Baccon P, Leroux D, Dascalescu C, et al. Novel evidence of a role for chromosome 1 pericentric heterochromatin in the pathogenesis of B-cell lymphoma and multiple myeloma. Gene Chromosome Canc. 2001;32(3):250-64.
26. Boyd KD, Ross FM, Tapper WJ, et al. The clinical impact and molecular biology of del(17p) in multiple myeloma treated with conventional or thalidomide-based therapy. Gene Chromosome Can. 2011;50(10):765-74.
27. Chng WJ, Price-Troska T, Gonzalez-Paz N, et al. Clinical significance of TP53 mutation in myeloma. Leukemia. 2007;21(3):582-4.
28. Fonseca R, Bergsagel PL, Drach J, et al. International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. Leukemia. 2009;23(12):2210-21.
29. Avet-Loiseau H, Leleu X, Roussel M, et al. Bortezomib plus dexamethasone induction improves outcome of patients with t(4;14) myeloma but not outcome of patients with del(17p). J Clin Oncol. 2010;28(30):4630-4.
30. Fonseca R, Van Wier SA, Chng WJ, et al. Prognostic value of chromosome 1q21 gain by fluorescent in situ hybridization and increase CKS1B expression in myeloma. Leukemia. 2006;20(11):2034-40.
31. Hanamura I, Stewart JP, Huang Y, et al. Frequent gain of chromosome band 1q21 in plasma-cell dyscrasias detected by fluorescence in situ hybridization: incidence increases from MGUS to relapsed myeloma and is related to prognosis and disease progression following tandem stem-cell transplantation. Blood. 2006;108(5):1724-32.
32. Shaughnessy JD Jr, Haessler J, van Rhee F, et al. Testing standard and genetic parameters in 220 patients with multiple myeloma with complete data sets: superiority of molecular genetics. Br J Haematol. 2007;137(6):530-6.
33. Avet-Loiseau H, Attal M, Moreau P, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. Blood. 2007;109(8):3489-95.
34. Kumar S, Fonseca R, Ketterling RP, et al. Trisomies in multiple myeloma: impact on survival in patients with high-risk cytogenetics. Blood. 2012;119(9):2100-5.
35. Neben K, Lokhorst HM, Jauch A, et al. Administration of bortezomib before and after autologous stem-cell transplantation improves outcome in multiple myeloma patients with deletion 17p. Blood. 2012;119(4):940-8.
36. Boyd KD, Ross FM, Chiecchio L, et al. A novel prognostic model in myeloma based on co-segregating adverse FISH lesions and the ISS: analysis of patients treated in the MRC Myeloma IX trial. Leukemia. 2012;26(2):349-55.
37. Chapman MA, Lawrence MS, Keats JJ, et al. Initial genome sequencing and analysis of multiple myeloma. Nature. 2011;471(7339):467-72.
38. Walker BA, Wardell CP, Melchor L, et al. Intraclonal heterogeneity and distinct molecular mechanisms characterize the development of t(4;14) and t(11;14) myeloma. Blood. 2012;120(5):1077-86.
39. Tiacci E, Trifonov V, Schiavoni G, et al. BRAF mutations in hairy-cell leukemia. N Engl J Med. 2011;364(24):2305-15.
40. Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature. 2008;456(7218):66-72.
41. Lee W, Jiang Z, Liu J, et al. The mutation spectrum revealed by paired genome sequences from a lung cancer patient. Nature. 2010;465(7297):473-7.
42. Treon SP, Xu L, Yang G, et al. MYD88 L265P somatic mutation in Waldenstrom's macroglobulinemia. N Engl J Med. 2012;367(9):826-33.
43. Ibrahim H, Wilusz J, Wilusz CJ. RNA recognition by 3'-to-5' exonucleases: the substrate perspective. Biochim Biophys Acta. 2008;1779(4):256-65.
44. van Haaften G, Dalgliesh GL, Davies H, et al. Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet. 2009;41:521-3.
45. Satyamoorthy K, Li G, Gerrero MR, et al. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Res. 2003;63(4):756-9.
46. White RM. The natural history of malignancies under conditions of BRAF inhibitor stimulation. Expert Opin Investig Drugs. 2011;20(1):135-6.
47. Ravnan MC, Matalka MS. Vemurafenib in patients with BRAF V600E mutation-positive advanced melanoma. Clin Ther. 2012;34(7):1474-86.
48. Chiecchio L, Dagrada GP, Ibrahim AH, et al. Timing of acquisition of deletion 13 in plasma cell dyscrasias is dependent on genetic context. Haematologica. 2009;94(12):1708-13.
49. Lopez-Corral L, Sarasquete ME, Bea S, et al. SNP-based mapping arrays reveal high genomic complexity in monoclonal gammopathies, from MGUS to myeloma status. Leukemia. 2012;26(12):2521-9.
50. Lopez-Corral L, Gutierrez NC, Vidriales MB, et al. The progression from MGUS to smoldering myeloma and eventually to multiple myeloma involves a clonal expansion of genetically abnormal plasma cells. Clin Cancer Res. 2011;17(7):1692-700.
51. Lopez-Corral L, Mateos MV, Corchete LA, et al. Genomic analysis of high-risk smoldering multiple myeloma. Haematologica. 2012;97(9):1439-43.
52. Nik-Zainal S, Van Loo P, Wedge DC, et al. The life history of 21 breast cancers. Cell. 2012;149(5):994-1007.
53. Yates LR, Campbell PJ. Evolution of the cancer genome. Nat Rev Genet. 2012;13(11):795-806.
54. Keats JJ, Chesi M, Egan JB, et al. Clonal competition with alternating dominance in multiple myeloma. Blood. 2012;120(5):1067-76.
55. Anderson K, Lutz C, van Delft FW, et al. Genetic variegation of clonal architecture and propagating cells in leukaemia. Nature. 2011;469(7330):356-61.

Treatment of relapsed and refractory multiple myeloma

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A B S T R A C T

Treatment options and outcomes for patients with relapsed myeloma have dramatically changed over the past ten years due in large part to the availability of novel agents such as bortezomib, thalidomide and lenalidomide. These have now been incorporated into the treatment approach for newly diagnosed patients and also raise questions about bow best to manage patients who relapse. In addition to existing and approved agents, several others have recently been or are soon to be approved, as well as new classes of agents in phase III trials that are likely to not only improve long-term outcomes, but that will also complicate treatment algorithms. We will review data on the optimal use of existing approaches for relapsed disease, as well as new agents under development for relapsed and refractory myeloma.

Learning goals

At the conclusion of this activity, participants should understand:
- when a patient needs therapy for relapsed myeloma;
- how best to use available treatment options for managing relapsed myeloma;
- which are the new agents under development and how to use them for the management of relapsed and refractory myeloma.

Introduction

Multiple myeloma (MM) is a clonal plasma cell malignancy characterized by bone, renal, hematologic, and often neurological complications.[1] The overall survival for patients with symptomatic myeloma has dramatically improved over the last decade due to the broad use of high-dose therapy and autologous transplant for suitable patients, as well as the availability of novel agents whose mechanisms of action are distinctly different from alkylators or steroids. However, even with these advances, most patients will eventually die of complications associated with the development of resistant disease.[2] Plasma cells spend their time in the marrow microenvironment supported by autocrine and paracrine secretion of growth factors such as IL-6, TNFα, IGF-1 and VEGF,[3] as well as direct interaction of the bone marrow microenvironment with plasma cells via integrins and cell adhesion molecules which promote growth and inhibit apoptosis.[4] However, the practical delivery of anti-myeloma therapy in the context of relapsed or refractory disease is a constantly evolving area of research, and one which needs to take into consideration factors of clinical importance. These include: i) which disease setting the patient comes to us in (early *vs*. late); ii) disease specific biology (standard- or high-risk); iii) prior therapies; and iv) prior toxicities from therapy. Through clinical integration of these factors, a treatment strategy can be defined for patients with relapsed or refractory myeloma with the highest chance of response and good tolerance for any given patient.

How to define relapse

Response criteria in myeloma represent an evolving work in progress. While the definition of complete response (CR) continues to become more and more stringent, the definition of relapse or progression has been relatively constant. Relapse from a CR is defined as reappearance of the serum or urinary paraprotein, of 5% or over bone marrow plasma cells, new lytic bone lesions/soft tissue plasmacytomas, an increase in size of residual bone lesions, and/or development of hypercalcemia (corrected serum calcium >11.5 mg/dL) not attributable to another cause.[5] Following the increased depth of response seen with new therapies, the ‘CR penalty’ was addressed by allowing patients who have achieved a CR to be defined as relapsed when they develop a protein of at least 0.5 gm/dL rather than the historical definition of immunofixation negative to immunofixation positive.[6] Criteria for progressive disease (PD) when a CR has not been achieved include new or expanding bone lesions, hypercalcemia, and a more than 25% increase in either serum monoclonal paraprotein concentration, 24-h urinary light chain excretion, or plasma cells within a bone marrow. Relapsed MM refers to the circumstance wherein a patient treated to the point of maximal response experiences PD, whereas refrac-

tory MM refers to a clinical scenario in which a patient is either unresponsive to current therapy or progresses within 60 days of last treatment. It is important to recall that 'patients with refractory disease' has historically referred to patients who were resistant to dexamethasone and alkylators and, given the short duration of response to both alone, patients often developed refractory disease. More recently, the availability of different classes of agents including proteasome inhibitors and immunomodulatory agents, the generic term 'refractory' requires more specificity. Currently, the term 'refractory' requires a description of what the patient is refractory to, i.e. steroids, bortezomib or lenalidomide. In addition, there may be biological differences between patients who are defined as refractory by progression on treatment *versus* those who progress within 60 days of stopping therapy and, as such, they should be specified when base-line patients' characteristics are described in clinical trials. Patients who fail to achieve any response to induction therapy (< minimal response, MR) and then progress on therapy are an especially challenging category of patients with primary refractory myeloma.[7]

As we now have more tools with which to approach relapsed disease, the decision as to when to treat this continues to be an issue. The current International Myeloma Working Group guidelines (IMWG) state that patients should be observed until they develop symptomatic relapse (the same criteria used to differentiate smoldering myeloma from symptomatic myeloma). This raises the issue of differences between patients with biochemical relapse (blood or urine protein only) *versus* those patients with symptomatic relapse (new evidence of end organ damage according to the CRAB criteria). Most clinical trials require patients to have symptomatic relapse prior to study entry, and it is clear that there are patients who can have long-term low-level disease burden and who do not require therapy. Early initiation of salvage therapy in those patients would not necessarily offer benefit, while there may be others with high risk or aggressive relapse for whom waiting till there is evidence of end organ damage may ultimately limit the efficacy of therapy. At this point, it remains prudent to observe patients who have 'biochemical relapse' only, unless there are other factors (prior history of rapid relapse, high-risk genetics, etc.) that suggest to the clinician that delaying therapy may cause the patient harm.

How to systematically approach a relapsed patient

While there is no clear simple algorithm to define how a patient should be treated in the relapsed setting, there are some general principles that can guide the choice of therapy.[8] For patients with indolent or relapse early in their disease course, the use of single agents, depending upon what was used in their initial therapy as well as treatment-related toxicity, is a reasonable approach. For patients who received thalidomide or lenalidomide-based induction therapy, switching to bortezomib-based salvage makes sense in order to switch drug class at the time of relapse. Similarly, patients who received bortezomib-based induction may gain benefit from switching to an immunomodulatory agent in the relapsed setting. In addition, for patients who did not have a transplant as part of their initial treatment, or for patients with long duration of remission following transplant, salvage autologous transplant could be considered. Incorporation of patient- or treatment-related AEs (existing cytopenias, neuropathy, or thrombosis) should also play a part in the choice of agents in the relapsed disease setting. For patients with more advanced relapse, or with aggressive disease biology, the use of combinations of agents or novel agents in combination with cytotoxic agents may be a more appropriate approach. Even among patients with aggressive relapse, the use of salvage transplant has a role if cytopenias are limiting treatment options, as long as some form of maintenance therapy is used afterwards in an effort to stave off early or rapid relapse. Disease biology can also influence the choice of therapy for relapsed/refractory MM, and regimens including bortezomib or lenalidomide are preferred in individuals with the higher risk t(4;14) disease as well as the use of some form of maintenance therapy, which appears to be of greater importance among patients with biologically defined high-risk disease. Importantly, the optimal therapy of patients with deletion of 17p (p53), who usually derived short benefit from available therapies outcomes, is not known at this time and thus for these patients aggressive combination therapy with aggressive maintenance treatment may be warranted.[9,10] More sophisticated biological correlatives for the selection of treatment are obviously desirable but are not currently available for routine clinical practice.

Interestingly, most of the novel combination approaches in MM explored to date have reliably produced response in the majority of patients, and CR/nCR is not uncommon. One unresolved question in MM therapy is whether use of combinations of novel agents to achieve high response rates is better than the sequential use of these agents alone or with corticosteroids. Emerging data from large phase III studies suggest that progression-free survival is superior with a 3-drug combination compared with a doublet, but so far no survival improvement has been noted.[11] Several currently ongoing phase III trials are evaluating a similar comparison (lenalidomide/dexamethasone +/- carfilzomib, lenalidomide/dexamethasone +/- elotuzumab, bortezomib/dexamethasone +/- panobinostat) and these data are critically important as we begin to evaluate the benefit of inducing deeper responses and their impact on long-term outcomes in the relapsed disease setting.

Second autologous transplant

For over 20 years, conventional chemotherapy and high-dose therapy (HDT) with either autologous stem cell support has been utilized in the management of relapsed and/or refractory MM. Regimens based on conventional chemotherapy have included: high-dose dexamethasone;[12,13] vincristine, doxorubicin, and dexamethasone (VAD);[14-18] vincristine, melphalan, cyclophosphamide, prednisone, vincristine, carmustine, doxorubicin, and prednisone (VMPC/VBAP);[19] and doxorubicin, vincristine, dexamethasone, etoposide, and cyclophosphamide (CEVAD).[20] The use of melphalan as high-dose therapy (HDT) in relapsed and/or refractory myeloma was introduced by McElwain and colleagues[21] and subsequently by Barlogie and colleagues, who demonstrated that

high-dose melphalan with stem cell support could overcome resistance to conventional-dose chemotherapy.[22] Available data on second autologous transplants for relapsed patients suggest that these procedures are relatively well-tolerated, with a 100-day mortality of less than 10%.[23-26] The overall response rates (ORR) in more recent studies, in which most patients have received novel agents, range from 55%-69%.[23,24,26,27] A recent analysis suggests that a relapse-free survival of more than 18 months after the first auto-SCT is the most reliable predictor of clinical outcome after a second auto-SCT,[28,29] though the impact of planned post-transplant maintenance therapy on the duration of first response post-auto transplant is currently unknown.

As such, for patients who experienced an initial duration of remission of more than 24 months following their initial autologous transplant, the use of a second autologous transplant can potentially offer clinical benefit following a short course of salvage therapy to re-induce some level of a response. Additionally, for patients who have significant cytopenias as a consequence of salvage therapy, the use of a salvage autologous transplant may provide a method by which more normal hematopoiesis can be re-established even in the setting of short duration of remission from previous autologous transplants. This may allow patients to receive additional salvage therapy that would ultimately be limited by low blood counts.

Novel agents in relapse

The emergence of novel therapies over the past decade has dramatically altered the therapeutic landscape and natural history of relapsed and refractory myeloma. The ability of the immunomodulatory drugs (IMiDs) thalidomide, lenalidomide, and pomalidomide, as well as the proteasome inhibitors bortezomib and carfilzomib, to overcome drug resistance was clearly demonstrated in pre-clinical models and confirmed in the context of clinical trials leading to US Food and Drug Administration (FDA) approval of these compounds in the treatment of MM. This review will elaborate on the role of novel agents in the treatment of relapsed and refractory MM, with discussion of other emerging compounds that may yet have a further impact on the field.

Specific therapeutic agents

Thalidomide

Thalidomide was one of the first novel agents to be evaluated in relapsed and refractory patients.[30,31] A recent review from Glasmacher *et al.* demonstrated that thalidomide alone produced a partial response or better in 30% of relapsed patients, with a 1-year survival of 60% and median survival of 14 months.[31] While the depth and duration of response may seem short by current standards, at the time this represented a major step forward for patients with few other options. Toxicities of thalidomide included sedation, constipation, and increased risk of venous thromboembolism (VTE), as well as peripheral neuropathy. It was noted later on that the incidence of peripheral neuropathy (PN) increased if the daily dose of thalidomide exceeded 200 mg or if administered for six months or more.[32,33] It was subsequently noted that the addition of steroids to thalidomide increases the overall response rate to 50%, typically with prolonged remission duration, as is now known to be the case for all the immunomodulatory agents.[34]

Thalidomide has also been combined with conventional

Table 1. Selected thalidomide combinations in relapsed or refractory MM.

Author/year	N.	Regimen	Overall response rate (%)	CR/nCR rate (%)	Median PFS (mos)	Median OS (mos)
Kropff 2003[35]	60	Hyper CDT	72		11.0 (EFS)	19.0
Garcia-Sanz 2004[36]	71	TCD	57			
Offidan 2006[38]	50	T/PLD/D	76		22.0	NYR
Palumbo 2006[37]	24	MPT	42		9.0	14.0

T: thalidomide; C: cyclophosphamide; D: dexamethasone; PLD: pegylated liposomal doxorubicin; P: prednisone; M: melphalan; CR: complete remission; nCR: near CR; PFS: progression-free survival; OS: overall survival; EFS: event-free survival; NYR: not yet reached; mos: months.

Table 2. Selected bortezomib combinations in relapsed or refractory MM.

Author/year	N.	Regimen	Overall response rate (%)	CR/nCR rate (%)	Median PFS (mos)	Median OS (mos)
Pineda-Romané 2008[39]	85	VTD	63	22	--	22
Biehn 2007[40]	22	V + PLD	63	36	9.3 (TTP)	38.3
Terpos 2005[41]	60	VMPT	59	11	9.5	--
Reece 2008[42]	13	V+C+P	85	54	>12	>12

T: thalidomide; C: cyclophosphamide; D: dexamethasone; PLD: pegylated liposomal doxorubicin; P: prednisone; M: melphalan; CR: complete remission; nCR: near CR; PFS: progression-free survival; OS: overall survival; EFS: event-free survival; NYR: not yet reached; mos: months.

cytotoxic drugs (Table 1)[35-37] and anthracyclines,[38] as well as with novel agents such as bortezomib,[39] in relapsed/refractory MM (Table 2).[39-42] Combination therapy with thalidomide improves the overall response rate and CR rates in several phase I-II studies. In a recent series from Garderet *et al.,* the 3-drug combination of VTD was superior to TD in the relapsed setting with a PFS of nearly 20 months (the longest reported in a phase III clinical trial in relapsed myeloma) compared with 12 months for TD alone. These data suggest that the combination of novel agents can be administered, and result in a very prolonged duration of remission, far superior to what is seen when single agents are administered separately. While there was no difference in overall survival, there is a trend favoring the group that received VTD salvage therapy.[11]

Thalidomide combinations carry an increased risk of venous thromboembolism (VTE) that requires some form of prophylaxis. Individuals with a prior history of VTE should be fully anti-coagulated, as should patients with other risk factors for the development of VTE. The use of aspirin, low molecular weight heparin (LMWH) and warfarin have all been evaluated,[43] and the International Myeloma Working Group has published guidelines based on a risk assessment model, with LMWH for patients with more than one risk factor, while aspirin (ASA) can be considered for those with lower risk profiles.[44] In a randomized trial from Palumbo and colleagues, patients who were receiving IMiD-based therapy (including thalidomide) were randomized to receive either LMWH, warfarin, or ASA. In this trial, patients received bortezomib containing regimens were used as a ‘low risk’ group for comparison. There was no statistically significant difference in incidence in VTE among the 3 randomized arms, and all 3 arms had a low incidence of VTE, supporting the equivalence and utility of ASA as a convenient oral antithrombotic agent in this setting.[45]

Lenalidomide

Lenalidomide is a 2nd generation immunomodulatory agent that is more potent than thalidomide, and has a very different safety profile. Similar to thalidomide, the efficacy of lenalidomide can be significantly enhanced through the co-administration of steroids. The primary registration trials for lenalidomide in the relapsed setting were the MM-009 and MM-010 trials. The dose of lenalidomide administered was 25 mg Days 1-21 of a 28-day schedule, with pulse dexamethasone given Days 1-4, 9-12 and 17-20 for the first 4 cycles; subsequently, the dose of dexamethasone was decreased to only Days 1-4 per cycle.[46,47] The results of the two trials were identical, with overall response rates of 60% and 61% for lenalidomide + dexamethasone compared with 20% and 24% with high-dose dexamethasone as a single agent. The median TTP was approximately 11 months in both trials, while the OS with the combination had not yet been reached in the North American trial (MM-090) at the time of the last report;[46] OS was 29.6 months in the European trial (MM-010).[47] Moreover, the benefit of lenalidomide + dexamethasone was apparent despite extensive crossover of patients from the dexamethasone arm to lenalidomide-based therapy.

The main toxicity of lenalidomide avoids some of the more common toxicities of thalidomide, such as somnolence, constipation and significant peripheral neuropathy. However, it is associated with an increased risk of VTE, similar to thalidomide, and thromboprophylaxis is required.[43,44] A retrospective analysis from Nooka *et al*. sought to validate the IMWG guidelines for lenalidomide thromboprophylaxis.[48] In this series, all patients being treated with lenalidomide in the relapsed or refractory setting were evaluated, and the incidence of VTE, with ASA prophylaxis, was low. Among patients who did develop VTE, each of them had more than 1 risk factor suggesting that their VTE episode was predicted by the International Myeloma Working Group (IMWG) guidelines.

Different to thalidomide, the most common adverse event associated with lenalidomide therapy is neutropenia and thrombocytopenia.[46,47,49] though these events do not typically limit the duration of therapy. If significant neutropenia occurs, either the dose of lenalidomide can be reduced, or intermittent dosing of granulocyte-colony stimulating factor (G-CSF) can be administered. In the experience reported at Princess Margaret Hospital, Toronto, Canada, an average of four doses of G-CSF per cycle is usually sufficient, and is typically given twice weekly starting Day 15 of each cycle.[50] Interestingly, at least when used as initial therapy, more neutropenia was observed in patients given a low-dose weekly, rather than full-dose pulse dexamethasone.[51]

Lenalidomide combinations have also been studied, mostly in phase I and II studies, but have also documented improved overall and depth of response when combinations are used. These include combinations with doxorubicin or pegylated liposomal doxorubicin[52,53] and cyclophosphamide[54] (Table 3). Lenalidomide + bortezomib +/- dexamethasone has shown especially encouraging activity and excellent tolerability in this context.[56] The maximum tolerated doses of this regimen were bortezomib 1.0 mg/m^2 on Days 1, 4, 8 and 11 and lenalidomide

Table 3. Selected lenalidomide combinations in relapsed or refractory MM.

Author/year	N.	Regimen	Overall response rate (%)	CR/nCR rate (%)	Median PFS (mos)	Median OS (mos)
Knop 2009[53]	66	LDoD	73	15	8	88% (1year)
Reece 2009[54]	15	LCP	74	45 (VGPR)	--	--
Baz 200652	52	L/PLD/Vi/D	75	29 (nCR)	61% (1year)	84% (1year)
Anderson 2009[55]	62	LVD Ph II	69	26	12	29

CR: complete remission; nCR: near CR; TTP: time to progression; OS: overall survival; L: lenalidomide; Do: doxorubicin; D: dexamethasone; C: cyclophosphamide; P: prednisone; PLD: pegylated liposomal doxorubicin; Vi: vincristine; V: bortezomib; VGPR: very good partial remission; Ph: phase; mos: months.

15 mg on Days 1-14 of a 21-day cycle with an overall response rate (ORR) of 60% and an encouraging median OS of 37 months. This combination has been evaluated with dexamethasone 20 mg on the day of and the day after bortezomib for the first 4 cycles and 10 mg on the same days after cycle 4, with the phase II trial of this regimen reporting at least a partial remission (PR) in 54%, including near CR in 6%, very good PR (>90% reduction in serum monoclonal protein) in 30%, and minimal response (MR) in 18%.[55]

Bortezomib

Bortezomib is a proteasome inhibitor with potent antimyeloma activity as a single agent.[57-59] The randomized APEX trial demonstrated the superiority of bortezomib on Days 1, 4, 8 and 11 of a 21-day cycle over pulse dexamethasone in MM patients with relapsed/refractory disease who had early relapse (1-3 prior lines of therapy). The overall response rate was 38% with a median time to progression (TTP) of 6.2 months, compared with only 18% and 3.5 months with high-dose dexamethasone.[60] Further follow up yielded a response rate of 43% with bortezomib, and a longer median overall survival of 29.8 *versus* 23.7 months for the high-dose dexamethasone-treated patients. This improvement in OS occurred despite the fact that over 60% of patients in the dexamethasone arm were allowed to cross-over to receive bortezomib.[61] Among a subset of patients treated in first relapse, the ORR for the bortezomib group was 51%.[61]

In the initial phase II studies, dexamethasone, was added for a suboptimal response or progression, with a resultant improvement in the degree of response in 18%-39% of patients.[62] Two smaller single arm phase II trials in relapsed and refractory patients have described the use of bortezomib +/- dexamethasone from the onset of therapy, with overall response rates ranging from 54%-74%, with a CR rate of 7% in both.[63,64]

The toxicity profile of bortezomib has been well-characterized, and includes nausea, diarrhea, cyclic reversible thrombocytopenia, and peripheral neuropathy.[57-60,65] Peripheral neuropathy occurs in approximately one-third of patients, and can be painful; however, with early intervention this can be reversible. Dose modification or discontinuation of bortezomib is required for moderate or severe neuropathy, especially if associated with pain; the neuropathy usually improves or resolves in a high proportion of affected individuals, although often over several months.[66] The use of subcutaneous and/or weekly dosing has changed the intensity and severity of bortezomib-induced PN. When used in combinations, weekly therapy results in a much lower incidence of grade 3/4 PN and also overall incidence of PN. In a randomized trial comparing intravenous (iv) and subcutaneous (sq) dosing of bortezomib in the relapsed disease setting, the overall response rate, time to progression (TTP) and OS were similar between each dosing method with a significant reduction in severity and overall incidence of PN. These data have led to a wholesale change in the route of administration of bortezomib with a resultant improvement in related toxicity.[67,68]

Bortezomib is an attractive agent to use in combination with other drugs and can safely be used in the setting of renal insufficiency.[69,70] Many bortezomib combinations have been evaluated in phase I-II trials (Table 2).[39-42] These combinations generally produce high overall response rates, in the range of 50%-80%, with encouraging duration of response and OS. The best example of bortezomib + chemotherapy in the relapsed setting is the trial comparing bortezomib alone with bortezomib + pegylated liposomal doxorubicin. This trial demonstrated the superiority of the combination in terms of TTP (9.3 *vs.* 6.5 months), and also overall survival.[71]

New agents

HDAC inhibitors

Histone deactylase inhibitors are known to be effective targets in several cancers and are thought to work primarily through epigenetic modification of gene expression. In the context of plasma cell disorders, the potential mechanism is thought to be related to the effects of HDAC inhibitors on HDAC 6, which is critical to the function of an alternative pathway of protein catabolism, the aggresome/autophagy pathway.[72] Inhibition of proteasome function results in activation of the alternative pathway, the aggresome pathway, and protein catabolism occurs via this. The combination of proteasome inhibition and HDAC 6 inhibition (accomplished using HDAC inhibitors, or tipifarnib[73]) results in pre-clinical synergy that has been demonstrated clinically. Preliminary data from phase I studies combining vorinostat with bortezomib demonstrated responses particularly among the patients who were defined as bortezomib resistant, with an overall response rate of 30%.[74,75] When this was tested in large phase II randomized trials, the use of vorinostat with bortezomib was found to be no different from bortezomib alone in terms of progression-free and overall survival, but there was significant toxicity associated with vorinostat administration at the dose and schedule used for the study that likely limited the ultimate durability of what was noted to be a higher overall response rate.[76,77] In a similar series of clinical trials, panobinostat (LBH589) was also tested alone and in combination with bortezomib.[78,79] While the single agent activity was limited, the activity in phase I and II studies combining panobinostat with bortezomib demonstrated encouraging response rates with what appears to be an improved safety profile when compared with vorinostat. The results of the randomized phase III Panorama 1 study are currently pending; this is testing bortezomib/dexamethasone *versus* panobinostat/bortezomib/dexamethasone in an early relapsed myeloma patient population, and should shed some light on the true efficacy of this approach in relapsed myeloma.

Antibodies

The effects of thalidomide and lenalidomide on immune function have been demonstrated in a number of animal and pre-clinical models, and include enhancement of NK cell function, $CD8^+$ T-cell activation, and increased secretion of IL2 and interferon-γ.[23,80,81] Data with antibodies directed against plasma cell and B-cell antigens such as CD40 and CS1 were evaluated in pre-clinical models with lenalidomide and demonstrated significant synergy.[82,83] Experience with the potent CS1 antibody elotuzumab (known as Huluc63) demonstrates that this target is relatively plasma cell specific, and that the functional activity of the CS1 antibody requires NK cells to be present for

activity.[84,85] A phase I/II study was designed to test the clinical efficacy of this approach combining lenalidomide with elotuzumab (HuLuc63) and low-dose dexamethasone.[86] In the phase I portion of the trial, patients received up to 20 mg/kg without experiencing DLT. The ORR for the phase I study was 82% with 95% of lenalidomide naïve patients achieving PR or better.[86] In a more recent phase II expansion of this study in which patients were randomized to receive either 10 or 20 mg/kg of elotuzomab in combination with lenalidomide and low-dose dexamethasone, a recent update of the data suggests not only does the high response rate hold up, but that the duration of response is very long as well. With a median follow up of nearly 21 months, the median PFS was 18 months for the group that received 20 mg/kg of elotuzomab *versus* 'still not reached' for the group that received 10 mg/kg.[87] When compared with historical cohorts of patients who were treated with lenalidomide and high-dose dexamethasone, there is the suggestion that the addition of elotuzomab enhanced the ORR and PFS in the context of relapsed myeloma. Follow up from the randomized phase III study (Eloquent 2) is clearly needed to better understand the clinical benefit for patients in the relapsed disease setting, but trends to date are very exciting.

While there are several other exciting antibody targets in myeloma with early data (including CD138, CD56, anti-BAFF, and anti-DKK-1), the antibody which does appear to provide very early and exciting response data is the anti-CD38 antibody daratumomab.[88] This was initially presented in 2012 in the context of a phase 1 single agent study; an encouraging overall response rate and complete remission rate was noted, suggesting that this may be the first antibody with single agent activity in the context of relapsed myeloma. Additional data are needed to understand more fully the extent of response in relapsed and refractory myeloma.

Carfilzomib

Carfilzomib is a 2nd generation proteasome inhibitor that has been studied in a number of different clinical settings, and was recently approved for use by the FDA in the setting of relapsed myeloma. The approval was based in part on clinical experience from several phase II studies evaluating the efficacy of carfilzomib in relapsed myeloma (Table 4).[89-94] Two phase II clinical studies evaluated carfilzomib in MM patients, the 003-A0 trial (n=46) in relapsed and refractory MM[89] and 004 trial (n=129) in an earlier relapsed myeloma patient population.[90] In both studies, patients received carfilzomib 20 mg/m^2 iv on Days 1, 2, 8, 9, 15 and 16 every 28 days for up to 12 cycles. The most common AEs were fatigue, anemia, thrombocytopenia, nausea, upper respiratory infections, increased creatinine, and diarrhea. PN occurred in fewer than 10% of patients with 1 grade 3 in a patient with pre-existing grade 2 PN. The treatment-emergent PN rate was low with grade 3/4 2.2%, despite the fact that 78% of patients had grade 1/2 PN at enrollment. The response rate in 003-A0 was 18% PR, 7% MR, and 41% stable disease (SD) in this cohort of refractory patients. Subsequently, a 'stepped-up' dosing schedule incorporated a higher dose of 20/27 mg/m^2 in order to maximize the clinical benefit of carfilzomib. Additional patients were enrolled to a 'stepped-up' dosing in the 003-A1 study and received 20 mg/m^2 for the first cycle and 27 mg/m^2 thereafter.[91] In the 003-A1 study, in which the increased dose was used in a refractory myeloma population, a total of 266 patients were enrolled and 257 were response-evalu-

Table 4. Carfilzomib trials.

Trials	Type/disease	N	Dose and schedule (every 28 days)	Results	Serious adverse event
PX-171-002 Alsina, et al.[94]	Phase I (hematologic malignancies)	37	1.2-27 mg/m^2 on Days 1,2, 8,9,15,16	PR 11% (MM) MR 3% (MM) SD 16% (6% MM; 10% NHL)	Hypoxia, thrombocytopenia; elevated creatinine
PX-171-003-A0 Jagannath, *et al.*[89]	Phase II (R/R myeloma)	46	CFZ: 20 mg/m^2 on Days 1,2,8,9,15,16	>PR 18%; MR 26%; 5.1 SD 41% median TTP mos; median DOR 7.4 mos	Anemia, thrombocytopenia neutropenia, fatigue, URTI; dyspnea
PX-171-003-A1 Siegel, *et al.*[91]	Phase IIb (R/R myeloma)	257	CFZ: Days 1,2,8,9,15,16 (20 mg/m^2 cohort 1; 27 mg/m^2 cohort 2-12)	>PR 24%; median DOR-7.4 mos >PR in pts with CTG abnormalities: 28%; median DOR-7.0m; median OS 15.5m	Anemia, thrombocytopenia neutropenia
PX-171-004 Vij, *et al.*[90]	Phase II (R or R myeloma; bortezomib naïve)	129	Cohort 1: 20/20 mg/m^2 Cohort 2 : 20/27 mg/m^2	Cohort 1:ORR 42%; CR 3%; VGPR 14%. Cohort 2: ORR 52%; CR 2%; VGPR 27%	Anemia, thrombocytopenia neutropenia, pneumonia, fatigue, dyspnea
PX-171-005 Badros, *et al.*[92]	Phase II (R/R myeloma with renal impairment)	39	CFZ: 15/20 mg/m^2 escalation to 27 mg/m^2 on Days 1,2,8,9,15,16	CBR 37% (PR 23%; MR 14%); SD 37%	Fatigue, anemia, diarrhea, nausea, thrombocytopenia, constipation
PX-171-006 Wang, *et al.*[93]	Phase Ib/II (R/R myeloma)	52	CFZ: 20/27 mg/m^2 on Days 1,2,8,9, 15, 16; L 25 mg Days 1-21; D 40 mg Days 1,8,15,22	ORR 78% (PR 38%; VGPR 22%, CR/sCR 18%)	Neutropenia, anemia thrombocytopenia

CFZ: carfilzomib; L: lenalidomide D: dexamethasone; PR: partial response; MR: minimal response; SD: stable disease; TTP: time to progression; DOR: duration of response; CR: complete response; nCR: near CR; sCR: stringent complete response; OS: overall survival; ORR: overall response rate; VGPR: very good partial response; CBR: clinical benefit response; MM: multiple myeloma; NHL: non-Hodgkin's lymphoma; CTG: clinical trials group; URTI: upper respiratory tract infection; mos: months.

able. ORR was 24% with median duration of response of 7.4 months. The most common treatment-related AEs were predominately hematologic events with a very low incidence of neuropathy.

As a consequence of the higher response rate seen with the 'stepped-up' dosing in the 003 trial, the 004 trial was also modified to increase the dose and assess responses.[90] A subsequent cohort of patients were treated with 'stepped-up' dosing with 20 mg/m^2 for the first cycle and 27 mg/m^2 thereafter in cohort 2 (n=70). The study included patients who were naïve to bortezomib treatment (n=129) and patients who had received prior bortezomib (n=35). The subset of patients who were bortezomib-treated patients had an ORR of 18%. The overall response rate for cohorts 1 and 2 were ORR 42% and 52%, with VGPR rates of 17% and 29% or over, and CR rates of 3% and 2%, respectively. The median PFS was 8.3 months for all patients, and the most common grade 3/4 toxicities were lymphopenia 14% and 19%, anemia 12% and 17%, thrombocytopenia 15% and 11%, neutropenia 12% and 14%, pneumonia 14% and 11%, fatigue 12% and 1%, and dyspnea 5% and 6%, respectively. Interestingly, grade 1/2 PN was seen in only 14% and 19% patients and grade 3/4 in only 2% and 0%, respectively.

An additional area of exploration for carfilzomib was the efficacy and safety in the context of renal dysfunction. Badros and colleagues performed a phase II trial to assess the safety and efficacy of carfilzomib in relapsed myeloma patients with varying degrees of renal insufficiency.[92] Patients were enrolled on the basis of base-line renal function broken down into normal (CrCl 80 mL/min), mild (50-79), moderate (30-49), and severe (<30) renal function. Patients received iv carfilzomib at a dose of 15 mg/m^2 on Days 1, 2, 8, 9, 15 and 16 every 28 days for cycle 1, escalating to 20 mg/m^2 in cycle 2 and to 27 mg/m^2 in cycle 3. Grade 3/4 AEs include anemia, thrombocytopenia, fatigue, increased creatinine, and mental status changes. Dose adjustments were not required suggesting manageable toxicity in renal failure patients.

Finally, as single agent activity was defined, combination therapy represented the next step. In this process, one of the first combinations to be used was the phase I/II trial combining carfilzomib with lenalidomide and dexamethasone.[93] Dosing consisted of carfilzomib 20 mg/m^2 on Days 1-2 of cycle 1; 27 mg/m^2 thereafter is administered on Days 1, 2, 8, 9, 15 and 16, oral (PO) lenalidomide 25 mg Days 1-21, and 40 mg dexamethasone PO (Days 1, 8, 15, 22) in a 28-day cycle. Among the 52 patients enrolled, no DLTs were reported and 11.5% of patients had serious AEs (6 of 52). Hematologic AEs, including grade 3/4 neutropenia (n=12), anemia (n=8), and thrombocytopenia (n=8), were manageable. The ORR was 78% (Cr/sCR 18%, VGPR 22%, PR 38%, MR 2%, SD 8%) and toxicities with prolonged administration of this regimen were manageable (14-23 months). This trial was used as the basis for the recently completed phase III ASPIRE trial in which patients were randomized to receive either CRd (carfilzomib/lenalidomide/dexamethasone) *versus* Rd (lenalido-

Table 5. Pomalidomide trials.

Trials	Type/disease	N	Dose and schedule (every 28 days)	Median prior therapy	Overall response, ≥PR
Schey[95]	Phase I	24	POM 1,2,5,10 mg Days 1-28 q 28	3 (1-6)	54%
Richardson[96]	Phase I	38	POM 2,3,4,5 mg Days 1-21 q 28 D 40 mg weekly	6 (2-17)	25%
Richardson[97]	Phase II	221	POM 4 mg Days 1-21 q 28 POM 4mg + Days 40 mg/wk Days 1-21 q 28	5 (2-13)	13% 34%
Leleu[98]	Phase II	84	POM 4 mg + D 40 mg/wk Days 1-28 q 28 POM 4 mg + D 40 mg/wk Days 1-21 q 28	5 (1-13)	35% 34%
Lacy[99]	Phase II	35	POM 4mg + D 40 mg/wk Days 1-28 q 28	6	29%
Shah[103]	Phase I	30	POM 4mg + D 40 mg/wk Days 1-28 q 28 CFZ escalating dose	6 (1-15)	50%
Richardson[101]	Phase I	15	POM 1-4 mg Days 1-14 q 21 D 20 mg day of and after V V 1-1.3 mg/m^2 Days 1,4,8,11	2 (1-4)	73%
Palumbo[102]	Phase 1	55	POM 1-2.5 mg continuous P 50 mg qod C 50 mg qod	3 (1-3)	51%

PR: partial response; POM: pomalidomide; D: dexamethasone; CFZ: carfilzomib; V: bortezomib; P: prednisone; C: cyclophosphamide;

mide/dexamethasone) in the setting of relapsed myeloma. Results are currently not available from the trial but enrollment has been completed.

Pomalidomide

Pomalidomide is the newest of the immunomodulatory class of agents that is now being evaluated in larger phase clinical trials. Data initially presented by Schey and colleagues demonstrated favorable tolerability and activity of this agent in myeloma patients with early relapse (Table 5).[95-102] Trials initially from the Mayo Clinic group demonstrated a good overall response rate in the context of relapsed myeloma, and this has now been tested in a number of different dosages and schedules.[103,104] It appears that, across the board, the response rate among patients with lenalidomide-resistant disease using pomalidomide and dexamethasone is 30%. This was seen in trials from the US[99,105] as well as in Europe.[98] In recently reported data from a European study comparing pomalidomide/dexamethasone with high-dose dexamethasone alone among refractory myeloma patients, not only was the overall response rate and progression-free survival superior for pomalidomide/dexamethasone, the overall survival also favored the use of pomalidomide/dexamethasone.[106] Trials have varied the starting dose of pomalidomide (2-4 mg) as well as the dosing schedule (continuous *vs*. '3 weeks on/1 week off') with different results, but most studies testing the use of pomalidomide in the relapsed setting are using 4 mg on a '3 weeks on/1 week off' schedule, though efficacy with fewer side effects has been seen at the 2 mg dose. Similar to data with lenalidomide and thalidomide, there are now trials that demonstrate very encouraging overall response rates when pomalidomide is combined. Trials were recently reported combining pomalidomide with cyclophosphamide as well as bortezomib and carfilzomib with very encouraging response rates. In the cyclophosphamide combination trial, the overall response rate was 51% with a maximum dose of pomalidomide of 2.5 mg in combination with 50 mg of cyclophosphamide given every other day and 50 mg of prednisone also every other day.[102] In the bortezomib combination trial, the overall response rate was 73% with full-dose bortezomib at the standard dose and schedule with pomalidomide 4 mg given on Days 1-14.[101] In the carfilzomib combination, the overall response rate was 50% with full-dose carfilzomib (20/27 mg/m^2) and pomalidomide at 4 mg on Days 1-21.[100] Many other studies are ongoing combining pomalidomide with existing agents or agents under development, and it is clear that this agent has single agent activity, as well as good potential in combined therapies.

Conclusion

Combinations of agents in relapsed and refractory MM are clearly moving forward. Advantages of combination therapy include higher overall response rates and, in many cases, better depth of responses, as well as the ability to revisit classical agents such as immunomodulatory agents and proteasome inhibitors that have formed the backbone of earlier treatment approaches. The question about sequencing *versus* combination therapy in the relapsed setting has yet to be settled, but emerging data suggest that combinations are more effective, and that as patients develop more and more resistant disease, the sensitivity to any agent begins to decline. This supports the use of combination therapy in the context of initial diagnosis, and this is now also being tested in the relapsed setting. In the balance is the concept that sequential therapy may be associated with less toxicity than is seen with combination therapy, and since few (if any) of these patients are cured of their disease, whether to treat a patient with single agents in sequence rather than combinations is an area of active study and ongoing debate. It is also clear that there are many new tools to use at our disposal and many more will be developed. As such, the use of genomics and whole genome sequencing will be critically important as we seek to tailor therapy to a given patient's disease, and to maximize duration of response while minimizing treatment-related toxicity.

References

1. Kyle RA, Rajkumar SV. Multiple myeloma. N Engl J Med. 2004;351:1860-73.
2. Harousseau JL, Shaughnessy J Jr, Richardson P. Multiple myeloma. Hematology Am Soc Hematol Educ Program. 2004:237-56.
3. Podar K, Tai YT, Hideshima T, Vallet S, Richardson PG, Anderson KC. Emerging therapies for multiple myeloma. Expert Opin Emerg Drugs. 2009;14:99-127.
4. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood. 2004;104:607-18.
5. Blade J, Samson D, Reece D, et al. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. Br J Haematol. 1998;102:1115-23.
6. Durie BG, Harousseau JL, Miguel JS, et al. International uniform response criteria for multiple myeloma. Leukemia. 2006;20:2220.
7. Anderson KC, Kyle RA, Rajkumar SV, Stewart AK, Weber D, Richardson P. Clinically relevant end points and new drug approvals for myeloma. Leukemia. 2008;22:231-9.
8. Lonial S, Mitsiades CS, Richardson PG. Treatment options for relapsed and refractory multiple myeloma. Clin Cancer Res. 2011;17:1264-77.
9. Mikhael JR, Goodwin J, Qi X, et al. p53 Deletion Yields High Response Rates but Rapid Progression and Poor Overall Survival in Multiple Myeloma Patients Undergoing Autologous Stem Cell Transplantation. ASH Annual Meeting Abstracts. 2007;110:953.
10. Kaufman J, Nooka A, Muppidi S, et al. Survival outcomes of early autologous stem cell transplant (ASCT) followed by lenalidomide, bortezomib, and dexamethasone (RVD) maintenance in patients with high-risk multiple myeloma (MM). In: ASCO Annual Meeting 2012, p. 8100.
11. Garderet L, Iacobelli S, Moreau P, et al. Superiority of the Triple Combination of Bortezomib-Thalidomide-Dexamethasone Over the Dual Combination of Thalidomide-Dexamethasone in Patients With Multiple Myeloma Progressing or Relapsing After Autologous Transplantation: The MMVAR/IFM 2005-04 Randomized Phase III Trial From the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. J Clin Oncol. 2012;30: 2475-82.
12. Alexanian R, Barlogie B, Dixon D. High-dose glucocorticoid treatment of resistant myeloma. Ann Intern Med. 1986;105:8-11.
13. Gertz MA, Garton JP, Greipp PR, Witzig TE, Kyle RA. A phase II study of high-dose methylprednisolone in refractory or relapsed multiple myeloma. Leukemia. 1995;9:2115-8.
14. Barlogie B, Smith L, Alexanian R. Effective treatment of advanced multiple myeloma refractory to alkylating agents. N Engl J Med. 1984;310:1353-6.
15. Anderson H, Scarffe JH, Ranson M, et al. VAD chemotherapy

as remission induction for multiple myeloma. Br J Cancer. 1995;71:326-30.
16. Gertz MA, Kalish LA, Kyle RA, Hahn RG, Tormey DC, Oken MM. Phase III study comparing vincristine, doxorubicin (Adriamycin), and dexamethasone (VAD) chemotherapy with VAD plus recombinant interferon alfa-2 in refractory or relapsed multiple myeloma. An Eastern Cooperative Oncology Group study. Am J Clin Oncol. 1995;18:475-80.
17. Lokhorst HM, Meuwissen OJ, Bast EJ, Dekker AW. VAD chemotherapy for refractory multiple myeloma. Br J Haematol. 1989;71:25-30.
18. Phillips JK, Sherlaw-Johnson C, Pearce R, et al. A randomized study of MOD versus VAD in the treatment of relapsed and resistant multiple myeloma. Leuk Lymphoma. 1995;17:465-72.
19. Durie BG, Dixon DO, Carter S, et al. Improved survival duration with combination chemotherapy induction for multiple myeloma: a Southwest Oncology Group Study. J Clin Oncol. 1986;4:1227-37.
20. Giles FJ, Wickham NR, Rapoport BL, et al. Cyclophosphamide, etoposide, vincristine, adriamycin, and dexamethasone (CEVAD) regimen in refractory multiple myeloma: an International Oncology Study Group (IOSG) phase II protocol. Am J Hematol. 2000;63:125-30.
21. McElwain TJ, Powles RL. High-dose intravenous melphalan for plasma-cell leukaemia and myeloma. Lancet. 1983;2:822-4.
22. Barlogie B, Alexanian R, Dicke KA, et al. High-dose chemoradiotherapy and autologous bone marrow transplantation for resistant multiple myeloma. Blood. 1987;70:869-72.
23. Burzynski JA, Toro JJ, Patel RC, et al. Toxicity of a second autologous peripheral blood stem cell transplant in patients with relapsed or recurrent multiple myeloma. Leuk Lymphoma. 2009;50:1442-7.
24. Elice F, Raimondi R, Tosetto A, et al. Prolonged overall survival with second on-demand autologous transplant in multiple myeloma. Am J Hematol. 2006;81:426-31.
25. Krivanova A, Hajek R, Krejci M, et al. Second autologous transplantation for multiple myeloma patients relapsing after the first autograft -- a pilot study for the evaluation of experimental maintenance therapies. Report of the prospective non-randomized pilot study of the Czech Myeloma Group. Onkologie. 2004;27:275-9.
26. Olin RL, Vogl DT, Porter DL, et al. Second auto-SCT is safe and effective salvage therapy for relapsed multiple myeloma. Bone Marrow Transplant. 2009;43:417-22.
27. Qazilbash MH, Saliba R, De Lima M, et al. Second autologous or allogeneic transplantation after the failure of first autograft in patients with multiple myeloma. Cancer. 2006;106:1084-9.
28. Alvares CL, Davies FE, Horton C, Patel G, Powles R, Morgan GJ. The role of second autografts in the management of myeloma at first relapse. Haematologica. 2006;91:141-2.
29. Shah N, Lonial S. Evidence-based mini-review: treatment options for patients with relapsed/refractory myeloma previously treated with novel agents and high-dose chemotherapy and autologous stem-cell transplantation. Hematology Am Soc Hematol Educ Program. 2010;2010:310-3.
30. Barlogie B, Desikan R, Eddlemon P, et al. Extended survival in advanced and refractory multiple myeloma after single-agent thalidomide: identification of prognostic factors in a phase 2 study of 169 patients. Blood. 2001;98:492-4.
31. Glasmacher A, Hahn C, Hoffmann F, et al. A systematic review of phase-II trials of thalidomide monotherapy in patients with relapsed or refractory multiple myeloma. Br J Haematol. 2006;132:584-93.
32. Mileshkin L, Stark R, Day B, Seymour JF, Zeldis JB, Prince HM. Development of neuropathy in patients with myeloma treated with thalidomide: patterns of occurrence and the role of electrophysiologic monitoring. J Clin Oncol. 2006;24:4507-14.
33. Richardson P, Schlossman R, Jagannath S, et al. Thalidomide for patients with relapsed multiple myeloma after high-dose chemotherapy and stem cell transplantation: results of an open-label multicenter phase 2 study of efficacy, toxicity, and biological activity. Mayo Clin Proc. 2004;79:875-82.
34. Dimopoulos MA, Zervas K, Kouvatseas G, et al. Thalidomide and dexamethasone combination for refractory multiple myeloma. Ann Oncol. 2001;12:991-5.
35. Kropff MH, Lang N, Bisping G, et al. Hyperfractionated cyclophosphamide in combination with pulsed dexamethasone and thalidomide (HyperCDT) in primary refractory or relapsed multiple myeloma. Br J Haematol. 2003;122:607-16.
36. Garcia-Sanz R, Gonzalez-Porras JR, Hernandez JM, et al. The oral combination of thalidomide, cyclophosphamide and dexamethasone (ThaCyDex) is effective in relapsed/refractory multiple myeloma. Leukemia. 2004;18:856-63.
37. Palumbo A, Avonto I, Bruno B, et al. Intravenous melphalan, thalidomide and prednisone in refractory and relapsed multiple myeloma. Eur J Haematol. 2006;76:273-7.
38. Offidani M, Corvatta L, Marconi M, et al. Low-dose thalidomide with pegylated liposomal doxorubicin and high-dose dexamethasone for relapsed/refractory multiple myeloma: a prospective, multicenter, phase II study. Haematologica. 2006;91:133-6.
39. Pineda-Roman M, Zangari M, van Rhee F, et al. VTD combination therapy with bortezomib-thalidomide-dexamethasone is highly effective in advanced and refractory multiple myeloma. Leukemia. 2008;22:1419-27.
40. Biehn SE, Moore DT, Voorhees PM, et al. Extended follow-up of outcome measures in multiple myeloma patients treated on a phase I study with bortezomib and pegylated liposomal doxorubicin. Ann Hematol. 2007;86:211-6.
41. Terpos E, Anagnostopoulos A, Kastritis E, et al. The Combination of Bortezomib, Melphalan, Dexamethasone and Intermittent Thalidomide (VMDT) Is an Effective Treatment for Relapsed/Refractory Myeloma: Results of a Phase II Clinical Trial. ASH Annual Meeting Abstracts. 2005;106:363.
42. Reece DE, Rodriguez GP, Chen C, et al. Phase I-II trial of bortezomib plus oral cyclophosphamide and prednisone in relapsed and refractory multiple myeloma. J Clin Oncol. 2008;26(29):4777-83.
43. Eby CS. Bleeding and thrombosis risks in plasma cell dyscrasias. Hematology Am Soc Hematol Educ Program. 2007;2007:158-64.
44. Palumbo A, Rajkumar SV, Dimopoulos MA, et al. Prevention of thalidomide- and lenalidomide-associated thrombosis in myeloma. Leukemia. 2008;22:414-23.
45. Palumbo A, Cavo M, Bringhen S, et al. A Phase III Study of Enoxaparin vs Aspirin vs Low-Dose Warfarin as Thromboprophylaxis for Newly Diagnosed Myeloma Patients Treated with Thalidomide Based-Regimens. ASH Annual Meeting Abstracts. 2009;114:492.
46. Weber D, Knight R, Chen C, et al. Prolonged Overall Survival with Lenalidomide Plus Dexamethasone Compared with Dexamethasone Alone in Patients with Relapsed or Refractory Multiple Myeloma. ASH Annual Meeting Abstracts. 2007;110:412.
47. Dimopoulos M, Spencer A, Attal M, et al. Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma. N Engl J Med. 2007;357:2123-32.
48. Nooka AK, Kaufman JL, Heffner LT, Gleason CL, Lonial S. Thromboembolic Events (TEE) with Lenalidomide-Based Therapies for Multiple Myeloma (MM): Emory Experience. ASH Annual Meeting Abstracts. 2009;114:3888.
49. Richardson PG, Blood E, Mitsiades CS, et al. A randomized phase 2 study of lenalidomide therapy for patients with relapsed or relapsed and refractory multiple myeloma. Blood. 2006;108:3458-64.
50. Reece DE, Masih-Khan E, Chen C, et al. Use of Lenalidomide (Revlimid(R) +/- Corticosteroids in Relapsed/Refractory Multiple Myeloma Patients with Elevated Baseline Serum Creatinine Levels. ASH Annual Meeting Abstracts. 2006;108:3548.
51. Rajkumar SV, Jacobus S, Callander N, Fonseca R, Vesole D, Greipp P. A Randomized Phase III Trial of Lenalidomide Plus High-Dose Dexamethasone Versus Lenalidomide Plus Low-Dose Dexamethasone in Newly Diagnosed Multiple Myeloma (E4A03): A Trial Coordinated by the Eastern Cooperative Oncology Group. ASH Annual Meeting Abstracts. 2006;108:799.
52. Baz R, Walker E, Karam MA, et al. Lenalidomide and pegylated liposomal doxorubicin-based chemotherapy for relapsed or refractory multiple myeloma: safety and efficacy. Ann Oncol. 2006;17:1766-71.
53. Knop S, Gerecke C, Topp MS, et al. Lenalidomide (RevlimidTM), Adriamycin and Dexamethasone Chemotherapy (RAD) Is Safe and Effective in Treatment of Relapsed Multiple Myeloma - First Results of a German Multicenter Phase I/II Trial. ASH Annual Meeting Abstracts. 2006;108:408.
54. Reece DE, Masih-Khan E, Khan A, et al. Phase I-II Trial of Oral Cyclophosphamide, Prednisone and Lenalidomide (Revlimid(R)) (CPR) for the Treatment of Patients with Relapsed and Refractory Multiple Myeloma. ASH Annual Meeting Abstracts. 2009;114:1874.
55. Anderson K, Jagannath S, Jakubowiak A, et al. Lenalidomide, bortezomib, and dexamethasone in relapsed/refractory multiple myeloma (MM): Encouraging outcomes and tolerability in

a phase II study. ASCO Annual Meeting 2009, p. 8536.
56. Richardson PG, Weller E, Jagannath S, et al. Multicenter, phase I, dose-escalation trial of lenalidomide plus bortezomib for relapsed and relapsed/refractory multiple myeloma. J Clin Oncol. 2009;27:5713-9.
57. Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med. 2003;348:2609-17.
58. Richardson PG, Barlogie B, Berenson J, et al. Extended follow-up of a phase II trial in relapsed, refractory multiple myeloma:: final time-to-event results from the SUMMIT trial. Cancer. 2006;106:1316-9.
59. Jagannath S, Barlogie B, Berenson J, et al. A phase 2 study of two doses of bortezomib in relapsed or refractory myeloma. Br J Haematol. 2004;127:165-72.
60. Richardson PG, Sonneveld P, Schuster MW, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N Engl J Med. 2005;352:2487-98.
61. Richardson PG, Sonneveld P, Schuster M, et al. Extended follow-up of a phase 3 trial in relapsed multiple myeloma: final time-to-event results of the APEX trial. Blood. 2007;110:3557-60.
62. Jagannath S, Richardson PG, Barlogie B, et al. Bortezomib in combination with dexamethasone for the treatment of patients with relapsed and/or refractory multiple myeloma with less than optimal response to bortezomib alone. Haematologica. 2006;91:929-34.
63. Mikhael JR, Belch AR, Prince HM, et al. High response rate to bortezomib with or without dexamethasone in patients with relapsed or refractory multiple myeloma: results of a global phase 3b expanded access program. Br J Haematol. 2009;144:169-75.
64. Kropff MH, Bisping G, Wenning D, et al. Bortezomib in combination with dexamethasone for relapsed multiple myeloma. Leuk Res. 2005;29:587-90.
65. Lonial S, Waller EK, Richardson PG, et al. Risk factors and kinetics of thrombocytopenia associated with bortezomib for relapsed, refractory multiple myeloma. Blood. 2005;106:3777-84.
66. Richardson PG, Briemberg H, Jagannath S, et al. Frequency, characteristics, and reversibility of peripheral neuropathy during treatment of advanced multiple myeloma with bortezomib. J Clin Oncol. 2006;24:3113-20.
67. Moreau P, Pylypenko H, Grosicki S, et al. Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study. Lancet Oncol. 2011;12:431-40.
68. Palumbo A, Bringhen S, Rossi D, et al. Bortezomib-melphalan-prednisone-thalidomide followed by maintenance with bortezomib-thalidomide compared with bortezomib-melphalan-prednisone for initial treatment of multiple myeloma: a randomized controlled trial. J Clin Oncol. 2010;28:5101-9.
69. Chanan-Khan AA, Kaufman JL, Mehta J, et al. Activity and safety of bortezomib in multiple myeloma patients with advanced renal failure: a multicenter retrospective study. Blood. 2007;109:2604-6.
70. Jagannath S, Barlogie B, Berenson JR, et al. Bortezomib in recurrent and/or refractory multiple myeloma. Initial clinical experience in patients with impared renal function. Cancer. 2005;103:1195-200.
71. Orlowski RZ, Nagler A, Sonneveld P, et al. Randomized phase III study of pegylated liposomal doxorubicin plus bortezomib compared with bortezomib alone in relapsed or refractory multiple myeloma: combination therapy improves time to progression. J Clin Oncol. 2007;25:3892-901.
72. Catley L, Weisberg E, Kiziltepe T, et al. Aggresome induction by proteasome inhibitor bortezomib and alpha-tubulin hyperacetylation by tubulin deacetylase (TDAC) inhibitor LBH589 are synergistic in myeloma cells. Blood. 2006;108:3441-9.
73. David E, Schafer-Hales K, Marcus AI, Kaufman JL, Lonial S. Combination of Bortezomib with Tipifarnib (R115777) Induces Synergistic Myeloma Cell Apoptosis Via Down-Regulation of HDAC6 and Inhibition of Aggresome Formation. ASH Annual Meeting Abstracts. 2007;110:1520.
74. Badros A, Philip S, Niesvizky R, et al. Phase I Trial of Suberoylanilide Hydroxamic Acid (SAHA) + Bortezomib (Bort) in Relapsed Multiple Myeloma (MM) Patients (pts). ASH Annual Meeting Abstracts. 2007;110:1168.
75. Weber DM, Graef T, Hussein M, et al. Phase I trial of vorinostat combined with bortezomib for the treatment of relapsing and/or refractory multiple myeloma. Clin Lymphoma Myeloma Leuk. 2012;12:319-24.
76. Dimopoulos MA, Jagannath S, Yoon S-S, et al. Vantage 088: Vorinostat in Combination with Bortezomib in Patients with Relapsed/Refractory Multiple Myeloma: Results of a Global, Randomized Phase 3 Trial. ASH Annual Meeting Abstracts. 2011;118:811.
77. Siegel DS, Dimopoulos MA, Yoon S-S, et al. Vantage 095: Vorinostat in Combination with Bortezomib in Salvage Multiple Myeloma Patients: Final Study Results of a Global Phase 2b Trial. ASH Annual Meeting Abstracts. 2011;118:480.
78. Wolf JL, Siegel D, Goldschmidt H, et al. Phase II trial of the pan-deacetylase inhibitor panobinostat as a single agent in advanced relapsed/refractory multiple myeloma. Leuk Lymphoma. 2012;53(9):1820-3.
79. Richardson PG, Alsina M, Weber DM, et al. Phase II Study of the Pan-Deacetylase Inhibitor Panobinostat in Combination with Bortezomib and Dexamethasone in Relapsed and Bortezomib-Refractory Multiple Myeloma (PANORAMA 2). ASH Annual Meeting Abstracts. 2011;118:814.
80. Chang DH, Liu N, Klimek V, et al. Enhancement of ligand-dependent activation of human natural killer T cells by lenalidomide: therapeutic implications. Blood. 2006;108:618-21.
81. Davies FE, Raje N, Hideshima T, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood. 2001;98:210-6.
82. Tai YT, Catley LP, Mitsiades CS, et al. Mechanisms by which SGN-40, a humanized anti-CD40 antibody, induces cytotoxicity in human multiple myeloma cells: clinical implications. Cancer Res. 2004;64:2846-52.
83. Tai YT, Li X, Tong X, et al. Human anti-CD40 antagonist antibody triggers significant antitumor activity against human multiple myeloma. Cancer Res. 2005;65:5898-906.
84. Hsi ED, Steinle R, Balasa B, et al. CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma. Clin Cancer Res. 2008;14:2775-84.
85. Tai YT, Soydan E, Song W, et al. CS1 promotes multiple myeloma cell adhesion, clonogenic growth, and tumorigenicity via c-maf-mediated interactions with bone marrow stromal cells. Blood. 2009;113:4309-18.
86. Lonial S, Vij R, Harousseau JL, et al. Elotuzumab in combination with lenalidomide and low-dose dexamethasone in relapsed or refractory multiple myeloma. J Clin Oncol. 2012;30:1953-9.
87. Richardson PG, Jagannath S, Moreau P, et al. A Phase 2 Study of Elotuzumab (Elo) in Combination with Lenalidomide and Low-Dose Dexamethasone (Ld) in Patients (pts) with Relapsed/Refractory Multiple Myeloma (R/R MM): Updated Results. ASH Annual Meeting Abstracts. 2012;120:202.
88. Plesner T, Lokhorst H, Gimsing P, Nahi H, Lisby S, Richardson PG. Daratumumab, a CD38 Monoclonal Antibody in Patients with Multiple Myeloma - Data From a Dose-Escalation Phase I/II Study. ASH Annual Meeting Abstracts. 2012;120:73.
89. Jagannath S, Vij R, Stewart AK, et al. An open-label single-arm pilot phase II study (PX-171-003-A0) of low-dose, single-agent carfilzomib in patients with relapsed and refractory multiple myeloma. Clin Lymphoma Myeloma Leuk. 2012;12:310-8.
90. Vij R, Wang M, Kaufman JL, et al. An open-label, single-arm, phase 2 (PX-171-004) study of single-agent carfilzomib in bortezomib-naive patients with relapsed and/or refractory multiple myeloma. Blood. 2012;119:5661-70.
91. Siegel DS, Martin T, Wang M, et al. A phase 2 study of single-agent carfilzomib (PX-171-003-A1) in patients with relapsed and refractory multiple myeloma. Blood. 2012;120:2817-25.
92. Badros AZ, Vij R, Martin T, et al. Phase I Study of Carfilzomib in Patients (Pts) with Relapsed and Refractory Multiple Myeloma (MM) and Varying Degrees of Renal Insufficiency. ASH Annual Meeting Abstracts. 2009;114: 3877.
93. Wang M, Bensinger W, Martin T, et al. Interim results from PX-171-006, a phase (Ph) II multicenter dose-expansion study of carfilzomib (CFZ), lenalidomide (LEN), and low-dose dexamethasone (loDex) in relapsed and/or refractory multiple myeloma (R/R MM). ASCO Annual Meeting 2011, p. 8025.
94. Alsina M, Trudel S, Furman RR, et al. A phase I single-agent study of twice-weekly consecutive-day dosing of the proteasome inhibitor carfilzomib in patients with relapsed or refractory multiple myeloma or lymphoma. Clin Cancer Res. 2012; 18:4830-40.
95. Schey SA, Fields P, Bartlett JB, et al. Phase I study of an immunomodulatory thalidomide analog, CC-4047, in relapsed or refractory multiple myeloma. J Clin Oncol. 2004;22:3269-76.
96. Richardson PG, Siegel D, Baz R, et al. A Phase 1/2 Multi-Center, Randomized, Open Label Dose Escalation Study to

Determine the Maximum Tolerated Dose, Safety, and Efficacy of Pomalidomide Alone or In Combination with Low-Dose Dexamethasone In Patients with Relapsed and Refractory Multiple Myeloma Who Have Received Prior Treatment That Includes Lenalidomide and Bortezomib. ASH Annual Meeting Abstracts. 2010;116:864.

97. Richardson PG, Siegel DS, Vij R, et al. Randomized, Open Label Phase 1/2 Study of Pomalidomide (POM) Alone or in Combination with Low-Dose Dexamethasone (LoDex) in Patients (Pts) with Relapsed and Refractory Multiple Myeloma Who Have Received Prior Treatment That Includes Lenalidomide (LEN) and Bortezomib (BORT): Phase 2 Results. ASH Annual Meeting Abstracts. 2011;118:634.
98. Leleu X, Attal M, Arnulf B, et al. High Response Rates to Pomalidomide and Dexamethasone in Patients with Refractory Myeloma, Final Analysis of IFM 2009-02. ASH Annual Meeting Abstracts. 2011;118:812.
99. Lacy MQ, Kumar SK, LaPlant BR, et al. Pomalidomide Plus Low-Dose Dexamethasone (Pom/Dex) in Relapsed Myeloma: Long Term Follow up and Factors Predicing Outcome in 345 Patients. ASH Annual Meeting Abstracts. 2012;120:201.
100. Shah JJ, Stadtmauer EA, Abonour R, et al. A Multi-Center Phase I/II Trial of Carfilzomib and Pomalidomide with Dexamethasone (Car-Pom-d) in Patients with Relapsed/Refractory Multiple Myeloma. ASH Annual Meeting Abstracts. 2012;120:74.
101. Richardson PG, Hofmeister CC, Siegel D, et al. MM-005: A Phase 1, Multicenter, Open-Label, Dose-Escalation Study to Determine the Maximum Tolerated Dose for the Combination of Pomalidomide, Bortezomib, and Low-Dose Dexamethasone in Subjects with Relapsed or Refractory Multiple Myeloma. ASH Annual Meeting Abstracts. 2012;120:727.
102. Palumbo A, Larocca A, Montefusco V, et al. Pomalidomide Cyclophosphamide and Prednisone (PCP) Treatment for Relapsed/Refractory Multiple Myeloma. ASH Annual Meeting Abstracts. 2012;120:446.
103. Lacy MQ, Gertz MA, Hayman SR, et al. Pomalidomide (CC4047) Plus Low Dose Dexamethasone (Pom/dex) Is Active and Well Tolerated in Lenalidomide Refractory Multiple Myeloma (MM). ASH Annual Meeting Abstracts. 2009;114:429.
104. Richardson P, Siegel D, Baz R, et al. A Phase 1/2 Multi-Center, Randomized, Open Label Dose Escalation Study to Determine the Maximum Tolerated Dose, Safety, and Efficacy of Pomalidomide Alone or in Combination with Low-Dose Dexamethasone in Patients with Relapsed and Refractory Multiple Myeloma Who Have Received Prior Treatment That Includes Lenalidomide and Bortezomib. ASH Annual Meeting Abstracts. 2009;114:301.
105. Jagannath S, Hofmeister CC, Siegel DS, et al. Pomalidomide (POM) with Low-Dose Dexamethasone (LoDex) in Patients (Pts) with Relapsed and Refractory Multiple Myeloma Who Have Received Prior Therapy with Lenalidomide (LEN) and Bortezomib (BORT): Updated Phase 2 Results and Age Subgroup Analysis. ASH Annual Meeting Abstracts. 2012;120:450.
106. Dimopoulos MA, Lacy MQ, Moreau P, et al. Pomalidomide in Combination with Low-Dose Dexamethasone: Demonstrates a Significant Progression Free Survival and Overall Survival Advantage, in Relapsed/Refractory MM: A Phase 3, Multicenter, Randomized, Open-Label Study. ASH Annual Meeting Abstracts. 2012;120:LBA-6.

Allogeneic stem cell transplantation in multiple myeloma patients

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A B S T R A C T

Allogeneic stem cell transplantation (allo-SCT) as a treatment option for multiple myeloma (MM) patients is heavily debated. In this educational review, results from prospective and retrospective studies of allo-SCT in myeloma are presented. Although the introduction of reduced intensity conditioning (RIC) has lowered the high treatment-related mortality associated with myeloablative conditioning, there is no convincing evidence that allo-RIC improves survival compared to autologous stem cell transplantation. New strategies are discussed aimed at lowering transplant toxicity and boosting the graft-versus-myeloma effect, and these are urgently needed to make allo-RIC safer and more effective for myeloma patients. But until this is achieved, allo-RIC in myeloma should only be recommended in the context of clinical trials.

Learning goals

At the conclusion of this activity, participants should be able to:
- discuss treatment options for high-risk myeloma with the emphasis on a possible role for allogeneic stem cell transplantation;
- discuss which conditioning regimens are most appropriate for patients with myeloma;
- speculate on the post-allogeneic stem cell transplantation options to boost the graft-*versus*-myeloma effect.

Introduction

Allogeneic stem cell transplantation (allo-SCT) is probably the only treatment for multiple myeloma (MM) with a curative potential. This is likely due to the graft-*versus*-myeloma effect (GvM) which was proven by the achievement of sustained complete remissions by donor lymphocyte infusions (DLI) without any other therapy in patients with a relapse after allo-SCT.[1-5] Clinical responses to DLI after myeloablative and non-myeloablative conditioning have been reported in up to 50% of patients, including 20% of patients with a complete remission (CR).[4,5] In several patients, these CRs were durable for more than ten years. The role of allo-SCT in multiple myeloma, however, is still a subject of debate due to the high mortality and morbidity related with this procedure. Particularly myeloablative conditioning in MM has an unacceptably high transplant-related mortality (TRM).[6-12] Due to better selection of patients, improved surveillance and therapies for opportunistic infections, and the use of peripheral blood stem cells (PBSC) instead of bone marrow stem cells, the European Bone marrow Transplantation (EBMT) group reported a reduction in TRM from 46% to 30%. However, survival at three years improved from 35% to 56% without a plateau in progression-free survival (PFS) or overall survival (OS) curves.[13]

Reduced intensity conditioning

Retrospective studies and phase II studies

The initial promising results of transplantations with non-myeloablative conditioning (NMA) have renewed interest in allo-SCT as a treatment option for multiple myeloma. Establishing donor lympho-hematopoieses without the acute toxicity associated with ablative SCT could be a major advantage for a disease like multiple myeloma that affects older patients and may be sensitive to allo-reactivity as shown by DLI. The pioneering studies in multiple myeloma were performed by the Seattle group who showed that donor engraftment could be achieved with the combination of low-dose total body irradiation (TBI) only (2Gy) and high-dose immune suppressive drugs ciclosporin and mycophenolic acid (MMF).[14] The Seattle group introduced the strategy of tandem autologous transplantation followed 2-4 months later by a reduced intensity conditioning (RIC) allograft. In 52 patients treated with this tandem modality, a CR was achieved in 48% of them and progression-free (PFS) and overall survival (OS) at 48 months were 48% and 69%, respectively. However, TRM was still 22%.[14] A wide variety of conditioning regimens for MM have since then been pioneered and published, ranging from low-intensity schemes such as cyclophosphamide/fludarabine, to fairly dose-intensive regimens with moderately high-dose

melphalan. In a review by the EBMT, 26 different conditioning schemes with and without T-cell depletion were identified for myeloma. No definite conclusions, however, could be drawn from these studies as most were retrospective evaluations of small numbers of patients who in many cases had been heavily pre-treated.[15]

Prospective studies of NMA allo-SCT as part of first-line therapy

The definite value of NMA allo-SCT for MM should be determined by prospective phase III studies with newly diagnosed patients that include a biological randomization (donor *vs*. no donor comparison). Five larger studies of this kind have been published. In the French IFM study, patients with an HLA identical sibling donor and high-risk MM defined by beta2(β2)-microglobulin 3 mg/L and deletion of chromosome 13 were candidates for auto-SCT followed by allo-SCT after NMA with busulfan, fludarabine and 5-day antithymocyte globulin (ATG). Patients without a sibling donor were treated with double auto-SCT. The intention to treat analysis showed no significant difference in event-free survival (EFS) (25 months auto/allo *vs*. 30 months double auto) and OS (35 auto/allo *vs.* 41 months double auto). A major criticism of this study design was the use of high-dose ATG included in the conditioning that results in profound *in vivo* T-cell depletion. The beneficial effects of this *in vivo* T-cell depletion are the low incidence of acute and chronic graft-*versus*-host disease (GvHD). However, the detrimental effect is the elimination of the GvM effect.[16]

A more positive result was published by Bruno *et al*. In this study, 58 patients with an HLA identical sibling donor assigned to be treated with tandem auto/RIC (conditioning low-dose TBI only) achieved higher CR (55% *vs*. 26%), and after a median follow up of 45 months had significant prolonged EFS (35 *vs*. 29 months) and OS (80 *vs*. 54 months) as compared to the 59 patients assigned to be treated with double myeloablative auto-SCT. The criticisms of this study were the small number of patients and the relatively inferior outcome of the double autologous arm.[17] An encouraging note was, however, given by the fact that TRM of RIC in the up-front setting may be strongly reduced (IFM 10.9%, Bruno *et al*. 11%). Of the 357 patients in the prospective EBMT study, 108 patients with an HLA-identical sibling donor were allocated to the auto-allo arm and 249 patients without a matched sibling donor were allocated to the auto arm.[18] Conditioning for the auto arm was melphalan 200 mg/m^2; conditioning for the allo arm was TBI 2 Gy plus fludarabine 30 mg/m^2/d for three days. After a median follow-up time of 61 months, PFS at 60 months was seen to be significantly better with auto/allo than with auto-SCT alone (35% *vs*. 18%; *P*=0.001), as was the risk of death and of relapse in the long term (*P*=0.047 and *P*=0.003, respectively). Overall survival at 60 months was 65% *versus* 58%, and relapse incidence was 49% *versus* 78%. Dutch HOVON performed a donor *versus* no-donor analysis of patients treated in the HOVON-50 study, a study that was originally designed to examine thalidomide combined with intensive therapy.[19] In this study, 138 patients without an HLA-identical sibling donor and 122 patients with a donor were evaluated after a median follow up of 77 months. There was no significant difference in complete remission, PFS, or OS between the 2 groups. PFS at six years was 28% for patients with a donor *versus* 22% for patients without a donor (*P*=0.19) and OS at six years from high-dose melphalan was 55%, irrespective of having a donor (*P*=0.68) (Figure 1). Cumulative incidence of non-relapse mortality at six years after autologous-SCT was 16% in the donor group *versus* 3% in the no-donor group (*P*<0.001). However, PFS was significantly prolonged in the 99 patients who actually proceeded to allo-SCT compared with the 115 patients who continued maintenance or received a second high-dose melphalan therapy, but the difference did not translate into a prolonged survival benefit. The large US multi-center trial from the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) including 710 patients also compared tandem autologous transplants with autologous-reduced intensity allografts based on biological randomization criteria.[20] Patients were classified as standard risk or high-risk on the basis of cytogenetics and β2-microglobulin concentrations**.** There was no difference in 3-year PFS or OS between the 2 groups: 43% *versus* 46% in the auto/auto group (*P*=0.7) and 77% *versus* 80% in the auto/allo group (*P* =0.19), respectively. The results of these prospective studies do not support a general application of allo-SCT as part of first-line therapy in standard-risk myeloma patients. This was confirmed by two recent meta-analyses that showed no benefit for auto-allo in the up-front setting with regard to OS compared to single or double auto-SCT. [20-22]

The results of the prospective donor *versus* no donor studies are summarized in Table 1.

Allo-SCT in high-risk myeloma

High-risk myeloma defined by cytogenetic abnormalities

An important question is whether patients with high-risk myeloma might benefit from a donor mediated graft-*versus*-myeloma effect. As mentioned above, in the IFM study, patients with high-risk disease, defined as β2-microglobulin less than 3 mg/L and chromosome 13 deletion (by fluorescence *in situ* hybridization, FISH) did not benefit from auto/allo-SCT as compared to the patients who were treated with double auto. The registry of the Société Française de Greffe de Moelle et de Thérapie Cellulaire, performed a retrospective study including 143 myeloma patients transplanted between 1999 and 2008.[23] When patients were grouped according to the presence of any of the poor prognosis cytogenetic abnormalities t(4;14), del(17p) or t(14;16) (n=53) or their absence (n=32), no difference in outcomes was observed between these 2 groups. The authors conclude that allo-SCT could potentially be of benefit to high-risk myeloma patients**.**

The incidence and impact of achievement of molecular remission (mCR) and high-risk cytogenetics was prospectively investigated in 73 patients after tandem auto/allo-SCT including 16 patients with high-risk cytogenetic features defined by positive FISH for del(17p13) and/or t(4;14).[24] Response, including CR and molecular CR (mCR), were equal irrespective of risk features. After a median follow up of six years, overall 5-year PFS was 29%, with no significant difference between patients harboring del 17p, t(4;14) and others (*P*=0.70). The 5-year progression-free survival differed substantially according

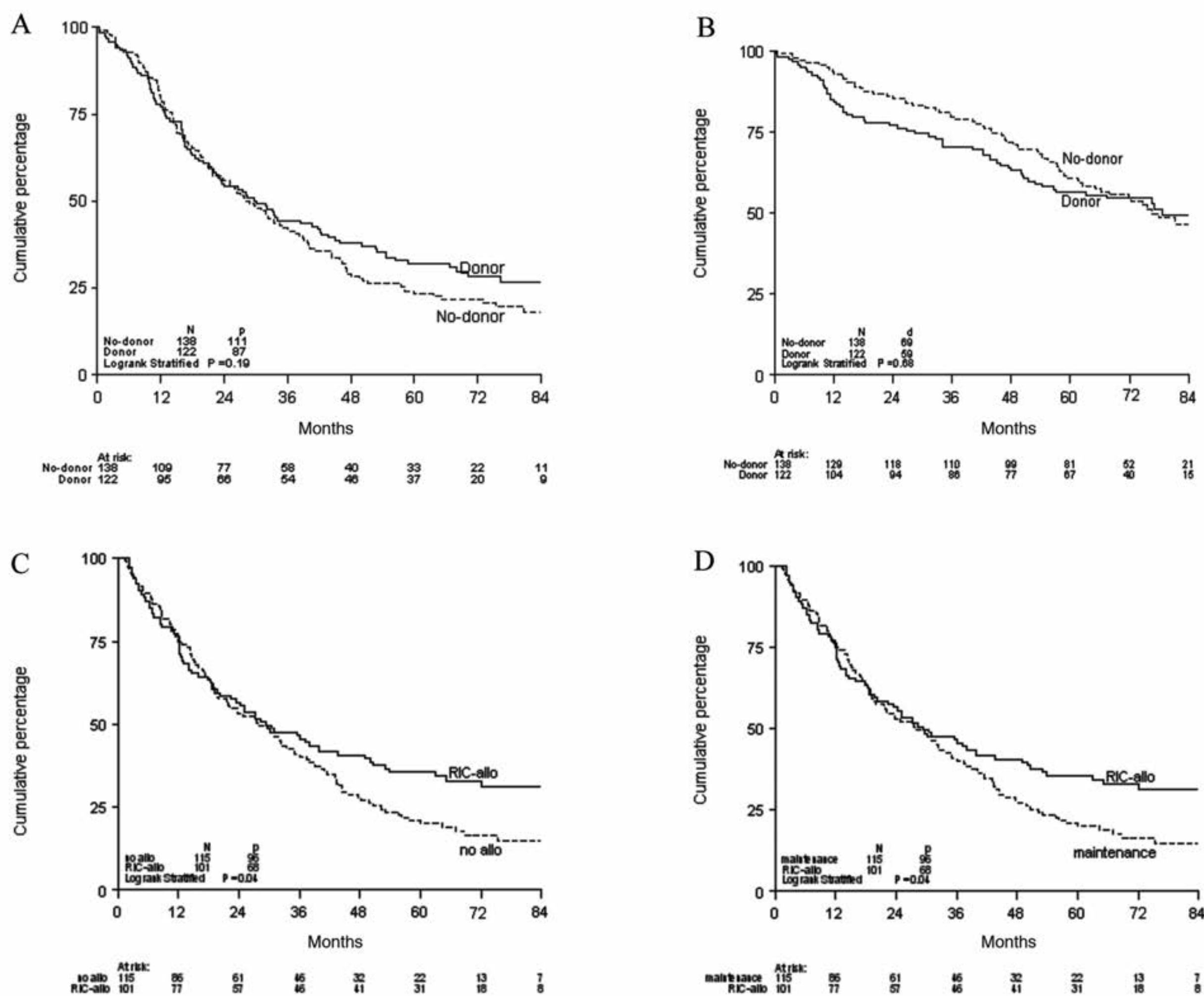

Figure 1. Progression-free survival (PFS) (A) and overall survival (OS) (B) according to availability of an HLA-identical sibling in the HOVON 50 study. PFS (C) and OS (D) according to treatment started after auto-SCT, *i.e.* allo-RIC *versus* maintenance with thalidomide or α-interferon. RIC, reduced intensity conditioning

Table 1. Prospective donor *versus* no donor studies in myeloma.

Study group	Number	TRM	CR	PFS	OS
IFM[16]					
Donor	65	11%	33%	0% / 5yr	33% / 5yr
No donor	219	5%	33%	0% / 5 yr	44% / 5yr
Bruno *et al.*[17]					
Donor	82	10%	55%	42% / 4yr	75% / 4 yr
No donor	80	4%	26%	20% / 4 yr	53% / 5 yr
HOVON[19]					
Donor	122	16%	37%	26% / 6 yr	55% / 6 yr
No donor	138	3%	43%	22% / 6 yr	55% / 6yrs
EBMT[18]					
Donor	108	12% (24 months)	51%	55% / 5 yr	65% / 5 yr
No donor	249	3%	41%	22% / 5 yr	58% / 5 yr
BMT-CTN[20]					
Donor	189	11% (36 months)	58%	43% / 3 yr	77% / 3 yr
No donor	436	4% (36 months)	48%	46% / 3 yr	80% / 3 yr

to the achieved remission rates: 17% for PR, 41% for CR, 57% for mCR, and 85% for sustained mCR. The authors suggest that auto/allo-SCT may overcome the negative prognostic effect of del(17p13) and/or t(4;14), and that achievement of molecular remission resulted in long-term freedom from disease.

More solid data about the effect of allo-SCT in high-risk MM may come from the prospective EMN-02/HOVON 95 study. Patients with high-risk features based on FISH included in this study are candidates for auto/allo after induction therapy and may be compared to patients without a donor treated according to the other 2 arms of the protocol (Figure 2).

High-risk myeloma defined by early relapse after autologous stem cell transplantation

Patients with relapsed disease after first-line therapy have a poor prognosis. This is illustrated by the median survival of only 19 months after relapse from thalidomide maintenance of patients who were included in the HOVON 50 study.[25] Relapse after auto-SCT may be even more challenging when the novel anti-myeloma agents like bortezomib and/or lenalidomide were part of the induction and maintenance. The design of the recently started EMN05/HOVON 108 study is based on a conditioning regimen with maximal T-cell depletion by alemtuzumb to create a platform for subsequent immunotherapy with preventive DLI and mHag dendritic cell vaccination in patients with a suitable mHag mismatch. To prevent early relapse after allo-SCT, patients start with a short course of consolidation therapy three months after stem cell infusion to prevent early relapse. Figure 3 shows the study design. A similar study led by Perez in first-relapsed patients has been started in Spanish transplantation centers. No data from this study are available yet.

Post-transplant strategies to improve the graft-*versus*-multiple myeloma effect

A role for the novel anti-myeloma agents?

Lenalidomide is an immunomodulatory drug with potent stimulatory effects on host anti-tumor T and natural killer (NK) cell immunity and cytotoxicity. Lenalidomide is 50-2000 times more potent than its analog thalidomide. It increases proliferation, secretion of IFN-γ by T cells and enhances cytotoxic T-cell and NK-cell mediated killing of MM tumors.[26-28] Given these properties, lenalidomide is expected to have a significant impact on the GvM effect.

Bortezomib is a proteasome inhibitor and blocks the activation of NF-κB, a highly important pathway for MM-cell survival. Since proteasomes and the NF-κB pathway are also important components of antigen processing and cell-mediated cytokine responses, a number of studies explored the influence of bortezomib on the cellular immune system. Bortezomib inhibited *in vitro* allo-reactive mixed lymphocyte responses but increased the T-cell dependent killing of the tumor cells.[29] Consistently, in a murine BMT model, bortezomib down-regulated cytokine production, induced T-cell apoptosis and prevented GvHD, while the GvT effect was preserved.[29] Nonetheless, delayed bortezomib administration appears to accelerate GvHD, underscoring the need for further careful studies on the timing and schedule of administration.[30] On the other hand, bortezomib can down-regulate HLA class I on MM cells, while this is not the case for normal cells like B cells, lymphocytes, monocytes, CD34 progenitor cells and dendritic cells.[26] Thus, bortezomib sensitizes tumor cells toward NK-cell mediated lysis by down-regulating MHC class I. Taken together, these results indicate that bortezomib may be highly beneficial

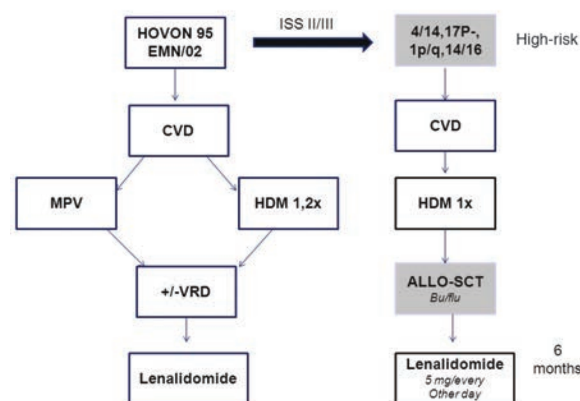

Figure 2. Patients included in the EMN02/HOVON 95 study with high-risk disease may be allocated to auto/allo-SCT following induction therapy with cyclophosphamide, velcade, dexamethasone (CVD).

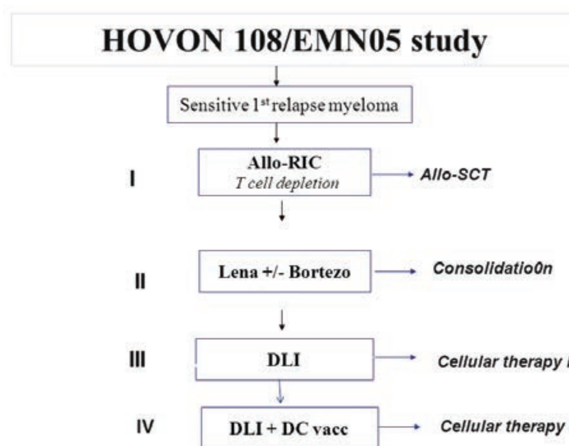

Figure 3. Patients with a first chemo-sensitive relapse following auto-SCT may be included. The allo-RIC is performed after conditioning with fludarabine, busilvix and alemtuzumab. Patients with a suitable mHag mismatch are candidates for a second DLI combined with vaccination with dendritic cell (DC) loaded with the relevant mHag peptide. DLI: donor lymphocyte infusion.

in tempering the potential immune side effects of lenalidomide, in particular GvHD.

Efficacy of novel anti-myeloma agents in relapsed myeloma after allo-SCT

Clinical observations support a possible role for the novel anti-myeloma agents as post-allo immune modulating agents stimulating graft-*versus*-myeloma. Thalidomide improved the response to DLI, apparently without enhancing GvHD.[31] Lenalidomide as salvage therapy in a group of patients with progressive symptomatic disease after allo-RIC induced responses in 14 of 15 patients, including 3 patients with a CR.[32] Unfortunately, some of these responses were accompanied by fulminating GvHD. These favorable results were recently confirmed in a multicenter retrospective study including 52 patients receiving lenalidomide alone or in combination with dexamethasone as salvage therapy after allo-SCT.[33] Lenalidomide induced a high response rate of 83% (including 29% complete response). However, 16 patients developed or exacerbated acute graft-*versus*-host disease and 2 patients (4%) died, one from treatment toxicity and one to graft-*versus*-host disease.

In MM patients, bortezomib has been reported to both stimulate and improve GvHD.[29-30] In the study recently published by Corradini *et al*., bortezomib was remarkably effective in patients with relapsed disease after allo-SCT, without apparent excessive stimulation of GvHD.[34] The dissociation of GvHD and GvM is of vital importance in improving the efficacy of allo-SCT and DLI. The novel anti-MM agents in the post allo-SCT setting may favor such a separation. However, exact mechanisms of action, as well as the optimal timing of these agents post- transplant, have yet to be determined.

Lenalidomide maintenance following tandem auto-SCT/allo-SCT

In the HOVON 76 study, lenalidomide 10 mg daily maintenance therapy was started 1-6 months after tandem auto-allo-SCT that had been part of first-line therapy for patients aged 65 years and under.[35] Thirty-five eligible patients were enrolled of whom 30 started with lenalidomide. After 2 cycles, 14 patients (47%) had to stop treatment, mainly because of the development of acute GVHD; 5 patients (17%) had to stop treatment because of other adverse events, and 5 patients (17%) because of progression. Responses improved in 37% of patients, and the estimated 1-year PFS from start of maintenance was 69%. Lenalidomide increased the frequency of human leukocyte antigen-DR$^+$ T cells and regulatory T cells, without correlation with clinical parameters.

Kroger *et al*. performed a prospective phase I/II study to define the dose-limiting toxicity and the immunological effects of lenalidomide given early (Day 100-180) after allograft for 2 cycles in patients with MM.[36] Also in this study, acute GvHD was a major problem and maximum tolerable dose was 5 mg/daily. GvHD after start of lenalidomide occurred in 38% of patients after a median of 22 days, and was a leading reason to discontinue the study in 29% of the patients. Natural killer (NK) cells isolated from the peripheral blood of patients demonstrated a significantly improved anti-myeloma activity after lenalidomide treatment. Although strong positive immune effects may be induced, however, the conclusion from these studies must be that lenalidomide maintenance after non-myeloablative allo-SCT with unmanipulated grafts is not feasible in MM patients, mainly because of the induction of acute GVHD.

Other strategies to boost the GvM effect

Other strategies that can be explored are NK-cell therapies, adoptive T-cell therapy and vaccination studies.[37-45]

Adoptive T-cell therapy and vaccination

A crucial role for professional antigen presenting cells in the graft-versus-tumor effect

Over the past years, several experimental studies demonstrated that the development of GvHD, as well as of graft-*versus*-tumor (GVT) effect, is not only dependent on the presence of donor CD4$^+$ and CD8$^+$ T cells in the transplant but also on the presence of professional antigen presenting cells (APCs) capable of properly activating these T cells.[46-49] Murine studies also showed that DLI applied in a mixed-chimeric status induced GvT due to the presence of host APCs but had no beneficial effect after the achievement of full donor-chimerism, a condition in which host APCs were no longer present.[50,51] In agreement with these results, clinical studies showed that the development of GvT was indeed dependent on a *mixed-chimeric* status in the patients.[52-54] Thus, there is now compelling evidence that the presence of professional APCs capable of presenting host antigens is crucial for the development of effective GvT responses after DLI. This strongly suggests that the GvT responses after DLI could be specifically improved by co-injection of professional APCs loaded with GvT-associated antigens.

Dendritic cells: key APCs for initiation of T-cell responses

A rare population of blood cells, named dendritic cells (DCs), are currently the best known APCs to T cells. Over the past decade, the development of *in vitro* DC-culture techniques has facilitated several DC-based therapeutic vaccination studies for cancer.[55] Many clinical trials have demonstrated that vaccination with antigen pulsed and optimally-maturated DCs is safe, well tolerated and results in enhanced immunity to the loaded antigens.[56]

Minor histocompatibility antigens (mHags): major target antigens of GvT in HLA-matched allo-SCT

In allo-SCT, the GvHD-risk can be significantly reduced by matching the recipient and the donor for HLA antigens. Nonetheless, strong GvT effects can develop even after a transplant between fully HLA-identical donor-recipient pairs.[57] This illustrates the prime importance of the so-called ‘minor histocompatibility antigens’ (mHags) in GvT after HLA-identical allo-SCT. In fact, mHags are polymorphic peptides presented by HLA molecules to donor T cells.[58] These peptides are derived from cellular proteins that are not entirely identical in the donor and the recipient. Many of these differences are due to non-synonymous single nucleotide polymorphisms (SNP) in allelic genes.[59] The clinical relevance of mHags has been demonstrated by several studies (reviewed by Spaapen and Mutis[60]) showing that:

i) mHag specific T cells can be readily isolated from patients with good clinical anti-tumor responses;

ii) virtually all mHag-specific T cells tested so far can lyse malignant cells from leukemia, lymphoma and myeloma patients;

iii) the emergence and expansion of mHag specific CTLs in the circulation is frequently associated with clinical responses after DLI.

GvT-associated, hematopoietic system specific mHags: tools for improving GvT with low risk of GvHD

Since mHags are important for both GvT and GvHD, initially it seemed impossible to utilize mHags for therapeutic purposes. However, in the mid-1990s, it was discovered that some mHags were solely expressed in hematopoietic cells, including their malignant counterparts. The mHags HA-1 and HA-2 were the first identified hematopoietic mHags. They are broadly expressed in all hematologic malignancies. Subsequently, several other hematopoietic-restricted mHags were identified: the B-cell associated mHags PANE-1 and HB-1 and ACC-1, ACC-2, LRH-1 and UTA2-1 which are expressed by various myeloid and lymphoid malignancies, including MM.[61] Finally, we have recently identified the first genuine hematopoietic mHag presented by HLA class II molecules to $CD4^+$ CTLs.[62] This antigen is encoded by a single amino acid substitution on the B-cell specific molecule CD19, which is expressed by almost all B-cell malignancies with the exception of MM.

Although the HLA restriction of mHags and the requirement of a mHag mismatch between the recipient and the donor are main restrictions for the general applicability of mHag-based therapies, the availability of these 8 antigens now makes it possible to offer this therapy to 23% of the patients in the HLA-identical sibling setting, and to a minimum of 40% of patients in the HLA-matched MUD allo-SCT setting.

Clinical responses to host DC vaccinations in MM patients

We recently performed a DC vaccination trial to improve the GvT effects in DLI non-responder MM patients. In this trial, a second DLI was combined with infusion of *ex vivo* generated but antigen unloaded host-DCs. Eight patients received DC vaccinations combined with DLI. A fraction of DC vaccines were pulsed with a foreign protein (KLH) in order to evaluate the potency of vaccination. In 5 of 6 patients, skin tests two weeks after the last vaccination revealed strong positive indurations against host-DCs. In all patients, functional assays (T-cell proliferation, IFN-γ release) revealed the development of T-cell responses against KLH and host-DCs starting from the second or last vaccination. In one patient, we also detected T-cell responses against host PBMCs as well as against malignant cells. Although vaccination did not result in an objective GvT effect in 6 patients, one patient, who had a very low level of tumor load at the time of vaccination, entered into remission and is still free of disease after 48 months. These results indicated that DC vaccinations combined with DLI is feasible, safe and capable of inducing T-cell responses. However, a more efficient targeting of the donor T cells toward malignant cells is necessary to achieve better overall clinical responses. The next step logical step is the loading of the autologous DC cells with myeloma associated, hematopoietic system specific mHags to improve the clinical responses of MM patients to DLI with low risk of GvHD. Such a study was recently initiated in DLI non-responding patients with relapsed or residual disease after allo-SCT.

Future aspects

A future role for allo-SCT in myeloma will depend mainly on 2 aspects. Are we able to reduce morbidity and mortality of the conditioning regimen and can we substantially boost the graft-*versus*-tumor effect. One attractive approach seems to be that of depleting T cells from the graft to reduce GvHD and create a platform for immune manipulation to enhance GvM.[63] Tools to create this may be CD34 selection, CD3/CD19 depletion or αβ T-cell depletion of the graft.[64-66] We apply alemtuzumab in the conditioning regimen as cited above. However, the first results show that there is probably a very narrow window between too great a depletion of T cells resulting in post-transplant severe infections and graft failure, and insufficient T-cell depletion resulting in acute and chronic GvHD. Both situations impede the subsequent immunotherapy of the protocol. It is also not clear what is the best way to specifically boost the GvM effect. For this, greater insight is still needed into the role of the different T-cell subsets involved, including Treg cells and mechanisms of immune resistance, like the role of PDL-1, IDO in MM cells and the contribution of the myeloma microenvironment. The final conclusion is that a possible future role for allo-SCT in MM can only be established by prospective studies exploring novel approaches.

References

1. Lokhorst HM, Schattenberg A, Cornelissen JJ, van Oers MH, Fibbe W, Russell I, et al. Donor lymphocyte infusions for relapsed multiple myeloma after allogeneic stem-cell transplantation: predictive factors for response and long-term outcome. J Clin Oncol. 2000;18:3031-71.
2. Zeiser R, Bertz H, Spyridonidis A, Houet L, Finke J. Donor lymphocyte infusions for multiple myeloma:clinical results and novel perspectives.Bone Marrow Transplant. 2004; 34:923-8.
3. Lokhorst HM, Wu K, Verdonck LF, Laterveer LL, van de Donk NW, van Oers MH, et al. The occurrence of graft-versus-host disease is the major predictive factor for response to donor lymphocyte infusions in multiple myeloma. Blood. 2004;103:4362-4.
4. van de Donk NW, Kröger N, Hegenbart U, Corradini P, San Miguel JF, Goldschmidt H, et al. Prognostic factors for donor lymphocyte infusions following non-myeloablative allogeneic stem cell transplantation in multiple myeloma. Bone Marrow Transplant. 2006;12:1135-4.
5. Verdonck LF, Lokhorst HM, Dekker AW, Nieuwenhuis HK, Petersen EJ. Graft-versus-myeloma effect in two cases. Lancet. 1996;347:800-1.
6. Gahrton G, Tura S, Ljungman P, Bladé J, Brandt L, Cavo M, et al. Prognostic factors in allogeneic bone marrow transplantation for multiple myeloma. J.Clin Oncol. 1995;13:1312-22.
7. Russell NH, Miflin G, Stainer C, McQuaker JG, Bienz N, Haynes AP, et al. Allogeneic bone marrow transplant for multiple myeloma. Blood 1997;89:2610-11.
8. Reece DE, Shepherd JD, Klingemann HG, Sutherland HJ, Nantel SH, Barnett MJ, et al. Treatment of myeloma using intensive therapy and allogeneic bone marrow transplantation. Bone Marrow Transplant. 1995;15:117-23.
9. Couban S, Stewart AK, Loach D, Panzarella T, Meharchand J. Autologous and allogeneic transplantation for multiple myeloma at a single centre. Bone Marrow Transplant. 1997;19:783-9.

10. Russell N, Bessell E, Stainer C, Haynes A, Das-Gupta E, Byrne J. Allogeneic haemopoietic stem cell transplantation for multiple myeloma or plasma cell leukaemia using fractionated total body radiation and high-dose melphalan conditioning. Acta Oncol. 2000;39:837-41.
11. Björkstrand BB, Ljungman P, Svensson H, Hermans J, Alegre A, Apperley J, et al. Allogeneic bone marrow transplantation versus autologous stem cell transplantation in multiple myeloma: a retrospective case-matched study from the European Group for Blood and Marrow Transplantation. Blood 1996;88:4711-8.
12. Lokhorst HM, Segeren CM, Verdonck LF, van der Holt B, Raymakers R, van Oers MH, et al. Partially T-cell-depleted allogeneic stem-cell transplantation for first-line treatment of multiple myeloma: a prospective evaluation of patients treated in the phase III study HOVON 24 MM. J.Clin Oncol. 2003;13:1728-33.
13. Gahrton G, Iacobelli S, Bandini G, Björkstrand B, Corradini P, Crawley C, et al. Peripheral blood or bone marrow cells in reduced-intensity or myeloablative conditioning allogeneic HLA identical sibling donor transplantation for multiple myeloma. Haematologica. 2007;92:1513-8.
14. Maloney DG, Molina AJ, Sahebi F, Stockerl-Goldstein KE, Sandmaier BM, Bensinger W, et al. Allografting with nonmyeloablative conditioning following cytoreductive autografts for the treatment of patients with multiple myeloma. Blood. 2003;102:3446-54.
15. Crawley C, Iacobelli S, Björkstrand B, Apperley JF, Niederwieser D, Gahrton G. Reduced-intensity conditioning for myeloma: lower nonrelapse mortality but higher relapse rates compared with myeloablative conditioning. Blood. 2007;109:3588-94.
16. Garban F, Attal M, Michallet M, Hulin C, Bourhis JH, Yakoub-Agha I, et al. Prospective comparison of autologous stem cell transplantation followed by dose-reduced allograft (IFM99-03 trial) with tandem autologous stem cell transplantation (IFM99-04 trial) in high-risk de novo multiple myeloma. Blood 2006;107:3474-80.
17. Bruno B, Rotta M, Patriarca F, Mordini N, Allione B, Carnevale-Schianca F, et al. A comparison of allografting with autografting in myeloma. N Engl J Med. 2007;356:1110-20.
18. Björkstrand B, Iacobelli S, Hegenbart U, Gruber A, Greinix H, Volin L, et al. Tandem autologous/reduced-intensity conditioning allogeneic stem-cell transplantation versus autologous transplantation in myeloma: long-term follow-up. J Clin Oncol. 2011;29:3016-22.
19. Lokhorst HM, van der Holt B, Cornelissen JJ, Kersten MJ, van Oers M, Raymakers R, et al. Donor versus no-donor comparison of newly diagnosed myeloma patients included in the HOVON-50 multiple myeloma study. Blood. 2012;119:6219-25.
20. Krishnan A, Pasquini MC, Logan B, Stadtmauer EA, Vesole DH, Alyea E 3rd, et al. Autologous haemopoietic stem-cell transplantation followed by allogeneic or autologous haemopoietic stem-cell transplantation in patients with multiple myeloma (BMT CTN 0102): a phase 3 biological assignment trial. Lancet Oncol. 2011;12:1195-203.
21. Armeson KE, Hill EG, Costa LJ.Tandem autologous vs autologous plus reduced intensity allogeneic transplantation in the upfront management of multiple myeloma: meta-analysis of trials with biological assignment. Bone Marrow Transplant. 2012 Sep 10. [Epub ahead of print]
22. Kharfan-Dabaja MA, Hamadani M, Reljic T, Nishihori T, Bensinger W, Djulbegovic B, et al. Comparative efficacy of tandem autologous versus autologous followed by allogeneic hematopoietic cell transplantation in patients with newly diagnosed multiple myeloma: a systematic review and meta-analysis of randomized controlled trials. J Hematol Oncol. 2013;10:1186.
23. Roos-Weil D, Moreau P, Avet-Loiseau H, Golmard JL, Kuentz M, Vigouroux S, et al. Impact of genetic abnormalities after allogeneic stem cell transplantation in multiple myeloma: a report of the Société Française de Greffe de Moelle et de Thérapie Cellulaire. Haematologica. 2011;96:1504-11.
24. Kröger N, Badbaran A, Zabelina T, Ayuk F, Wolschke C, Alchalby H, et al. Impact of High-Risk Cytogenetics and Achievement of Molecular Remission on Long-Term Freedom from Disease after Autologous-Allogeneic Tandem Transplantation in Patients with Multiple Myeloma. Biol Blood Marrow Transplant. 2012 Oct 16. [Epub ahead of print]
25. Lokhorst HM, van der Holt B, Zweegman S, Vellenga E, Croockewit S, van Oers MH, et al. A randomized phase 3 study on the effect of thalidomide combined with adriamycin, dexamethasone, and high-dose melphalan, followed by thalidomide maintenance in patients with multiple myeloma. Blood. 2010;115:1113-20.
26. Teo SK. Properties of thalidomide and its analogues: implications for anticancer therapy. AAPS J. 2005;7(1):E14-9.
27. Davies,FE, Raje N, Hideshima T, Lentzsch S, Young G, Tai YT, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood 2001;98:210-6.
28. Chang,DH., Liu N, Klimek V, Hassoun H, Mazumder A, Nimer SD, et al. Enhancement of ligand-dependent activation of human natural killer T cells by lenalidomide: therapeutic implications. Blood. 2006:108:618-21.
29. Sun K, Welniak LA, Panoskaltsis-Mortari A, O'Shaughnessy MJ, Liu H, Barao I, et al. Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib. Proc Natl Acad Sci USA. 2004;101(21):8120-5.
30. Sun K, Wilkins DE, Anver MR, Sayers TJ, Panoskaltsis-Mortari A, Blazar BR, et al. Differential effects of proteasome inhibition by bortezomib on murine acute graft-versus host disease (GVHD): delayed administration of bortezomib results in increased GVHD-dependent gastrointestinal toxicity. Blood. 2005;106:3293-9.
31. Kroger N, Shimoni A, Zagrivnaja A, Ayuk F, Lioznov M, Schieder H, et al. Low-dose thalidomide and donor lymphocyte infusion as adoptive immunotherapy after allogeneic stem cell transplantation in patients with multiple myeloma. Blood. 2004;104(10):3361-3.
32. Minnema MC, van der Veer MS, Aarts T, Emmelot M, Mutis T, Lokhorst HM. Lenalidomide alone or in combination with dexamethasone is highly effective in patients with relapsed multiple myeloma following allogeneic stem cell transplantation and increases the frequency of CD4+Foxp3+ T cells. Leukemia. 2009;23(3):605-7.
33. Coman T, Bachy E, Michallet M, Socie' G, Uzunov M, Bourhis JH, et al. Lenalidomide as salvage treatment for multiple myeloma relapsing after allogeneic hematopoietic stem cell transplantation: a report from the SFGM-TC. Haematologica. 2012 Nov 9. [Epub ahead of print]
34. Montefusco V, Spina F, Patriarca F, Offidani M, Bruno B, Montanari M, et al. Bortezomib Plus Dexamethasone Followed by Escalating Donor Lymphocyte Infusions for Patients with Multiple Myeloma Relapsing or Progressing after Allogeneic Stem Cell Transplantation. Biol Blood Marrow Transplant. 2012 Nov 8. [Epub ahead of print]
35. Kneppers E, van der Holt B, Kersten MJ, Zweegman S, Meijer E, Huls G, et al. Lenalidomide maintenance after nonmyeloablative allogeneic stem cell transplantation in multiple myeloma is not feasible: results of the HOVON 76 Trial. Blood. 2011;118(9):2413-9.
36. Wolschke C, Stübig T, Hegenbart U, Schönland S, Heinzelmann M, Hildebrandt Y, et al. Postallograft lenalidomide induces strong NK cell-mediated antimyeloma activity and risk for T cell-mediated GvHD: results from a phase I/II dose-finding study. Exp Hematol. 2013;41(2):134-42.
37. Bendandi M, Rodriguez-Calvillo M, Inoges S, Lopez-Diaz de CA, Perez-Simon JA, Rodriguez-Caballero A, et al. Combined vaccination with idiotype-pulsed allogeneic dendritic cells and soluble protein idiotype for multiple myeloma patients relapsing after reduced-intensity conditioning allogeneic stem cell transplantation. Leuk.Lymphoma 2006;47:29-37.
38. Neelapu SS, Munshi NC, Jagannath S, Watson TM, Pennington R, Reynolds C, et al. Tumor antigen immunization of sibling stem cell transplant donors in multiple myeloma. Bone Marrow Transplant. 2005;36:315-23.
39. Fujio K, Misaki Y, Setoguchi K, Morita S, Kawahata K, Kato I, et al. Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex. J Immunol. 2000;165:528-32.
40. Stanislawski T, Voss RH, Lotz C, Sadovnikova E, Willemsen RA, Kuball J, et al. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. Nat.Immunol. 2001;2:962-70.
41. van der Veken L, Hoogeboom M, de Paus RA, Willemze R, Falkenburg JH, Heemskerk MH. HLA class II restricted T-cell receptor gene transfer generates CD4+ T cells with helper activity as well as cytotoxic capacity. Gene Ther. 2005;12: 1686-95.
42. Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P, et al. Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. Gene Ther. 2000;7:1369-77.
43. Kershaw MH, Westwood JA, Hwu P. Dual-specific T cells combine proliferation and antitumor activity. Nat.Biotechnol.

2002;20:1221-27.
44. Song W, van der Vliet HJ, Tai YT, Prabhala R, Wang R, Podar K, et al. Generation of antitumor invariant natural killer T cell lines in multiple myeloma and promotion of their functions via lenalidomide: a strategy for immunotherapy Clin Cancer Res. 2008;14:6955-62.
45. Alici E, Konstantinidis KV, Sutlu T, Aints A, Gahrton G, Ljunggren HG, et al. Anti-myeloma activity of endogenous and adoptively transferred activated natural killer cells in experimental multiple myeloma model. Exp Hematol. 2007;35:1839-46.
46. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science. 1999;285:412-5.
47. Duffner UA, Maeda Y, Cooke KR, Reddy P, Ordemann R, Liu C, et al. Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. J Immunol. 2004;172:7393-8.
48. Sykes M, Sheard MA, Sachs DH. Graft-versus-host-related immunosuppression is induced in mixed chimeras by alloresponses against either host or donor lymphohematopoietic cells. J Exp Med. 1988;168:2391-6.
49. Georges GE, Storb R, Thompson JD, Yu C, Gooley T, Bruno B, et al. Adoptive immunotherapy in canine mixed chimeras after nonmyeloablative hematopoietic cell transplantation. Blood. 2000;95:3262-9.
50. Mapara MY, Kim YM, Marx J, Sykes M. Donor lymphocyte infusion-mediated graft-versus-leukemia effects in mixed chimeras established with a nonmyeloablative conditioning regimen: extinction of graft-versus-leukemia effects after conversion to full donor chimerism. Transplantation. 2003;76:297-305.
51. Pelot MR, Pearson DA, Swenson K, Zhao G, Sachs J, Yang YG, et al. Lymphohematopoietic graft-vs.-host reactions can be induced without graft-vs.-host disease in murine mixed chimeras established with a cyclophosphamide-based nonmyeloablative conditioning regimen. Biol Blood Marrow Transplant. 1999;5:133-43.
52. Mapara MY, Kim YM, Wang SP, Bronson R, Sachs DH, Sykes M. Donor lymphocyte infusions mediate superior graft-versus-leukemia effects in mixed compared to fully allogeneic chimeras: a critical role for host antigen-presenting cells. Blood. 2002;100:1903-9.
53. Billiau AD, Fevery S, Rutgeerts O, Landuyt W, Waer M. Crucial role of timing of donor lymphocyte infusion in generating dissociated graft-versus-host and graft-versus-leukemia responses in mice receiving allogeneic bone marrow transplants. Blood. 2002;100:1894-902.
54. Peggs KS, Thomson K, Hart DP, Geary J, Morris EC, Yong K, et al. Dose-escalated donor lymphocyte infusions following reduced intensity transplantation: toxicity, chimerism, and disease responses Blood. 2004;103:1548-56.
55. Tacken PJ, de Vries IJ, Torensma R, Figdor CG. Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. Nat Rev Immunol. 2007;7:790-802.
56. Lesterhuis WJ, Aarntzen EH, De Vries IJ, Schuurhuis DH, Figdor CG, Adema GJ, Punt CJ. Dendritic cell vaccines in melanoma: from promise to proof? Crit Rev Oncol Hematol. 2008;66:118-34.
57. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990;75:555-62.
58. Goulmy E. Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. Immunol Rev. 1997;157:125-40.
59. Mutis T, Goulmy E. 2002. Hematopoietic system-specific antigens as targets for cellular immunotherapy of hematological malignancies. Semin Hematol. 2002;39:23-31.
60. Spaapen R, Mutis T. 2008. Targeting hematopoietic-specific minor histocompatibility antigens to distinguish graft-versus-tumour effects from graft-versus-host disease. Best Pract Res Clin Haematol. 2008;21:543-57.
61. Oostvogels R, Minnema MC, van Elk M, Spaapen RM, Te Raa GD, Giovannone B, et al. Towards effective and safe immunotherapy after allogeneic stem cell transplantation: identification of hematopoietic-specific minor histocompatibility antigen UTA2-1. Leukemia. 2012 Oct 1. [Epub ahead of print]
62. Spaapen RM, Lokhorst HM, van den Oudenalder K, Otterud BE, Dolstra H, Leppert MF, et al. Toward targeting B cell cancers with CD4+ CTLs: identification of a CD19-encoded minor histocompatibility antigen using a novel genome-wide analysis. J Exp Med. 2008;205:2863-72.
63. Marcu-Malina V, Heijhuurs S, van Buuren M, Hartkamp L, Strand S, Sebestyen Z, et al. Redirecting αβ T cells against cancer cells by transfer of a broadly tumor-reactive γδT-cell receptor. Blood. 2011;118:50-9.
64. Pasquini MC, Devine S, Mendizabal A, Baden LR, Wingard JR, Lazarus HM, et al. Comparative outcomes of donor graft CD34+ selection and immune suppressive therapy as graft-versus-host disease prophylaxis for patients with acute myeloid leukemia in complete remission undergoing HLA-matched sibling allogeneic hematopoietic cell transplantation. J Clin Oncol. 2012;30:3194-201.
65. Levenga H, Schaap N, Maas F, Esendam B, Fredrix H, Greupink-Draaisma A, et al. Partial T cell-depleted allogeneic stem cell transplantation following reduced-intensity conditioning creates a platform for immunotherapy with donor lymphocyte infusion and recipient dendritic cell vaccination in multiple myeloma. Biol Blood Marrow Transplant. 2010;16: 320-32.
66. Handgretinger R . Negative depletion of CD3(+) and TcRαβ(+) T cells. Curr Opin Hematol. 2012;19:434-9.

Recent advances in molecular genetics of myelodysplastic syndromes as revealed by massively parallel sequencing

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A B S T R A C T

During the past ten years, major mutational gene targets of myelodysplastic syndromes (MDS) have been revealed relying on high-throughput genomic technologies such as massively parallel sequencing of MDS genomes. Frequent mutations in epigenetic regulators and RNA splicing machinery are among most relevant discoveries, although their functional impacts on MDS pathogenesis has not been fully clarified. The spectrum of gene mutations is largely overlapped to that found in other myeloid neoplasms including acute myeloid leukemia (AML), myelodysplastic syndromes/myeloproliferative neoplasms (MDS/MPN) and myeloproliferative neoplasms (MPN), indicating common molecular pathogenesis among them. Meanwhile, some mutations, including spliceosome mutations, are more prevalent in MDS and therefore more tightly linked to MDS-specific pathogenesis. Many MDS patients show multiple subclones originated from a common ancestor at diagnosis that are dynamically shaped by acquisition of new gene mutations and subsequent clonal selection, progressing to secondary acute myeloid leukemia (sAML). Deep sequencing of recurrent gene mutations in multiple cases indicates the presence of a hierarchy among different mutations, which might be relevant to disease progression and prognostication of clinical outcomes. Understanding of gene mutations and their impacts on the pathogenesis and clinical parameters of MDS should provide knowledge essential to improving the clinical outcomes of MDS.

Learning goals

At the conclusion of this activity, participants should have an understanding of:
- a spectrum of gene mutations found in myelodysplastic syndromes (MDS) and their phenotype correlations;
- the functional relevance of major mutations to the pathogenesis of MDS/myeloid neoplasms, especially roles of mutations in epigenetic regulators and RNA splicing factors;
- Intratumor heterogeneity and hierarchy of gene mutations and their relevance to development and progression of MDS.

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of chronic myeloid neoplasms characterized by varying degrees of cytopenia with dysplastic blood cell morphology, frequently terminating in acute myeloid leukemia (AML).[1] Reflecting their heterogeneity, MDS show varying degrees of lineage involvement, blast/ring sideroblast counts and cytogenetic lesions, depending on which the current WHO classification distinguishes several subtypes of MDS, including refractory cytopenias with unilineage (RCUD) or multilineage (RCMD) dysplasia, refractory anemia with ring sideroblasts (RARS), refractory anemia with excess blasts (RAEB), MDS with isolated del(5q) and other subtypes.[2] However, the discrimination between MDS and other myeloid cancers, such as acute myeloid leukemia (AML), myeloproliferative neoplasms (MPN) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN), and among different MDS subtypes is frequently obscured in many borderline cases, suggesting common underlying mechanisms shared by these apparently different entities of myeloid malignancies. Meanwhile, dramatic advances in high-throughput genome technology of recent years have provided a novel opportunity to understand the molecular genetics/pathogenesis of MDS and other myeloid malignancies in terms of gene mutations through comprehensive detection of genetic lesions using massively parallel sequencing.[3-8] Moreover, deep sequencing of large numbers of somatic mutations detected by whole genome sequencing in consecutively obtained tumor specimens has enabled detailed analysis of the intratumoral structure of gene mutations and their clonological behaviors that shapes progression from MDS to secondary AML (sAML) in terms of clonal evolution. In this review, recent progress in molecular genetics of MDS and its relevance to pathogenesis will be discussed.

Spectrum of gene mutations in MDS

Somatic gene mutation in MDS was first

described in 1987 by Hirai *et al.,* in which mutant *NRAS* alleles were isolated through *in vivo* selection of NIH3T3 cells transduced with patients' genomic DNA in nude mice.[9] However, since then, only a few common gene targets of somatic mutations, such as *KRAS*, *TP53* and *RUNX1*, had been reported until early 2000, hampering our understanding of molecular pathogenesis of MDS.[10-12] The situation has been dramatically improved during the past ten years in which major targets of gene mutations in MDS have been exhaustively identified using high-throughput genomics, including mutations in additional RAS pathway genes (*PTPN11*, *NF1* and *CBL)*[13-17] and other signal transducing molecules (*c-KIT, JAK2, FLT3* and *cMPL)*[18-21] as well as hematopoietic transcription factor genes (*CEBPA and ETV6*) (Figure 1).[22,23] Among others, the discovery of frequent mutations of epigenetic regulators and RNA splicing factors represents the most significant progress, shedding light on novel aspects of the molecular pathogenesis of MDS.

Mutations in epigenetic regulators

Frequent mutation of epigenetic regulators is one of the major discoveries in recent cancer genome studies and has been commonly observed in a wide variety of human cancers, suggesting that epigenetic alterations in cancers may be caused, at least in part, by primary gene mutations.[24] In myeloid neoplasms, major mutational targets of epigenetic regulators include *TET2* and *IDH1/2*, *DNMT3A*, and several components of the polycomb repressive complex 2 (PRC2) (*EZH2, EED, SUZ12* and *JARID2*) and other polycomb-related proteins (*ASXL1* and *BCOR/BCOR1*).

TET2

TET2 was originally identified within a microdeletion at 4q21 as a mutational target in loss of heterozygosity (LOH) in 4q arm.[25,26] TET2 belongs to a TET family of proteins encoding α-ketoglutarate (αKG)-dependent oxidases engaged in hydroxylation of 5'-methylcytosine (5mC) to 5hmC,[27] which is now believed to be a critical step of demethylation of methylated CpG (Figure 2).[28,29] Mutations are most frequently found in CMML (approx. 40-50%) and MDS (approx. 25%), but are also common in *de novo* AML and MPN.[25,26,30,31] Most of the mutations are either nonsense/frameshift changes or involving the catalytic domains, and more likely to be hemizygous, leading to haploinsufficiency of TET2 function. In fact, *TET2*-mutated leukemic cells showed decreased 5hmC levels, although it is less clear whether *TET2*-mutated cells showed DNA hypermethylation.[32] *TET2*-deficient mice develop severe myeloproliferation, indicating the leukemogenic role of TET2 dysfunction.[33,34] Interestingly, a recent study indicated that TET2 is also involved in histone modification (O-GlcNAcylation) during gene transcription.[35]

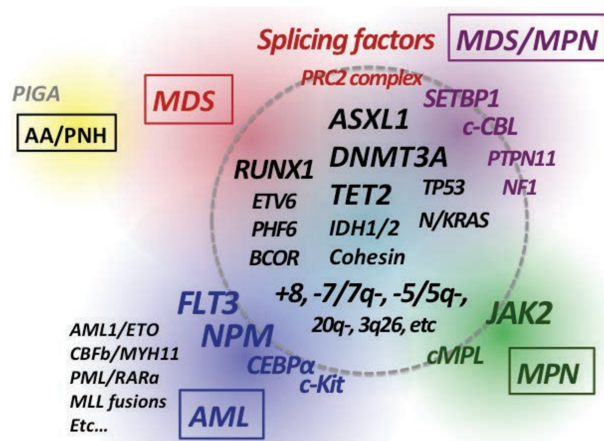

Figure 1. Spectrum of gene mutations in MDS and other myeloid neoplasms. More than 30 genes have been reported to be recurrently mutated in MDS. Most of these mutations, and other chromosomal lesions, such as -5/5q-, -7/7q-, 20q-, 13q-, 17p-, are also prevalent in other myeloid neoplasms, suggesting common mechanisms operate in myeloid leukemogenesis, although their frequencies show considerable variations depending on disease type. Some gene mutations are more specific to particular diseases, such as *JAK2* mutations in MPN, *NPM-1* and *FLT3* mutations in AML, splicing factor mutations in MDS and MDS/MPN.

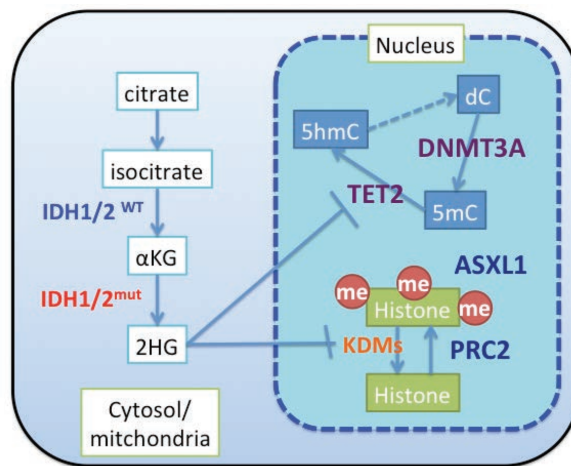

Figure 2. Mutations in epigenetic regulators. Mutation of epigenetic regulators is a common feature in human cancers. In MDS (and other myeloid neoplasms), major mutational targets include DNMT3A and TET2, which regulate DNA methylation, and components of PRC2 (EZH2, EED, SUZ12 and JARID2) and other polycomb-related proteins (ASXL1, BCOR), which are involved in gene repression through di/trimethylation of H3K27 and ubiquitination of H2A. Mutated IDH1/2 produces an oncometabolite, 2HG, which inhibits multiple αKG-dependent oxidases by competing with αKG, including TET2. These mutations are thought to deregulate expression of critical genes involved in epigenetic regulation of stem-cell functions, differentiation and cell cycle regulation of hematopoietic progenitors, leading to myeloid leukemogenesis. KDM: lysine(K) demethylase.

IDH1/2

Mutations of *IDH1* or *IDH2* were first documented in secondary glioma (approx. 85%) and *de novo* AML (approx. 15%), and were shown to be also common in MDS (approx. 10%).[7,36,37] The *IDH1* and *IDH2* genes encode isocitrate dehydrogenases that catalyze conversion of isocitrate to αKG in cytosol and mitochondria, respectively. Mutations exclusively involved R132 (IDH1) or R140/R172 (IDH2) sites, indicating that they represented gain-of-function mutations.[38] In fact, mutated enzymes catalyze conversion of αKG to 2 hydroxyglutarate (2HG), rather than isocitrate to αKG, leading to aberrant accumulation of the latter metabolite, which in turn inhibits many αKG-dependent oxidases, including TET2 protein (Figure 2).[39-41] In accordance with this, mutations of *TET2* and *IDH1/2* occurred in a largely mutually exclusive manner, although no *TET2* mutations have been reported in glioma and 2HG inhibits not only TET2 but also other oxidases such as KDMs, which also use αKG as a substrate.

DNMT3A

DNA methylation occurs at CpG sites, which is mediated by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B. The latter two are responsible for *de novo* DNA methylation, while DNMT1 takes care of maintenance methylation during DNA replication (Figure 2).[42] Mutations of DNMT3A were first reported in *de novo* AML via high-throughput sequencing of AML genomes almost exclusively involving M4/M5 subtypes,[6,43,44] but also found in approx. 10-15% of MDS and CMML and to a lesser extent MPN cases.[45,46] Most mutations are found in the C-terminal catalytic domain to abrogate DNMT activity and approximately two-thirds of mutations affected a highly conserved R822 residue. Conditional deletion of *Dnmt3a* in mouse bone marrow progressively impairs hematopoietic stem cell (HSC) differentiation over serial transplantations, while simultaneously expanding HSC numbers in the bone marrow, which is associated with upregulation of HSC multipotency genes and downregulation of differentiation factors.[47]

PRC2 and other polycomb-related genes

Polycomb group (PcG) proteins are engaged in diverse biological functions including differentiation, maintaining cell identity and proliferation, and stem-cell plasticity in a wide variety of organ systems, including hematopoiesis.[48] These diverse functions are mediated in large part by transcriptional repression, which is accomplished by co-ordinated functions of several discrete protein complexes, including polycomb repressive complex 1 (PRC1), PRC2 and PHO-repressive complex (PhoRC), and polycomb repressive deubiquitinase (PR-DUB).[49-51] PRC2 catalyzes methylation of histone H3 at lysine 27 (H3K27) to H3K27me3, to which PRC1 is thought to be recruited to mediate monoubiquitination of Lys 119 of histone H2A (H2AK119ub). PR-DUB, on the other hand, removes monoubiquitin from H2A via the ubiquitin carboxy-terminal hydrolase, BAP1, that binds to another component of PR-DUB, ASX in *Drosophila*.[50] In a subset of MDS, several components of PRC2 are mutated/deleted, including *EZH2* within del(7q) region,[52,53] and, less frequently, *EED, SUZ12* and *JARID2*, which were mutually exclusive among mutated cases.[54,55] *ASXL1* is a mammalian homolog of *ASX* and frequently mutated in MDS and other myeloid neoplasms at high frequencies (approx. 20-25%), although no mutation have been reported in *BAP1* in myeloid neoplasms. Even though no PRC1 components have been reported to be mutated, mutations of *BCOR* (and its homolog *BCORL1*), which is associated with some PCR1 components (RING1 and RNF2), are mutated in approximately 10% cases of AML and MDS.

Splicing factor mutations

The RNA splicing machinery is also a new class of mutational target in human cancers, which were unexpectedly discovered through whole exome sequencing of MDS and chronic lymphocytic leukemia (CLL).[4,8,56-62] Papaemmanuil *et al.* identified *SF3B1* mutations in 6 of the 8 discovery RARS cases with whole exome sequencing and this was confirmed in the subsequent large scale mutation analysis in MDS (72 of 354; 20%) but rare in other myeloid neoplasms, including AML (3 of 57; 5%), CML (0 of 53) and MPNs (12 of 420, 3%).[8] Moreover, Yoshida *et al.* analyzed 29 cases with different subtypes of myelodysplasia and identified mutations of multiple components of the RNA splicing machinery, including *SF3B1, U2AF35, SRSF2, ZRSR2, SF3A1* and *PRPF40B* in 16 cases.[4] Affecting at least 8 components of the RNA splicing machinery, mutations were found in 130 of 228 MDS (57%), 48 of 88 CMML (55%) and 16 of 62 sAML (25.8%) cases, but relatively rare in *de novo* AML (10 of 151; 6.6%) and MPNs (5 of 53; 9.4%).[4] These observations were confirmed in subsequent studies,[30,57,63-73] although splicing factor mutations seem to be rare in pediatric myeloid neoplasms including juvenile myelomonocytic leukemia.[74,75]

RNA splicing provides a basic cellular mechanism for expression of genetic information.[76,77] Common to all eukaryotes, it allows for generating a large diversity of protein species in the face of a limited set of genes by way of alternative splicing.[78] RNA splicing is accomplished by recruitment and disengagement of multiple small nuclear ribonucleoprotein particle (snRNP) complexes and other protein factors to newly transcribed pre-mRNA, through which exon-intron boundaries are recognized and intronic sequences were correctly spliced out to generate mature mRNA.[76] RNA splicing is initiated by the recognition of 5' splice site by a U1 snRNP complex, followed by the recruitment of a complex consisting of a U2AF35/65 heterodimer, ZRSR2 and an SR protein, such as SRSF1 and 2, and other factors to recognize the 3' splice site. Finally, a U2 snRNP complex replaces SF1 bound to the branch-point sequence with one of its subcomponent, SF3B1, to establish a splicing A complex (Figure 3).[76,77,79] Of particular note, most of the mutated splicing factors in MDS are involved in this complex, in which mutations are largely mutually exclusive, indicating that the common functional target of these mutations should be the 3' splice site recognition.[4]

Another conspicuous feature of splicing factor mutations is the presence of mutational hot spots in major mutational targets, including *SF3B1, SRSF2* and *U2AF35*. In *U2AF35*, the mutations almost exclusively involved highly conserved two amino acid positions, S34 and Q157, within the N- and C-terminal zinc finger domains, while almost all *SRSF2* mutations are missense changes at

P95 or deletions involving this amino acid position.[4,30,67,69] Less conspicuously but significantly, *SF3B1* mutations were confined to 5-7 amino acid positions within the domains corresponding to exons 12-16, of which approx. 50% of the mutations were accounted for by K700E.[4,8] No homozygous mutations have been reported for these three genes. The presence of hot spots and the absence of nonsense or frameshift changes strongly indicated that they could be associated with some gain-of-function rather than representing simple loss of functions. In contrast, mutations of *ZRSR2* on the X chromosome were distributed along the entire coding region.[4] Approximately two-thirds of mutations were either nonsense or frameshift changes, causing a premature stop codon.[4,30,69] The majority of the *ZRSR2* mutated cases were male in whom single mutations resulted in complete loss of functions.[4]

Compromised 3' splice site recognition seems to be a common consequence of different splicing factor mutations. In fact, forced expression of mutant U2AF35 alleles *in vitro* induces global defects in RNA splicing, including aberrant exon skipping and alternative exon usage, misrecognition of splice sites, especially in 3' splice sites, and increased intron retention.[4,56] However, no functional gene targets for abnormal splicing that explain MDS pathogenesis have yet been clarified. In addition, the presence of strong genotype/phenotype associations among different splicing factor mutations suggests discrete gene targets for different mutations, which is most prominent for *SF3B1* mutations and ring sideroblasts. *SF3B1* mutations were found in 68-82% of refractory anemia with ring sideroblasts (RSRS) and 57-76% of refractory cytopenia with ring sideroblasts (RCMD-RS).[4,8,57,64] Malcovati *et al.* reported that, regardless of disease type, *SF3B1* mutations strongly predicted the presence of ring sideroblasts with 97.7% and 98.7% of positive and negative predictive values, respectively, although the cases examined did not necessarily satisfy the criteria for RARS or RCMD-RS (*i.e.* >15% of all erythroblasts).[80] Less prominently, *SRSF2* mutations were more frequently found in CMML (30.7-47%) than in other subtypes of myeloid neoplasms.[4,67] Interestingly, *SF3B1* mutations, but not other splicing factor mutations, have also been reported in 5-15% of chronic lymphocytic leukemia (CLL), especially in high-risk cases.[58-62] In addition, *SF3B1* are mutated in several solid cancers, including breast, bladder, endometrial and other cancers, although the mutation frequencies were low.[8,81] These genotype-phenotype associations may reflect gene-specific functions of individual mutations. For example, *SF3B1* was shown to be essential for Hox gene regulation through functionally interacting with polycomb and trithorax genes.[82] SRSF2 have been also implicated in genetic stability and their defects could lead to hypermutability.[83]

Several reports described the clinical impact of splicing factor mutations. However, there seemed to be some discrepancies in their impact among different studies. Initial reports indicated a significantly better overall survival for *SF3B1* mutated cases compared to unmutated cases in MDS,[8,80] while other studies showed no significant impact of the mutations on survival.[64,69,73] *SRSF2* mutations were reported to be associated with poor prognosis in univariate analysis, but may not be an independent prognostic predictor.[64,69,73] Also, *U2AF35* mutations were associated with a poor prognosis or higher risk of progression to AML in univariate analysis in some series[56,84] but not in others. To clarify the precise impact of splicing factor mutations, a well-designed control study is needed.

Other mutational targets

Cohesin complex

Cohesin is a multimeric protein complex conserved across species and composed of four core subunits, i.e. SMC1, SMC3, RAD21 and STAG proteins, together with a number of regulatory molecules.[85,86] Forming a ring-like structure, cohesin is engaged in cohesion of sister chromatids during cell division, post-replicative DNA repair, and regulation of global gene expression through long-range *cis*-interactions. Germline mutations in cohesin components lead to congenital multisystem malformation syndromes known as Cornelia de Lange syndrome and Robert's syndrome. Mutations of cohesin components were first reported in colon cancer, glioblastoma, and other solid cancers,[87,88] and more recently revealed via whole genome/exome/target deep sequencing of AML and other myeloid malignancies[89] (Kon *et al.,* unpublished data, 2013) in which *STAG2* was most frequently mutated, followed by *RAD21, SMC1A,* and *SMC3.* Most of the *STAG2* and *RAD21* mutations were either nonsense or frameshift changes, while *SMC1A* and *SMC3* mutations are more likely to be missense changes. Combined, mutations of cohesin components are found in 10-15% of the cases with AML, MDS, and CMML, in a mutually exclusive manner. As for the leukemogenic mechanism of cohesin mutations, many cohesin-mutated cases showed completely normal kary-

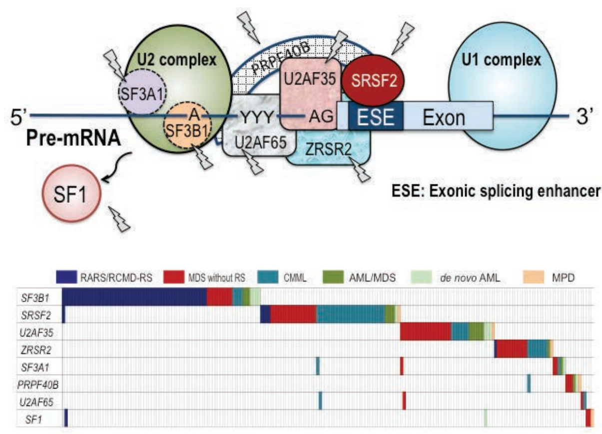

Figure 3. Pathway mutations in RNA splicing machinery. RNA splicing is initiated by the recognition of the 5' splice site by U1 snRNP complex. After the recognition of the 5' splice site, a protein complex consisting of a U2AF heterodimer, ZRSR2 and SRSF1/2 is recruited to the 3' splice boundary. SRSF1/2, members of the SR-family of proteins, bind to splicing enhancer sequences and also interact with other proteins through its SR domain. To the 5' upstream of the polypyrimidine tract lies a branchpoint sequence, to which another splicing factor, SF-1, binds and together with the U2AF heterodimer and other components, participates in the establishment of the splicing E complex. Once the splicing E complex is established, a U2 snRNP complex replaces SF-1 to generate the splicing A complex. Targets of gene mutation in MDS and other myeloid neoplasms are indicated by arrows.

otypes, suggesting that compromised cohesion of sister chromatids and consequent aneuploidy is not likely to play a major role, but deregulated expressional regulation and/or DNA hypermutability might be important for leukemogenesis.

SETBP1

SETBP1 is a newly identified target of somatic mutations in myeloid neoplasms, which are commonly mutated in all subtypes of MDS/MPN, including CMML, JMML and aCML, and also found in high-risk MDS and sAML, while rare in *de novo* AML cases[90] (Makishima *et al.* and Sakaguchi *et al.*, unpublished data, 2013). SETBP1 was initially identified as a 170 kD nuclear protein which binds to SET, a small protein inhibitor of putative tumor suppressors, PP2A and NM23-H1, and recently identified as the causative gene for Schinzel-Giedion syndrome (SGS), a highly recognizable congenital disease characterized by severe mental retardation, distinctive facial features, multiple congenital malformations and a higher-than-normal prevalence of tumors, typically neuroepithelial neoplasia.[91] Conspicuously, somatic mutations of *SETBP1* in myeloid neoplasms are identical to the hotspot germline mutations in SGS, including p.D686N/Y, p.G870S or p.I8781T, suggesting their gain-of-function nature. In fact, mutant *SETBP1*-transduced cells showed higher proliferation and less apoptosis than wild-type *SETBP1*-transduced cells which is thought to be due to increased protein stability. *SETBP1*-mutations were significantly associated with poor overall survivals.

Common and discrete gene mutations among different myeloid neoplasms

Although more than 30 recurrent mutations have been reported in MDS, only a handful of genes, including *TET2, SF3B1, SRSF2, ASXL1, RUNX1* and *DNMT3A,* are found in more than 10% of the cases, while dozens of other recurrent targets, which may or may not involve previously known pathways, are mutated at much lower frequencies. Many of these recurrent mutational targets in MDS, such as *TET2, IDH1/2, ASXL1, DNMT3A, RUNX1,* cohesin components and RAS pathway genes, are also commonly mutated in different myeloid neoplasms, suggesting that these mutations are involved in common pathophysiology among different myeloid neoplasms. In contrast, other gene mutations correlate well with specific phenotypes of myeloid neoplasms. For example, RNA splicing factor mutations are more specific to those subtypes showing myelodysplasia (MDS and MDS/MPN). A very strong association between *SF3B1* mutations and increased ring sideroblasts has already been mentioned. Mutations in *NPM1, IDH1/2, c-KIT* and *FLT3* are more common features of AML. Well-known gene fusions, such as, *CBF*-fusions, *PML/RAR* and *MLL*-fusions are almost pathognomonic of CBF leukemia, acute promyelocytic leukemia and MLL-leukemia, respectively. Similarly, *JAK2* gene mutations primarily characterize MPN and frequently co-exist with *SF3B1* mutation in RARS with thrombocytosis (RARS-T). RAS pathway mutations and *SETBP1* mutations are more typical features of MDS/MPN and RAEB subtypes and are associated with a high risk of transformation to secondary AML.

Intratumor heterogeneity and clonal evolution of MDS

Because MDS typically shows an indolent clinical course, frequently terminating in sAML, the clonological behavior of MDS in terms of relevant gene mutations, especially during progression to AML, is of particular importance in understanding the pathogenesis of MDS. In exome sequencing, on average approximately 10-15 non-silent mutations are detected per sample.[4,8] However, deep sequencing of these mutations discloses different allelic burdens of individual mutations, corresponding to a more complex clonal architecture showing multiple tumor subclones within the relevant tumor population. Such intratumor heterogeneity in MDS was best characterized by identifying large numbers of somatic mutations in clonologically obtained samples during evolution from MDS to sAML, followed by deep sequencing of each mutant alleles. Walter *et al.* demonstrated that MDS typically consists of multiple subclones at diagnosis, each of which was usually characterized by one or more driver mutations. Subsequent progression to sAML is dynamically shaped by multiple cycles of acquisition of mutations and other chromosomal lesions and by clonal selection.[92] Intratumor heterogeneity is also found in sAML, indicating that clonal evolution and selection persist even after progression to sAML. The process of clonal evolution/selection does not seem be totally random, but there could exist a hierarchy, if not very stringent, among common gene mutations with regard to their order. For example, mutations of splicing factors, *TET2* and *IDH1/2* tend to show highest allelic burdens, whilst RAS pathway mutations frequently show lower relative allele frequencies, indicating that the former could represent founder mutations involved in the initiation of MDS, while the latter mutations are more likely to be involved in disease progression (Figure 4). Thus, such a hierarchy of gene mutations could be closely related to the biological mechanism of disease progression and would be relevant to molecular diagnosis and prognostica-

Figure 4. Other gene mutations. Intratumor heterogeneity in terms of genetic lesions is quite common in MDS from diagnosis, in which the tumor population consists of multiple subclones derived from different progenies of ancestral/founder clones. These substructures of tumor population are generated by acquisition of new genetic lesions (such as mutations and chromosomal lesions) and subsequent clonal selection/expansion, leading ultimately to sAML. The order of gene mutations to be selected does not seem to be totally random, but selection of mutations shows a trend, in which some mutations tend to precede others in a hierarchical manner.

tion of clinical outcomes in MDS, although the exact order/role of all gene mutations in such a hierarchy has not been fully clarified.

Conclusion

Over the past ten years, our knowledge about the molecular genetics of MDS has been dramatically improved through identification of the major targets of gene mutations. Although spliceosome and other mutations are more prevalent in myelodysplasia, the majority of gene mutations are commonly found in other types of myeloid malignancies, suggesting a common molecular pathogenesis of myeloid neoplasms. Therefore, to understand the molecular pathogenesis of MDS, we have to clarify the commonality of the impact of these mutations on myeloid leukemogenesis, together with the molecular mechanism that explains MDS-specific pathogenesis. Frequent presence of intratumor heterogeneity and a dynamic temporal behavior seems to be an intrinsic feature of MDS, and an understanding of this would be indispensable not only to clarify the pathogenesis of MDS and transformation to sAML, but also for the development of better MDS diagnostics and therapeutics.

References

1. Corey SJ, Minden MD, Barber DL, Kantarjian H, Wang JC, Schimmer AD. Myelodysplastic syndromes: the complexity of stem-cell diseases. Nat Rev Cancer. 2007;7:118-29.
2. Brunning R, Orazi A, Germing U, Le Beau M, Porwit A, Baumann I, et al. Myelodysplastic Syndromes. Lyon: IARC Press; 2008.
3. Ley TJ, Mardis ER, Ding L, Fulton B, McLellan MD, Chen K, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature. 2008;456:66-72.
4. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. Nature. 2011;478:64-69.
5. Meyerson M, Gabriel S, Getz G. Advances in understanding cancer genomes through second-generation sequencing. Nat Rev Genet. 2010;11:685-96.
6. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010;363:2424-33.
7. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med. 2009;361: 1058-66.
8. Papaemmanuil E, Cazzola M, Boultwood J, Malcovati L, Vyas P, Bowen D, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. N Engl J Med. 2011;365:1384-95.
9. Hirai H, Kobayashi Y, Mano H, Hagiwara K, Maru Y, Omine M, et al. A point mutation at codon 13 of the N-ras oncogene in myelodysplastic syndrome. Nature. 1987;327:430-2.
10. Song WJ, Sullivan MG, Legare RD, Hutchings S, Tan X, Kufrin D, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat Genet. 1999;23:166-75.
11. Osato M, Asou N, Abdalla E, Hoshino K, Yamasaki H, Okubo T, et al. Biallelic and heterozygous point mutations in the runt domain of the AML1/PEBP2alphaB gene associated with myeloblastic leukemias. Blood. 1999;93:1817-24.
12. Sugimoto K, Hirano N, Toyoshima H, Chiba S, Mano H, Takaku F, et al. Mutations of the p53 gene in myelodysplastic syndrome (MDS) and MDS-derived leukemia. Blood. 1993;81:3022-6.
13. Lyons J, Janssen JW, Bartram C, Layton M, Mufti GJ. Mutation of Ki-ras and N-ras oncogenes in myelodysplastic syndromes. Blood. 1988;71:1707-12.
14. Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, Szpurka H, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res. 2008;68:10349-57.
15. Haferlach C, Bacher U, Haferlach T, Dicker F, Alpermann T, Kern W, et al. The inv(3)(q21q26)/t(3;3)(q21;q26) is frequently accompanied by alterations of the RUNX1, KRAS and NRAS and NF1 genes and mediates adverse prognosis both in MDS and in AML: a study in 39 cases of MDS or AML. Leukemia. 2011;25:874-7.
16. Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, Yamazaki S, et al. Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. Nature. 2009;460:904-8.
17. Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet. 2003;34:148-50.
18. Lorenzo F, Nishii K, Monma F, Kuwagata S, Usui E, Shiku H. Mutational analysis of the KIT gene in myelodysplastic syndrome (MDS) and MDS-derived leukemia. Leuk Res. 2006;30:1235-9.
19. Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadleigh M, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood. 2006;108:3472-6.
20. Steensma DP, Dewald GW, Lasho TL, Powell HL, McClure RF, Levine RL, et al. The JAK2 V617F activating tyrosine kinase mutation is an infrequent event in both "atypical" myeloproliferative disorders and myelodysplastic syndromes. Blood. 2005;106:1207-9.
21. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001;97:2434-9.
22. Bejar R, Stevenson K, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, et al. Clinical effect of point mutations in myelodysplastic syndromes. N Engl J Med. 2011;364:2496-506.
23. Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. CEBPA point mutations in hematological malignancies. Leukemia. 2005;19:329-34.
24. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer. 2012;12:599-612.
25. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet. 2009;41:838-42.
26. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009;360:2289-301.
27. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;324:930-5.
28. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, et al. Tet-Mediated Formation of 5-Carboxylcytosine and Its Excision by TDG in Mammalian DNA. Science. 2011; 333(6047):1303-7.
29. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, et al. Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine. Science. 2011; 333(6047):1300-3.
30. Damm F, Kosmider O, Gelsi-Boyer V, Renneville A, Carbuccia N, Hidalgo-Curtis C, et al. Mutations affecting mRNA splicing define distinct clinical phenotypes and correlate with patient outcome in myelodysplastic syndromes. Blood. 2012;119:3211-8.
31. Smith AE, Mohamedali AM, Kulasekararaj A, Lim Z, Gaken J, Lea NC, et al. Next-generation sequencing of the TET2 gene in 355 MDS and CMML patients reveals low-abundance mutant clones with early origins, but indicates no definite prognostic value. Blood. 2010;116:3923-32.
32. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature. 2010;468:839-43.
33. Moran-Crusio K, Reavie L, Shih A, Abdel-Wahab O, Ndiaye-Lobry D, Lobry C, et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell. 2011;20:11-24.
34. Quivoron C, Couronne L, Della Valle V, Lopez CK, Plo I, Wagner-Ballon O, et al. TET2 Inactivation Results in Pleiotropic Hematopoietic Abnormalities in Mouse and Is a Recurrent Event during Human Lymphomagenesis. Cancer Cell. 2011;20:25-38.

35. Chen Q, Chen Y, Bian C, Fujiki R, Yu X. TET2 promotes histone O-GlcNAcylation during gene transcription. Nature. 2013;493:561-4.
36. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008;321:1807-12.
37. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. N Engl J Med. 2009;360:765-73.
38. Dang L, Jin S, Su SM. IDH mutations in glioma and acute myeloid leukemia. Trends Mol Med. 2010;16:387-97.
39. Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell. 2011;19:17-30.
40. Chowdhury R, Yeoh KK, Tian YM, Hillringhaus L, Bagg EA, Rose NR, et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. EMBO Rep. 2011; 12:463-9.
41. Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, Abdel-Wahab O, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. Nature. 2012;483:474-8.
42. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer. 2003;3:253-66.
43. Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, et al. Array-based genomic resequencing of human leukemia. Oncogene. 2010;29:3723-31.
44. Yan XJ, Xu J, Gu ZH, Pan CM, Lu G, Shen Y, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet. 2011;43:309-15.
45. Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, et al. Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. Leukemia. 2011;25:1153-8.
46. Jankowska AM, Makishima H, Tiu RV, Szpurka H, Huang Y, Traina F, et al. Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: UTX, EZH2 and DNMT3A. Blood. 2011;118:3932-41.
47. Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet. 2012;44:23-31.
48. Saunthararajah Y, Maciejewski J. Polycomb segment myeloid malignancies. Blood. 2012;119:1097-8.
49. Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. Nature. 2011;469:343-9.
50. Scheuermann JC, de Ayala Alonso AG, Oktaba K, Ly-Hartig N, McGinty RK, Fraterman S, et al. Histone H2A deubiquitinase activity of the Polycomb repressive complex PR-DUB. Nature. 2010;465:243-7.
51. Martin-Perez D, Piris MA, Sanchez-Beato M. Polycomb proteins in hematologic malignancies. Blood. 2010;116:5465-75.
52. Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet. 2010;42:722-6.
53. Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tonnissen ER, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. Nat Genet. 2010;42:665-7.
54. Ueda T, Sanada M, Matsui H, Yamasaki N, Honda ZI, Shih LY, et al. EED mutants impair polycomb repressive complex 2 in myelodysplastic syndrome and related neoplasms. Leukemia. 2012; 26(12):2557-60.
55. Score J, Hidalgo-Curtis C, Jones AV, Winkelmann N, Skinner A, Ward D, et al. Inactivation of polycomb repressive complex 2 components in myeloproliferative and myelodysplastic/myeloproliferative neoplasms. Blood. 2012;119:1208-13.
56. Graubert TA, Shen D, Ding L, Okeyo-Owuor T, Lunn CL, Shao J, et al. Recurrent mutations in the U2AF1 splicing factor in myelodysplastic syndromes. Nat Genet. 2012;44:53-7.
57. Visconte V, Makishima H, Jankowska A, Szpurka H, Traina F, Jerez A, et al. SF3B1, a splicing factor is frequently mutated in refractory anemia with ring sideroblasts. Leukemia. 2012;26:542-5.
58. Rossi D, Bruscaggin A, Spina V, Rasi S, Khiabanian H, Messina M, et al. Mutations of the SF3B1 splicing factor in chronic lymphocytic leukemia: association with progression and fludarabine-refractoriness. Blood. 2011;118:6904-8.
59. Wang L, Lawrence MS, Wan Y, Stojanov P, Sougnez C, Stevenson K, et al. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. N Engl J Med. 2011;365:2497-506.
60. Damm F, Nguyen-Khac F, Fontenay M, Bernard OA. Spliceosome and other novel mutations in chronic lymphocytic leukemia, and myeloid malignancies. Leukemia. 2012;26:2027-31.
61. Quesada V, Conde L, Villamor N, Ordonez GR, Jares P, Bassaganyas L, et al. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. Nat Genet. 2012;44:47-52.
62. Rossi D, Rasi S, Spina V, Fangazio M, Monti S, Greco M, et al. Different impact of NOTCH1 and SF3B1 mutations on the risk of chronic lymphocytic leukemia transformation to Richter syndrome. Br J Haematol. 2012;158:426-9.
63. Cui R, Gale RP, Xu Z, Qin T, Fang L, Zhang H, et al. Clinical importance of SF3B1 mutations in Chinese with myelodysplastic syndromes with ring sideroblasts. Leuk Res. 2012;36:1428-33.
64. Damm F, Thol F, Kosmider O, Kade S, Loffeld P, Dreyfus F, et al. SF3B1 mutations in myelodysplastic syndromes: clinical associations and prognostic implications. Leukemia. 2012;26:1137-40.
65. Jeromin S, Haferlach T, Grossmann V, Alpermann T, Kowarsch A, Haferlach C, et al. High frequencies of SF3B1 and JAK2 mutations in refractory anemia with ring sideroblasts associated with marked thrombocytosis strengthen the assignment to the category of myelodysplastic/myeloproliferative neoplasms. Haematologica. 2012; 98(2):e15-e17.
66. Lasho TL, Finke CM, Hanson CA, Jimma T, Knudson RA, Ketterling RP, et al. SF3B1 mutations in primary myelofibrosis: clinical, histopathology and genetic correlates among 155 patients. Leukemia. 2012;26:1135-7.
67. Meggendorfer M, Roller A, Haferlach T, Eder C, Dicker F, Grossmann V, et al. SRSF2 mutations in 275 cases with chronic myelomonocytic leukemia (CMML). Blood. 2012;120(15):3080-8.
68. Patnaik MM, Lasho TL, Hodnefield JM, Knudson RA, Ketterling RP, Garcia-Manero G, et al. SF3B1 mutations are prevalent in myelodysplastic syndromes with ring sideroblasts but do not hold independent prognostic value. Blood. 2012;119:569-72.
69. Thol F, Kade S, Schlarmann C, Loffeld P, Morgan M, Krauter J, et al. Frequency and prognostic impact of mutations in SRSF2, U2AF1, and ZRSR2 in patients with myelodysplastic syndromes. Blood. 2012;119(15):3578-84.
70. Visconte V, Rogers HJ, Singh J, Barnard J, Bupathi M, Traina F, et al. SF3B1 haploinsufficiency leads to formation of ring sideroblasts in myelodysplastic syndromes. Blood. 2012;120(16):3173-86.
71. Wu SJ, Kuo YY, Hou HA, Li LY, Tseng MH, Huang CF, et al. The clinical implication of SRSF2 mutation in patients with myelodysplastic syndrome and its stability during disease evolution. Blood. 2012;120(15):3106-11.
72. Zhang SJ, Rampal R, Manshouri T, Patel J, Mensah N, Kayserian A, et al. Genetic analysis of patients with leukemic transformation of myeloproliferative neoplasms shows recurrent SRSF2 mutations that are associated with adverse outcome. Blood. 2012;119:4480-5.
73. Bejar R, Stevenson KE, Caughey BA, Abdel-Wahab O, Steensma DP, Galili N, et al. Validation of a Prognostic Model and the Impact of Mutations in Patients With Lower-Risk Myelodysplastic Syndromes. J Clin Oncol. 2012;30:3376-82.
74. Hirabayashi S, Flotho C, Moetter J, Heuser M, Hasle H, Gruhn B, et al. Spliceosomal gene aberrations are rare, coexist with oncogenic mutations, and are unlikely to exert a driver effect in childhood MDS and JMML. Blood. 2012;119:e96-e99.
75. Takita J, Yoshida K, Sanada M, Nishimura R, Okubo J, Motomura A, et al. Novel splicing-factor mutations in juvenile myelomonocytic leukemia. Leukemia. 2012;26:1879-81.
76. Wahl MC, Will CL, Luhrmann R. The spliceosome: design principles of a dynamic RNP machine. Cell. 2009;136:701-18.
77. Bonnal S, Vigevani L, Valcarcel J. The spliceosome as a target of novel antitumour drugs. Nat Rev Drug Discov. 2012;11:847-59.
78. Chen M, Manley JL. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. Nat Rev Mol Cell Biol. 2009;10:741-54.
79. Tronchere H, Wang J, Fu XD. A protein related to splicing factor U2AF35 that interacts with U2AF65 and SR proteins in splicing of pre-mRNA. Nature. 1997;388:397-400.
80. Malcovati L, Papaemmanuil E, Bowen DT, Boultwood J, Della Porta MG, Pascutto C, et al. Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms. Blood.

2011;118:6239-46.
81. Ellis MJ, Ding L, Shen D, Luo J, Suman VJ, Wallis JW, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature. 2012;486:353-60.
82. Isono K, Mizutani-Koseki Y, Komori T, Schmidt-Zachmann MS, Koseki H. Mammalian polycomb-mediated repression of Hox genes requires the essential spliceosomal protein Sf3b1. Genes Dev. 2005;19:536-41.
83. Xiao R, Sun Y, Ding JH, Lin S, Rose DW, Rosenfeld MG, et al. Splicing regulator SC35 is essential for genomic stability and cell proliferation during mammalian organogenesis. Mol Cell Biol. 2007;27:5393-402.
84. Makishima H, Visconte V, Sakaguchi H, Jankowska AM, Abu Kar S, Jerez A, et al. Mutations in the spliceosome machinery, a novel and ubiquitous pathway in leukemogenesis. Blood. 2012;119:3203-10.
85. Gruber S, Haering CH, Nasmyth K. Chromosomal cohesin forms a ring. Cell. 2003;112:765-77.
86. Nasmyth K, Haering CH. Cohesin: its roles and mechanisms. Annu Rev Genet. 2009;43:525-58.
87. Solomon DA, Kim T, Diaz-Martinez LA, Fair J, Elkahloun AG, Harris BT, et al. Mutational inactivation of STAG2 causes aneuploidy in human cancer. Science. 2011;333:1039-43.
88. Strom L, Karlsson C, Lindroos HB, Wedahl S, Katou Y, Shirahige K, et al. Postreplicative formation of cohesion is required for repair and induced by a single DNA break. Science. 2007;317:242-5.
89. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, et al. The origin and evolution of mutations in acute myeloid leukemia. Cell. 2012;150:264-78.
90. Piazza R, Valletta S, Winkelmann N, Redaelli S, Spinelli R, Pirola A, et al. Recurrent SETBP1 mutations in atypical chronic myeloid leukemia. Nat Genet. 2012;45:18-24.
91. Hoischen A, van Bon BW, Gilissen C, Arts P, van Lier B, Steehouwer M, et al. De novo mutations of SETBP1 cause Schinzel-Giedion syndrome. Nat Genet. 2010;42:483-5.
92. Walter MJ, Shen D, Ding L, Shao J, Koboldt DC, Chen K, et al. Clonal architecture of secondary acute myeloid leukemia. N Engl J Med. 2012;366:1090-8.

Diagnosis and biomarkers in myelodysplastic syndromes

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A B S T R A C T

Myelodysplastic syndromes (MDS) is a heterogeneous group of clonal myeloid disorders characterized by cytopenia, bone marrow dysplasia and a high risk for disease progression. The marked variation in clinical presentation sets high demands on the diagnostic process and on the possibility of predicting general and treatment-specific outcomes. While some patients experience a close to normal life expectancy without therapy, others are up-front candidates for allogeneic stem cell transplantation. This review describes the currently recommended diagnostic and prognostic systems and looks at newer tools with potential value for certain patient groups. The World Health Organization (WHO) classification is mandatory as a basis on which to evaluate the impact of additional methods. The rapid development of next generation sequencing has made it possible to detect a large number of somatic mutations, some of which are likely to add significantly to the understanding of MDS, and provide important tools for clinical decision-making. Most of these mutations, however, need further evaluation before introduction into current prognostic models. Age and concurrent non-hematologic disorders are also important outcome variables, and co-morbidity scores are introduced to help assess patients evaluated for therapeutic intervention.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe state-of-the-art diagnostic procedures for MDS;
- apply standard prognostic assessment for younger and older patients with MDS of all risk groups;
- discuss currently available molecular markers as tools for clinical management in MDS;
- discuss co-morbidity scores as tools for clinical management in MDS.

Introduction

The myelodysplastic syndromes (MDS) constitute a heterogeneous group of myeloid malignancies originating in the hematopoietic stem cell compartment of the bone marrow. The common clinical features, which allow MDS to be put together into one category of hematologic disorders, are cytopenia, morphological dysplasia of bone marrow precursors, and an increased risk for disease progression to more advanced MDS or to overt acute myeloid leukemia (AML). The current WHO classification is based on these features and, to a limited degree, on cytogenetic findings.[1] However, several new molecular lesions and aberrant pathways have recently been identified in MDS and it is likely that some of these will influence the diagnostic process within the next few years. Also, it may be anticipated that the use of biomarkers for diagnosis and prognosis, and for predicting outcome of specific treatment options will be introduced as a part of the basic investigation of MDS. How these newly identified mutations will influence existing classification and prognostic systems still have to be worked out, but without doubt, this will require considerable time, effort and large patient cohorts. Hence, for a number of years it will be necessary to adhere to the current systems and learn how to use individual new biomarkers as additive knowledge. Variables with impact on the overall outcome for patients with MDS are described here, while those influencing therapeutic strategies are mainly described in the paper "Risk-adapted treatment of myelodysplastic syndromes" by Dr Guillermo Sanz.

Diagnosis of myelodysplastic syndromes

Diagnosis as a result of multi-professional consensus

Myelodysplastic syndromes is considered by many to be a difficult diagnosis to make. There is no typical malignant cell population to identify, but rather more or less clear dysplasia of many cell types in the bone marrow and alterations in their relative frequencies. In order to classify a patient according to the WHO classification, clinical information is essential.[1] A detailed family history is required to exclude inherited bone marrow failures, such as telomere disorders and MDS secondary to familial platelet disorders and germ-line RUNX-1 mutations.[2,3] In order to divide patients into primary and therapy-related

MDS, the previous history of chemotherapy and radiation therapy is essential. Cytogenetics, morphology, histopathology, and to an increasing degree multiparameter flow cytometry, are cornerstones in the diagnostic process. In particular, the distinction between secondary cytopenia due to non-malignant disease and low-risk MDS with mild cytopenia and no ring sideroblasts requires good collaboration between all disciplines. It could be detrimental for an individual to be wrongly diagnosed with a malignant disorder. Table 1 summarizes the recommended diagnostic procedure in MDS.

World Health Organization classification: morphology and histopathology

The World Health Organization (WHO) classification constitutes the gold standard for diagnosis of MDS and mixed MDS/myeloproliferative neoplasms (MDS/MPN) and should serve as a basis, even when additional diagnostic and prognostic procedures are implemented.[1] The classification is built on three main procedures; morphology of blood and bone marrow smears, a bone marrow biopsy, and cytogenetics. The morphological examination of blood and bone marrow smears stained with May-Grünvald-Giemsa and iron staining should include a thorough evaluation of the degree of dysplasia within the respective hematopoietic lineages in order to distinguish between uni- and multilineage dysplasia. The evaluation of dysplastic features has recently been updated by an international working group.[4] A bone marrow biopsy at diagnosis is mandatory in order to assess megakaryocyte morphology, cellularity, and fibrosis. Hypoplastic MDS is a condition that may be difficult to distinguish from aplastic anemia and which usually requires several biopsies including immunohistochemistry for $CD34^+$ expression before a final diagnosis could be made.[5,6] Moderate to severe fibrosis is a validated marker for poor prognosis.[7,8] It is clinically relevant to distinguish hypoplastic from normo/-hyperplastic or fibrotic MDS, since treatment approaches for these entities may differ. In addition, the histopathological material allows assessment of pathological bone marrow topography, such as abnormal localization of immature progenitors (ALIPs).[9] The biopsy specimen is also useful for assessing immunohistochemistry, such as CD34 and CD117 expression, which could act as a surrogate marker for blasts and immature erythropoiesis, when the aspiration material is insufficient. Immune histopathology for specific protein markers, such as p53, has also a distinct prognostic value, as will be further discussed below.

Cytogenetics

A cytogenetic analysis of bone marrow aspirate should be performed in all patients with suspected MDS. At least 20 metaphases should be analyzed and described according to the International System for Human Cytogenetic Nomenclature (ISCN).[10] Importantly, certain karyotypic abnormalities, including but not confined to those involving chromosome 5, 7 and 17, can be sufficient to establish an MDS diagnosis in cases with persistent cytopenia of undetermined significance.[11] This is particularly important in younger patients who have been treated with chemotherapy and or irradiation and in whom a diagnosis of therapy-related MDS may lead to a rapid donor search for allogeneic stem cell transplantation (allo-SCT). The value of chromosomal analysis has been well established for more than two decades and cytogenetics constitutes a cornerstone in all current diagnostic and prognostic scoring systems. It is, therefore, important to include a chromosomal analysis in the workup of all patients with suspected MDS. The non-interventional prospective clinical European Union registry EU MDS will make it possible to follow up how this works in clinical practice.[12]

Fluorescence in situ hybridization

If the cytogenetic analysis fails due to absent or too few metaphases, specific questions about numeric abnormalities or loss of part of a chromosome may be addressed by interphase Fluorescence *in situ* hybridization (FISH) analysis.[13] For example, this may be the case in hypoplastic or severely fibrotic MDS. FISH has also been used to complement conventional cytogenetics when the karyotype is complex, but it is questionable whether this will remain an indication with genetic screening becoming more common. Balanced translocations are uncommon in MDS and are rarely investigated with this technique. Current prognostic scoring systems are based on conventional cytogenetics and FISH should not be regarded as a substitution for this method.[14] However, it may sometimes be indicated to ask specific questions such as the absence of chromosome 7 or chromosome location 5q.31 in cases where these specific MDS subtypes are suspected. FISH to detect targeted chromosomal abnormalities in interphase nuclei is recommended in the case of failure of standard G-banding (L Malcovati *et al.,* Diagnosis and treatment of primary myelodysplastic syndromes in adults. Recommendations from the European LeukemiaNet. Submitted paper).

Immunophenotyping by multiparametric flow cytometry

Flow cytometry allows the malignant MDS clone to be described in more detail, but the value of this technique has for a long time been hampered by considerable methodological variance between countries and individual centers. Standardization has markedly improved due to the ambitious process driven by the International Flow Cytometry Working Group within the European Leukemia Network, which over the past years has published several consensus papers on this topic. Standard methods as well as recommended combinations of antibodies for flow cytometry analysis of dysplasia in MDS have been established.[15-19]

Flow cytometry may evolve as a clinical tool to distinguish between low-risk MDS and non-malignant cytopenia, in cases when assessment of morphological dysplasia is difficult and no markers for clonality can be detected. By contrast, flow cytometry has limited value in the diagnostic process when a significant number of ring sideroblasts have been detected by morphology or when an aberrant karyotype has been confirmed.[19] The European LeukemiaNet Working Group describes surface markers, which in combination allow a detailed assessment of neutrophils, monocytes, myeloid progenitors and, to some extent, also erythroid progenitors[16] (Table 2). Hence, flow cytometry using these standardized protocols may distinguish non-sideroblastic anemia with normal karyotype from cytopenia of non-malignant origin. Furthermore, flow cytometry may be helpful in the assessment of high-risk MDS, both as a tool to quantify $CD34^+$ progenitors,

Table 1. Diagnostic procedure in myelodysplastic syndromes.

Methodology	Diagnostic value	Priority
Peripheral blood smear	Number of dysplastic lineages Percentage of blasts Number of monocytes	Mandatory
Bone marrow aspiration	Number of dysplastic lineages Percentage of blasts Percentage of ring sideroblasts	Mandatory
Bone marrow biopsy	Cellularity, fibrosis Immunohistochemistry (CD34) P53 staining*	Mandatory Recommended[1] Recommended[1]
Flow cytometry	Distinction between MDS and non-clonal cytopenia CD34+ assessment MRD assessment	Recommended[1] Recommended[1] Recommended[1]
Cytogenetic analysis	Chromosomal aberrations	Mandatory
FISH analysis	Specific chromosome re-arrangements	Recommended1
SNP arrays	Detailed analysis of chromosome regions	Not validated[2]
Sequencing	Mutations in candidate genes	In specific cases[3]

*[1]Recommended refers to the specific situations described in this review. [2]May be useful in certain situations and when used with experience. [3]Specific mutations may be very useful to predict prognosis and outcome to treatment. Not dependent on specific technology.*In MDS with karyotype including del(5q).*

Table 2. Proposed markers for flow cytometry analysis of dysplasia in MDS.[16]

General markers	Erythroid lineage	Hematopoietic progenitors	Maturing neutrophils	Monocyte lineage
CD45	CD45	CD45	CD45	CD45
	CD71			
	CD235a			
CD34		CD34	CD34	CD34
CD117	CD117	CD117	CD117	CD117
HLA-DR		HLA-DR	HLA-DR	HLA-DR
CD11b		CD11b	CD11b	CD11b
CD13		CD13	CD13	CD13
CD16			CD16	CD16
CD33			CD33	CD33
CD14			CD14	CD14
	CD36			CD36
			CD64	CD64
CD7		CD7		
CD56		CD56	CD56	CD56
CD19		CD19		
		CD5		
				CD2
		CD15	CD15	
			CD10	

and to detect aberrant expression of high-risk markers, such as CD7 on marrow blasts.[20]

A limitation of flow cytometry is when the bone marrow aspiration is diluted by blood, which may falsely reduce the relative numbers of immature cells. This is particularly common in patients with significant fibrosis, and in these cases immunohistopathology with staining for the same markers may be more useful.

Molecular genetics as part of the diagnostic process

The WHO classification does not recommend the use of specific mutational analyses in the workup of patients with suspected MDS or MDS/MPN. The long list of recently identified driver mutations that will be discussed below are yet to be evaluated in relation to the conventional diagnostic methods. In this review, gene mutations are, therefore, described with regard to their impact on prognosis rather than in their role in the diagnostic process. The only method that so far has been considered as an aid in the diagnostic process is single-nucleotide polymorphisms (SNP) that may be used for high-resolution genome-wide characterization of chromosomal aberrations without metaphase generation.[21] SNP array-based karyotyping has been applied in a range of studies in patients with MDS and other hematologic malignancies and may be used as a tool to identify chromosomal defects that are not detected by standard cytogenetics, or when karyotyping fails, such as in hypoplastic MDS and MDS with fibrosis.[22] The usefulness of this technique has been implicated in the distinction between hypoplastic MDS and aplastic anemia.[24] SNP arrays, however, do not provide the same level of detail as exome analysis or targeted sequencing and would need the same kind of standardization work that was necessary for flow cytometry to be clinically useful in a broad sense.

The advantages and disadvantages of the 2008 WHO classification of myelodysplastic syndromes

The most recent version of the WHO classification was published in 2008[1,11] and included a few major and several minor changes compared to the 2001 version. A major change was that the WHO 2001 category ‘refractory anemia’ (RA) with unilineage dysplasia was substituted by the broader category ‘refractory cytopenia with unilineage dysplasia’ (RCUD). This in turn is made up of three subcategories; RA, refractory neutropenia (RN), and refractory thrombocytopenia (RT). This made sense since many patients with isolated cytopenia do not have anemia or erythroid dysplasia. A diagnosis of RA may be difficult to separate from that of secondary anemia, and it may be difficult to differentiate RN and RT from immune-mediated neutropenia and thrombocytopenia, respectively, with normal karyotypes. In such cases, and in particular when the cytopenia is moderate, it is wise to wait and observe the course of the disease. Also, in these situations, flow cytometry may add significant diagnostic value. Moreover, the finding of recurrent mutations will probably add to the understanding of these borderline conditions.

The rapid development of molecular genetics during the last few years has already challenged some of the changes implemented in the WHO 2008 classification. The 2008 edition fused refractory cytopenia with multilineage dysplasia with or without ring sideroblasts into one category (RCMD). However, in 2011, a European consortium identified mutations in *SF3B1*, a core component in the spliceasome, in the vast majority of patients with MDS and ring sideroblasts, while finding these mutations in less than 10% of other types of MDS.[24,25] *SF3B1* mutations were significantly associated with the presence of ring sideroblasts and a typical gene expression pattern with underexpression of genes in the mitochondrial pathway. Hence, RCMD and RCMD-RS should probably remain two different MDS entities. Moreover, the MDS/MPN subcategory RARS with marked thrombocytosis, RARS-T, also carries *SF3B1* mutations. These most likely occur before the acquisition of *JAK2* mutations and make this category more similar to RARS and RCMD-RS than the other mixed MDS/MPN.[26]

Another less useful change was to put therapy-related MDS and AML together into one group, ‘therapy-related myeloid neoplasms’ without requiring a more detailed diagnosis of these patients with regard to cytogenetics and morphology. For AML, it has been convincingly demonstrated in large patient series that the value of specific chromosomal aberrations is retained, even in therapy-related AML.[27-29] Similar large MDS cohorts are not available, but several smaller studies show that therapy-related MDS respond as well to treatment with azacytidine as primary MDS, with the same cytogenetic pattern.[30,31] By analogy, the cytogenetic risk groups but not the division into primary and therapy-related MDS, showed impact on outcome in a large retrospective analysis of MDS patients undergoing SCT.[32]

Pediatric myelodysplastic syndromes

Myelodysplastic syndromes in children constitutes a rare but distinct entity, and since many of these children are candidates for curative treatment with allo-SCT, a correct and early diagnosis is essential. Childhood MDS cases are found in three different chapters in the WHO 2008 classification; juvenile myelomonocytic leukemia (JMML) in the mixed MDS/MPN category, RAEB 1 and 2 in the main MDS category for adult MDS, and refractory cytopenia of childhood (RCC) in a separate entity.[1] RCC often presents with a hypoplastic bone marrow and is sometimes treated with immunosuppression. In JMML, the current spectrum of commonly occurring mutations in *NF1, RAS*, *PTPN11* and *CBL* converge on the Ras/MAPK pathway, which has also emerged as a potential target for treatment.[33] The most common cytogenetic abnormality in this subgroup is monosomy 7.[1] Generally, children with MDS are more often treated upfront with allo-SCT than adult patients, and the clinical response and tolerance to chemotherapy differs from that of adult MDS. This review has no ambition to cover this complex and interesting topic, but very young adults with MDS may be more like children than elderly patients, so specific investigation and consultation with a pediatric MDS specialist may be advisable.

Prognostic assessment in myelodysplastic syndromes

The clinical course of MDS varies from indolent, with life expectancy similar to that of age-matched healthy individuals, to rapidly progressive myeloid disease, similar to AML.[34] It is, therefore, important to apply risk-adapted strategies, first by evaluating the prognosis without treatment, and then for deciding about the optimal choice of therapy. These categories of prognostic factors may overlap.

Prognostic systems developed for untreated patients

The current prognostic systems are based on degree of cytopenia, percentage of myeloblasts in the bone marrow, and cytogenetics, and are derived from patients who have not received disease-modifying approaches. Karyotype was first introduced in risk models for MDS by the Group Francophone des Myélodysplasies (GFM) and subsequently in the IPSS risk model from 1997.[14,35] The original IPSS divided karyotypes into three risk groups; low-risk (normal karyotype, -Y, and isolated del(5q) or del(20q)), high-risk (complex as defined as three or more aberrancies, monosomy 7 and del(7q)), and intermediate (any other abnormalities). This system was recently improved by a series of large investigations including over 2000 patients from the German-Austrian group.[36-38] First, the patient cohort was used to demonstrate the prognostic relevance of a more detailed subgrouping than in the previous IPSS system.[36] Then, in collaboration with the MD Anderson Cancer Center, the group showed that cytogenetics was underweighted as a prognostic marker in relation to marrow blast percentage and cytopenia.[37] Finally, the IPSS revision group published a new cytogenetic scoring system including five risk groups.[38] This scoring system was subsequently incorporated into the revised IPSS (IPSS-R) including over 7000 patients[39] (Table 3). Major advantages of the revised cytogenetic scoring system are: i) a subset of patients with more favorable prognosis has been defined; ii) isolated del(7q) has been identified as less unfavorable than monosomy 7; and iii) a very high-risk karyotype was defined as patients with more than 3 abnormalities, just as for AML. The latter category includes most patients with a so-called monosomal karyotype, which has been shown to confer a very poor outcome also in MDS.[32] In addition, several new small categories with distinct prognostic relevance have been identified. Interestingly, fibrosis did not prove to be an independent prognosis marker in the IPSS-R. This may be due to the low frequency (19%) of patients with adequate assessment of fibrosis, the variability in methodology, or the fact that the revised scoring system detects outcomes that previously were revealed by fibrosis.

The IPSS-R also introduced categorical cut offs for blast counts, thrombocytopenia, anemia and leukopenia. It still has to be confirmed whether morphological examination is a good enough tool for this level of blast count detail, and if concordance between observers in clinical routine practice is sufficient to allow for a distinction between less than 2%, 2% to less than 5%, and 5% or over marrow blasts. Moreover, it will be interesting to observe whether IPSS-R will become established among clinicians before the molecular markers influence the scenario.

Several risk factors with additional prognostic value have been identified over recent years. The Pavia group identified multi-lineage dysplasia as well as presence of a regular transfusion need as independent risk factors, and published the WPSS scoring system including also these

Table 3. Revised International Prognostic Scoring System (IPSS-R) for MDS: based on 7012 patients.

Variable	Points						
	0	0.5	1	1.5	2	3	4
Cytogenetics	Very Good		Good		Intermediate	Poor	Very Poor
BM blast	≤2%		>2%-<5%		5%-10%	>10%	
Hemoglobin	≥10		8-<10	<8			
Platelets	≥100	50-<100	<50				
ANC	≥0.8	<0.8					

IPSS-R risk group	Score
Very low	≤1.5
Low	>1.5-3
Intermediate	>3-4.5
High	>4.5-6
Very high	>6

Cytogenetic risk groups (% of patients); Very good (4%) -Y, del(11q); Good (72%) normal, del(5q), del(12p), del(20q), double including del(5q); Intermediate (13%) del(7q), +8, +19, i(17q), any other single or double; Poor (4%) -7, inv(3)/t(3q)/del(3q), double including -7/del(7q); and Very poor (7%) complex: ≥3 abnormalities.[39]

factors.[40] Later, the same group showed a prognostic impact of the degree of anemia, also in patients without transfusion need.[41] Recently, transfusion dependence was again identified as an adverse risk factor in the prospective European Registry for IPSS low and INT-1 risk MDS of 1000 patients.[12] Moreover, the presence of bone marrow fibrosis grade II or III confers a significantly worse prognosis to all patients with MDS, irrespective of IPSS risk group.[7,8] Generally speaking, these additional risk factors for untreated patients are more important in IPSS low and INT-1 risk MDS than in higher-risk MDS, and may impact the decision about allo-SCT in eligible patients. Patients with higher-risk MDS have a short expected survival even without these extra risk factors, and should be candidates for curative regimens when age and co-morbidities allow. Immunophenotyping by multiparametric flow cytometry may also provide prognostic information, as described above. A major role for flow cytometry would be if this tool could be used to assess minimal residual disease in remission or after allo-SCT.[42]

Somatic mutations as prognostic variables

Rapid methodological development within the field of next generation sequencing has made it feasible and relatively inexpensive to detect somatic mutations by exome sequencing. An even more cost-effective approach, which is developing into a potential clinical tool, is targeted sequencing, in which a specific number of selectively enriched genes are analyzed by direct (and/or deep) sequencing. This has opened up a totally new understanding of MDS and other hematologic malignancies. Molecular lesions in MDS are outlined in detail in the chapter "Recent advance in molecular genetics of myelodysplastic syndromes as revealed by massively parallel sequencing" by Seishi Ogawa. The number of genes in which recurrent mutations in coding regions occur in more than a few percent of patients with MDS seems to be approximately 25, according to a large recent report of more than 700 patients.[43] Only four genes, *SF3B1*, *TET2*, *SRSF2* and *ASXL1* were mutated in more than 10% of patients, while more than 30 genes were mutated in less than 5% of patients. Interestingly, there is a strong co-existence between certain mutations, while others almost never co-occur in the same patient. The most common mutations in MDS are found in genes involved in epigenetic regulation and in the splicing process. Mutations in regulators of methylation as well as chromatin remodeling show significant overlap between MDS and AML and are likely to affect the biology of these disorders.[44-49] By contrast, mutations in splice factor genes are strikingly more common in MDS than in AML and myeloproliferative neoplasms.[24,50-52] The downstream consequences of these mutations are yet to be worked out, but they seem to be associated with the morphological features of dysplasia.

As the majority of these mutations co-occur in the same tumor clone and in a large number of different patterns, it will be a monumental task to sort out the prognostic implications of the various patterns. Only one mutation has so far been associated with a specifically favorable prognosis and that is *SF3B1*, found in more than 75% of patients with lower-risk MDS and more than 15% ring sideroblasts (RARS, RCMD-RS, and RARS-T) but in less than 10% in other MDS categories.[24,25] One can argue that SF3B1 identifies a subgroup of MDS, which is already recognized as an indolent MDS subtype, but the identification of a driver mutation in this specific subtype will allow for a better understanding and future therapeutic development. As an example, altered exon usage and decreased expression of the mitochondrial iron transporter *ABCB7* has recently been identified as a key mediator of erythroid failure in RARS.[53] Other mutations, such as *TET2*, are common but do not seem to be associated with any particular outcome.[54] A number of mutations have in several studies been associated with a short survival and risk for AML transformation. In a pivotal paper by Bejar *et al.,*[55] 439 MDS patients were analyzed for 18 genes excluding the splice factor mutations, whereof five mutations (*TP53*, *EZH2*, *ETV6*, *RUNX1* and *ASXL1*) were independently associated with a worse outcome. Importantly, the IPSS category still retained a major prognostic value. Bejar *et al*. then assessed the same genes plus the splice factor mutations in 288 patients with lower-risk MDS and validated the independent prognostic value of *EZH2* in this cohort.[56] Some of these mutations have been verified as prognostic markers also in other studies of MDS, while others have not. It is clear that new molecular markers have to be tested in independent patient cohorts and in the multivariate setting before they could be introduced as potential prognostic tools in clinical practice. Moreover, the significance of these mutations probably lies in their role in the biology of disease rather than in the prognostic value of each mutation. Hence, this review deliberately refrains from producing a table summarizing the prognostic value of various molecular markers. Moreover, different WHO entities, such as the MDS/MPNs have to be assessed separately.[57] One important finding is that the number of driver mutations in itself is associated with outcome. In the large study by Papaemmanuil *et al.,* patients with 0-2 driver mutations have a better outcome than patients with 3 or more mutations.[43]

The spread of massive parallel sequencing methods will soon make it possible for clinicians to detect a broad range of somatic point mutations in their patients. The question is now what kind of recommendations should be given for their role in predicting outcome with and without therapy. A limited number of molecular markers are likely to appear within the next few years. A few, however, already seem to have proven their clinical importance.

Small subclones: the limitations of conventional sequencing

We recently showed that the presence of small *TP53* mutated subclones in lower-risk del(5q) MDS confer a strong negative impact on the probability for survival as well as for remaining in the low-risk MDS category.[58] The presence of such subclones was recently confirmed by two other studies, of which the latter supports *TP53* as strong adverse marker both in high-risk and in low-risk MDS with del(5q).[59,60] Moreover, *TP53* mutated high-risk del(5q) MDS was reported to be resistant to treatment with lenalidomide.[61] Similar results have been reported in AML,[62] and it seems clear that *TP53* mutation is one of the most unfavorable molecular markers in MDS and AML. Importantly, these subclones of heterozygously mutated

cells are relatively small. The median clone size in the first study was 11% (1-54%), hence a majority of these subclones would not have been detected by conventional Sanger sequencing.[58] In the case of *TP53*, p53 immunohistochemical staining (IHC) is presently evaluated for its role as a clinical biomarker in low-risk del(5q) MDS and with regard to its concordance with underlying *TP53* mutations. Other potentially important subclones are yet to be discovered with improved sequencing techniques and may eventually increase the understanding of relapse after initial successful treatment. One important conclusion of these investigations is that not only dominant driver mutations impact the outcome, but that also clones too small to be detected by conventional sequencing can be extremely important for therapeutic decision-making.

Co-morbidity evaluation

The median age of MDS diagnosis is close to 75 years, which means that most patients are elderly.[12,34,64] As a consequence, a high prevalence of non-hematologic disorders has been reported in MDS patients, with 50% of patients having one or more co-morbidities. The role of co-morbidities is stronger in lower-risk than in higher-risk MDS, where disease biology usually affects outcome irrespective of performance status. A more detailed pattern of co-morbidities will, in time, appear from the prospective European MDS Registry.[12] In general, the pattern of non-hematologic diseases follows that of the general population, with heart disease being the most frequent. It is possible, however, that the frequency of heart problems increases with severity and duration of anemia. Co-morbidity assessment is most critical when a patient is evaluated for a specific therapy.[65] For moderately toxic therapy, such as azacytidine, co-morbidity rather than age is the limiting factor for treatment. For more toxic therapy, such as allo-SCT, it is well known that both age and co-morbidity are strong determinants of transplant outcome. The Hematopoietic Cell Transplantation Comorbidity Index (HCT-CI) is a useful and validated tool to predict post-transplantation outcomes for patients with MDS and AML. Decision-making about SCT in MDS is a difficult task, and host and donor variables, the doctor's 'gut feeling', and, in the end, the patient's own expectations and perceptions about risks and possibilities all have to be taken into consideration. A more strict use of available decision models may make this process more transparent for those involved.

The present review does not aim to cover the field of co-morbidities in detail, nor risk factors that have been acquired during the course of disease, such as iron overload caused by transfusions.

Conclusion

Myelodysplastic syndromes may be a difficult diagnosis to make and it is important to adhere to current diagnostic and prognostic guidelines, including bone marrow morphology, histopathology and cytogenetics. If possible, patients should be discussed at multi-professional conferences. Immunophenotyping by multiparametric flow cytometry, and FISH may emerge as useful additional diagnostic methods for selected patients. Newer biomarkers and, in particular, the finding of specific acquired somatic mutations are likely to add to the understanding of these disorders and provide important tools for clinical decision-making. However, further evaluation will be needed before these are introduced into current prognostic models.

References

1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO Classification of Tumors Of Haematopoietic and Lymphoid Tissues. Lyon: IARC Press, 2008, pp. 89-107.
2. Calado RT, Young NS. Telomere diseases. N Engl J Med. 2009;361:2353-65.
3. Liew E, Owen C. Familial myelodysplastic syndromes: a review of the literature. Haematologica. 2011;96:1536-42.
4. Mufti GJ, Bennett JM, Goasguen J, Bain BJ, Baumann I, Brunning R, et al. Diagnosis and classification of myelodysplastic syndrome: International Working Group on Morphology of myelodysplastic syndrome (IWGM-MDS) consensus proposals for the definition and enumeration of myeloblasts and ring sideroblasts. Haematologica. 2008;93:1712-7.
5. Huang TC, Ko BS, Tang JL, Hsu C, Chen CY, Tsay W, et al. Comparison of hypoplastic myelodysplastic syndrome (MDS) with normo-/hypercellular MDS by International Prognostic Scoring System, cytogenetic and genetic studies. Leukemia. 2008;22:544-50.
6. Sloand EM. Hypocellular myelodysplasia. Hematol Oncol Clin North Am. 2009;23:347-60.
7. Buesche G, Teoman H, Wilczak W, Ganser A, Hecker H, Wilkens L, et al. Marrow fibrosis predicts early fatal marrow failure in patients with myelodysplastic syndromes. Leukemia. 2008;22:313-22.
8. Della Porta MG, Malcovati L, Boveri E, Travaglino E, Pietra D, Pascutto C, et al. Clinical relevance of bone marrow fibrosis and CD34-positive cell clusters in primary myelodysplastic syndromes. J Clin Oncol. 2009;27:754-62.
9. Verburgh E, Achten R, Maes B, Hagemeijer A, Boogaerts M, De Wolf-Peeters C, et al. Additional prognostic value of bone marrow histology in patients subclassified according to the International Prognostic Scoring System for myelodysplastic syndromes. J Clin Oncol. 2003;21:273-82.
10. Shaffer LG, Slovak ML, LJ C. ISCN (2009): International System of Human Cytogenetic Nomenclature. Basel S Karger AG, 2009.
11. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114:937-51.
12. de Swart L, Smith A, Fenaux P, Sanz G, Hellstrom-Lindberg E, Symeonidis A, et al. Early Mortality in 1000 Newly Diagnosed MDS Patients with Low- and Intermediate-1 Risk MDS in the European Leukemianet MDS (EUMDS) Registry. ASH Annual Meeting Abstracts. Blood;2012;120:3830.
13. Bernasconi P, Cavigliano PM, Boni M, Calatroni S, Klersy C, Giardini I, et al. Is FISH a relevant prognostic tool in myelodysplastic syndromes with a normal chromosome pattern on conventional cytogenetics? A study on 57 patients. Leukemia. 2003;17:2107-12.
14. Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood. 1997;89:2079-88.
15. van de Loosdrecht AA, Alhan C, Béné MC, Della Porta MG, Dräger AM, Feuillard J, et al. Standardization of flow cytometry in myelodysplastic syndromes: report from the first European LeukemiaNet working conference on flow cytometry in myelodysplastic syndromes. Haematologica. 2009;94:1124-34.
16. Westers TM, Ireland R, Kern W, Alhan C, Balleisen JS, Bettelheim P, et al. Standardization of flow cytometry in myelodysplastic syndromes: a report from an international consortium and the European leukemianet working group. Leukemia. 2012;26:1730-41
17. van de Loosdrecht AA, Ireland R, Kern W, Della Porta MG, Alhan C, Balleisen JS, et al. Rationale for the clinical application of flow cytometry in patients with myelodysplastic syn-

dromes: position paper of an International Consortium and the European LeukemiaNet Working Group. Leuk Lymphoma. 2012 Sep 14. [Epub ahead of print]
18. Della Porta MG, Picone C, Pascutto C, Malcovati L, Tamura H, Handa H, et al. Multicenter validation of a reproducible flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes: results of a European LeukemiaNET study. Haematologica. 2012;97:1209-17.
19. Ogata K, Kishikawa Y, Satoh C, Tamura H, Dan K, Hayashi A. Diagnostic application of flow cytometric characteristics of CD34+ cells in low-grade myelodysplastic syndromes. Blood. 2006;108:1037-44.
20. Ogata K, Kakumoto K, Matsuda A, Tohyama K, Tamura H, Ueda Y, et al. Differences in blast immunophenotypes among disease types in myelodysplastic syndromes: a multicenter validation study. Leuk Res. 2012;36:1229-36.
21. Tiu RV, Gondek LP, O'Keefe CL, Elson P, Huh J, Mohamedali A, et al. Prognostic impact of SNP array karyotyping in myelodysplastic syndromes and related myeloid malignancies. Blood. 2011;117:4552-60.
22. Maciejewski JP, Mufti GJ. Whole genome scanning as a cytogenetic tool in hematologic malignancies. Blood. 2008;112:965-74
23. Afable MG, Wlodarski M, Makishima H, Shaik M, Sekeres MA, Tiu RV, et al.SNP array-based karyotyping: differences and similarities between aplastic anemia and hypocellular myelodysplastic syndromes. Blood. 2011;117:6876-84.
24. Papaemmanuil E, Cazzola M, Boultwood J, Malcovati L, Vyas P, Bowen D, et al. Somatic mutation of SF3B1 in myelodysplasia with ring sideroblasts and other cancers. N Engl J Med. 2011;365:1384-95.
25. Malcovati L, Papaemmanuil E, Bowen DT, Boultwood J, Della Porta MG, Pascutto C, et al. Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms. Blood. 2011;118:6239-46.
26. Malcovati L, Della Porta MG, Pietra D, Boveri E, Pellagatti A, Galli A, et al. Molecular and clinical features of refractory anemia with ringed sideroblasts associated with marked thrombocytosis. Blood. 2009;114:3538-45.
27. Schoch C, Kern W, Schnittger S, Hiddemann W, Haferlach T. Karyotype is an independent prognostic parameter in therapy-related acute myeloid leukemia (t-AML): an analysis of 93 patients with t-AML in comparison to 1091 patients with de novo AML. Leukemia. 2004;18:120-5.
28. Kayser S, Döhner K, Krauter J, Köhne CH, Horst HA, Held G, et al; German-Austrian AMLSG. The impact of therapy-related acute myeloid leukemia (AML) on outcome in 2853 adult patients with newly diagnosed AML. Blood. 2011;117:2137-45.
29. Feldman EJ. Does therapy-related AML have a poor prognosis, independent of the cytogenetic/molecular determinants? Best Pract Res Clin Haematol. 2011;24:523-6.
30. Fianchi L, Criscuolo M, Lunghi M, Gaidano G, Breccia M, Levis A, et al Outcome of therapy-related myeloid neoplasms treated with azacitidine. J Hematol Oncol. 2012;5:5-44.
31. Klimek VM, Dolezal EK, Tees MT, Devlin SM, Stein K, Romero A, et al. Efficacy of hypomethylating agents in therapy-related myelodysplastic syndromes. Leuk Res. 2012;36:1093-7.
32. Deeg HJ, Scott BL, Fang M, Shulman HM, Gyurkocza B, Myerson D, et al. Five-group cytogenetic risk classification, monosomal karyotype, and outcome after hematopoietic cell transplantation for MDS or acute leukemia evolving from MDS. Blood. 2012;120:1398-408.
33. Loh ML. Recent advances in the pathogenesis and treatment of juvenile myelomonocytic leukaemia. Br J Haematol. 2011;152:677-87.
34. Malcovati L, Porta MG, Pascutto C, Invernizzi R, Boni M, Travaglino E, et al. Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: a basis for clinical decision making. Clin Oncol. 2005;23:7594-603.
35. Morel P, Hebbar M, Lai JL, Duhamel A, Preudhomme C, Wattel E, et al. Cytogenetic analysis has strong independent prognostic value in de novo myelodysplastic syndromes and can be incorporated in a new scoring system: a report on 408 cases. Leukemia.1993;7:1315-23.
36. Schanz J, Steidl C, Fonatsch C, Pfeilstöcker M, Nösslinger T, Tuechler H, et al. Coalesced multicentric analysis of 2,351 patients with myelodysplastic syndromes indicates an underestimation of poor-risk cytogenetics of myelodysplastic syndromes in the international prognostic scoring system. J Clin Oncol. 2011;29:1963-70.
37. Schanz J, Tüchler H, Solé F, Mallo M, Luño E, Cervera J, et al. New Comprehensive Cytogenetic Scoring System for Primary Myelodysplastic Syndromes (MDS) and Oligoblastic Acute Myeloid Leukemia After MDS Derived From an International Database Merge. J Clin Oncol. 2012;30:820-9.
38. Haase D, Germing U, Schanz J, Pfeilstöcker M, Nösslinger T, Hildebrandt B, et al. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. Blood. 2007;110:4385-95.
39. Greenberg PL, Tuechler H, Schanz J, Sanz G, Garcia-Manero G, Solé F, et al. Revised international prognostic scoring system for myelodysplastic syndromes. Blood. 2012;120:2454-65.
40. Malcovati L, Germing U, Kuendgen A, Della Porta MG, Pascutto C, Invernizzi R, et al. Time-Dependent Prognostic Scoring System for Predicting Survival and Leukemic Evolution in Myelodysplastic Syndromes. J Clin Oncol. 2007;25:3503-10.
41. Malcovati L, Della Porta MG, Strupp C, Ambaglio I, Kuendgen A, Nachtkamp K, et al. Impact of the degree of anemia on the outcome of patients with myelodysplastic syndrome and its integration into the WHO classification-based Prognostic Scoring System (WPSS). Haematologica. 2011;96:1433-40.
42. Ossenkoppele GJ, van de Loosdrecht AA, Schuurhuis GJ. Review of the relevance of aberrant antigen expression by flow cytometry in myeloid neoplasms. Br J Haematol. 2011;153:421-36.
43. Papaemmanuil E, Gerstung M, Malcovati L, Tauro S, Gundem G, Van Loo P, et al. High Throughput Targeted Gene Sequencing in 738 Myelodysplastic Syndromes Patients Reveals Novel Oncogenic Genes, Rare Driver Mutations and Complex Molecular Signatures with Potential Impact for Patient Diagnosis and Prognosis in the Clinic. ASH Annual Meeting Abstracts. Blood;2012;120:LBA-5.
44. Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet. 2009;41:838-42.
45. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Massé A, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009;360:2289-301.
46. Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tönnissen ER, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. Nat Genet. 2010;42:665-7.
47. Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet. 2010;42:722-6.
48. Boultwood J, Perry J, Pellagatti A, Fernandez-Mercado M, Fernandez-Santamaria C, Calasanz MJ, et al. Frequent mutation of the polycomb-associated gene ASXL1 in the myelodysplastic syndromes and in acute myeloid leukemia. Leukemia. 2010;24:1062-5.
49. Thol F, Friesen I, Damm F, Yun H, Weissinger EM, Krauter J, et al. Prognostic significance of ASXL1 mutations in patients with myelodysplastic syndromes. J Clin Oncol. 2011;29:2499-506.
50. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. Nature. 2011;478:64-9.
51. Graubert TA, Shen D, Ding L, Okeyo-Owuor T, Lunn CL, Shao J, et al. Recurrent mutations in the U2AF1 splicing factor in myelodysplastic syndromes. Nat Genet;44:53-7.
52. Damm F, Kosmider O, Gelsi-Boyer V, Renneville A, Carbuccia N, Hidalgo-Curtis C, et al. Mutations affecting mRNA splicing define distinct clinical phenotypes and correlate with patient outcome in myelodysplastic syndromes. Blood. 2012;119:3211-8.
53. Nikpour M, Scharenberg C, Liu A, Conte S, Karimi M, Mortera-Blanco T, et al. The transporter gene ABCB7 is an important mediator in the phenotype of acquired refractory anemia with ring sideroblasts. Leukemia. 2012 Oct 16. [Epub ahead of print].
54. Smith AE, Mohamedali AM, Kulasekararaj A, Lim Z, Gäken J, Lea NC, et al. Next-generation sequencing of the TET2 gene in 355 MDS and CMML patients reveals low-abundance mutant clones with early origins, but indicates no definite prognostic value. Blood. 2010;116:3923-32.
55. Bejar R, Stevenson K, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, et al. Clinical effect of point mutations in myelodysplastic syndromes. N Engl J Med. 2011;364:2496-

506.
56. Bejar R, Stevenson KE, Caughey BA, Abdel-Wahab O, Steensma DP, Galili N, et al. Validation of a prognostic model and the impact of mutations in patients with lower-risk myelodysplastic syndromes. J Clin Oncol. 2012;30:3376-82.
57. Grossmann V, Kohlmann A, Eder C, Haferlach C, Kern W, Cross NC, et al. Molecular profiling of chronic myelomonocytic leukemia reveals diverse mutations in >80% of patients with TET2 and EZH2 being of high prognostic relevance. Leukemia. 2011;25:877-9.
58. Jädersten M, Saft L, Smith A, Kulasekararaj A, Pomplun S, Göhring G, et al. TP53 mutations in low-risk myelodysplastic syndromes with del(5q) predict disease progression. J Clin Oncol. 2011;29:1971-9.
59. Sebaa A, Ades L, Baran-Marzack F, Mozziconacci MJ, Penther D, Dobbelstein S, et al. Incidence of 17p deletions and TP53 mutation in myelodysplastic syndrome and acute myeloid leukemia with 5q deletion. Genes Chromosomes Cancer. 2012;51:1086-92.
60. Kulasekararaj AG, Smith AE, Mian SA, Mohamedali AM, Krishnamurthy P, Lea NC, et al. TP53 mutations in myelodysplastic syndrome are strongly correlated with aberrations of chromosome 5, and correlate with adverse prognosis. Br J Haematol. 2013 Jan 9. [Epub ahead of print]
61. Möllgård L, Saft L, Bach Treppendahl M, Dybedal I, Maxwell Nørgaard J, et al. Clinical effect of high-dose Lenalidomide in High-Risk Myelodysplastic Syndrome and Acute Myeloid Leukemia with Chromosome 5 Abnormalities. Haematologica. 2011;96:963-71.
62. Rücker FG, Schlenk RF, Bullinger L, Kayser S, Teleanu V, Kett H, et al. TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. Blood. 2012;119:2114-21.
63. Goldberg SL, Chen E, Corral M, Guo A, Mody-Patel N, Pecora AL, et al. Incidence and clinical complications of myelodysplastic syndromes among United States Medicare beneficiaries. J Clin Oncol. 2010;28:2847-52.
64. Della Porta MG, Malcovati L. Clinical relevance of extra-hematologic comorbidity in the management of patients with myelodysplastic syndrome. Haematologica. 2009;94:602-6.
65. Della Porta MG, Malcovati L, Strupp C, Ambaglio I, Kuendgen A, Zipperer E, et al. Risk stratification based on both disease status and extra-hematologic comorbidities in patients with myelodysplastic syndrome. Haematologica. 2011;96:441-9.
66. Sorror ML, Sandmaier BM, Storer BE, Maris MB, Baron F, Maloney DG, et al. Comorbidity and disease status based risk stratification of outcomes among patients with acute myeloid leukemia or myelodysplasia receiving allogeneic hematopoietic cell transplantation. J Clin Oncol. 2007;25:4246-54.

Risk-adapted treatment of myelodysplastic syndromes

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A B S T R A C T

Treatment choice in patients with myelodysplastic syndromes (MDS) is not an easy task. MDS are heterogeneous clonal hematopoietic disorders mainly affecting the elderly. MDS are characterized by varying degrees of cytopenias, dysplastic morphological features of hematopoietic cell lines, and propensity to acute myeloblastic leukemia evolution. Consequently, prognosis of MDS patients varies widely, with overall survival ranging from a few weeks to several years depending on disease-related characteristics, mainly specific cytogenetic abnormalities, percentage of blast cells in bone marrow, severity and number of cytopenias, and patient-related factors such as age, performance status and comorbidities. Despite the recent availability of drugs able to modify the natural history of MDS, emergence of new modalities of allogeneic hematopoietic stem cell transplantation (allo-HSCT), which notably increase its applicability, development of better prognostic scoring systems, and evident progress in our knowledge on their molecular basis, treatment for most patients remains unsatisfactory. This review analyzes the prognostic factors and scoring systems that should be considered for therapy planning in MDS, critically evaluates the information on the different treatment strategies currently available, and, finally, suggests potential algorithms for risk-adapted treatment of MDS patients.

Learning goals

At the conclusion of this activity, participants should be able to:
- accurately estimate the individual prognosis of patients with MDS;
- describe treatment modalities currently available for patients with MDS;
- select appropriate treatment for patients with MDS based on individual risk assessment.

Introduction

The selection of treatment and timing for therapeutic intervention in patients with MDS is complicated by different factors. First, as expected for a group of disorders that encompasses very heterogeneous entities, the natural course of the disease is highly variable, both in terms of overall survival (OS) and risk of progression to acute myeloblastic leukemia (AML). Second, *de novo* MDS mainly affects elderly people (median age 75 years) who, in many instances, present poor performance status and relevant comorbidities. Additionally, allogeneic hematopoietic stem cell transplantation (allo-HSCT), the only curative treatment option currently available, carries high rates of morbidity and mortality that limit its applicability to younger fitter patients with an adequate donor. Finally, evidence for the efficacy of different therapeutic alternatives comes often from small, uncontrolled phase II clinical trials that use quite different response criteria. Due to all these facts, an individual risk-adapted treatment strategy, with therapy tailored to the expected outcome, is essential.

Furthermore, it should be emphasized that treatment for most patients with MDS remains disappointing and best supportive care continues to be an essential component of patient management. Despite the recent availability of new drugs able to modify the natural history of MDS, such as hypomethylating agents, the emergence of new modalities of allo-HSCT which have notably increased its use, the development of better prognostic scoring systems, and evident progress in our knowledge on the molecular basis of these disorders, most patients with MDS will die from the consequences of progressive bone marrow failure with or without overt AML evolution. Thus, treatment of patients with MDS should always be considered investigational, and patients should be included whenever possible in well-designed prospective clinical trials driven to gain insight into our understanding of MDS and to improve outcome.

Risk assessment in MDS

The natural history of MDS is extremely heterogeneous. In an attempt to accurately establish the expected outcome in individual patients, during the last three decades there has been considerable research into identifying prognostic factors and developing prognostic indexes capable of stratifying MDS patients in different risk groups. In addition, factors predictive of response to a specific treatment

alternative are also relevant for planning therapy. Thus, three different kinds of prognostic factors can be recognized and must be taken into account for risk-adapted treatment: 1) disease-related, i.e. those due to the characteristics of the abnormal MDS clone; 2) patient-related, i.e. reflecting specific patient's characteristics; and 3) treatment-related, i.e. linked to the probability of success of a particular treatment option.

Disease-related prognostic factors

Several earlier studies showed the independent prognostic impact of percentage of blast cells in bone marrow, cytogenetics, and number of cytopenias.[1-3] Since its publication in 1997, the International Prognostic Scoring System (IPSS),[4] which is based on those variables (Table 1), has been unanimously accepted as the reference for clinical decision-making as well as for the design and analysis of clinical trials in MDS. The IPSS is able to stratify patients into 4 risk groups (low-, intermediate-1-, intermediate-2-, and high-risk) with significant statistical differences in OS and rate of progression to AML. Its validity to predict the outcome of both untreated and treated patients has been demonstrated in independent series, and it is extremely simple to use. Moreover, it can be reapplied dynamically, both at diagnosis and during disease evolution.[5]

The presence of multilineage dysplasia, as defined by the World Health Organization (WHO) classification, and red blood cell (RBC) transfusion dependence have also shown independent prognostic relevance and were integrated, along with the IPPS cytogenetic risk categories, in the WHO classification-based prognostic scoring system (WPSS),[5,6] which is able to classify patients into 5 risk groups (very low-, low, intermediate, high, and very high-risk) with clearly different OS and probabilities of leukemic evolution both at diagnosis and during follow up (Table 2). The prognostic value of WPSS has also been validated in external series but this scoring system has not

Table 1. International Prognostic Scoring System (IPSS).[4]

Prognostic characteristic	Points				
	0	0.5	1	1.5	2
Blasts in bone marrow (%)	<5	5-10		11 - 20	21 - 30
Cytogenetic risk category[a]	Good	Intermediate	Poor		
Number of cytopenias[b]	0 or 1	2 or 3			

IPSS risk group	Score	Median overall survival (years)
Low	0	5.7
Intermediate-1	0.5-1.0	3.5
Intermediate-2	1.5-2.0	1.2
High	2.5-3.5	0.4

[a]*Good: normal, del(5q), del(20q), and –Y as single abnormalities; Poor: complex (≥3 abnormalities) or chromosome 7 abnormalities; Intermediate: other abnormalities.* [b]*Cytopenias: hemoglobin <10 g/dL, neutrophil count <1.8x10^9/L, platelet count < 100×10^9/L.*

Table 2. WHO classification-based Prognostic Scoring System (WPSS).[5]

Prognostic characteristic			Points		
	0	1		2	3
WHO 2001 category	RA, RARS, MDS with isolated deletion (5q)	RCMD	RAEB-1	RAEB-2	
Cytogenetic risk category[a]	Good	Intermediate	Poor	-	
RBC transfusion requirement[b,c]	No	Regular	-	-	

WPSS risk group	Score	Median overall survival (months)
Very low	0	103
Low	1	72
Intermediate	2	40
High	3-4	21
Very high	5-6	12

RA: refractory anemia; RARS: refractory anemia with ringed sideroblasts; 5q–: myelodysplastic syndrome with isolated del(5q); RCMD: refractory cytopenia with multilineage dysplasia; RCMD-RS: refractory cytopenia with multilineage dysplasia and ring sideroblasts; RAEB-1: refractory anemia with excess of blasts-1; RAEB-2: refractory anemia with excess of blasts-2. [a]*Good: normal, del(5q), del(20q), and –Y as single abnormalities; Poor: complex (≥3 abnormalities) or chromosome 7 abnormalities; Intermediate: other abnormalities.* [b]*RBC transfusion requirement was defined as having at least one RBC transfusion every eight weeks over a period of four months.* [c]*Severe anemia (hemoglobin <9 g/dL in males or <8 g/dL in females) can substitute regular RBC transfusion requirement.*[7]

been universally adopted, probably because it has not demonstrated substantial improvement over IPSS and the subjectivity inherent to the evaluation of morphological dysplasia and to start RBC transfusion support. In an attempt to deal with the later criticism, the Pavia group has recently shown that the level of hemoglobin (≤8 g/dL in women and ≤9 g/dL in men) could substitute RBC transfusion dependence in the WPSS with similar predictive power.[7]

In recent years, it has become clear that there is an independent association with outcome for other disease characteristics, such as raised serum ferritin (> 1000 ng/mL),[5,8] bone marrow fibrosis (grade 2 [moderate] to grade 3 [severe] by the European consensus guidelines),[9] and severe neutropenia (PMN count $<0.5\times10^9$/L)[10] and thrombocytopenia (platelet count $<30\times10^9$/L).[11] The use of these variables allows a more accurate definition of the prognosis of individual patients with MDS to be made, in particular those in low or intermediate-1 IPSS risk groups.[9-11] Furthermore, the analysis of very large series of patients has provided a better understanding of the prognostic impact of specific chromosomal abnormalities, has clarified the prognostic value of some specific abnormalities not recognized in IPSS, has shown that prognostic weight of cytogenetic risk categories in a prognostic index for MDS should be at least similar to the one assigned to the percentage of blast cells in bone marrow, and has defined five cytogenetic risk categories for OS.[12,13]

To examine the prognostic impact of these new clinical and cytogenetic variables and to refine the IPSS, the International Working Group for Prognosis in MDS project assembled a large multicenter database of 7012 untreated patients with MDS to generate a revised IPSS (IPSS-R) that is shown in Table 3.[14] The IPSS-R continues to be based on the same variables already present in IPSS but their categorization is rather different and, inevitably to improve accuracy, more complex. The new IPSS-R is able to stratify patients into 5 risk groups (very low-, low-, intermediate-, high- and very high-risk), with clear differences in OS and risk of progression to AML. Patient age was a significant additive feature for OS but not for AML evolution. Other potentially differentiating characteristics, such as serum ferritin and LDH level, RBC transfusion dependency, beta 2 (β2)-microglobulin, bone marrow fibrosis, and performance status could not be incorporated into the general prognostic model because they were not available in a substantial number of patients.

Several recent studies suggest that flow cytometry immunophenotyping[15,16] and gene mutations[17-20] may provide more accurate risk stratification of individual patients. However, further investigations are necessary to precisely determine the independent prognostic impact of these technologies and the advantages of their addition to already available scoring systems before recommending these approaches be performed on a routine daily basis and incorporated into clinical decision-making in MDS.

Currently, the use of the IPSS, WPSS, and IPSS-R is strongly recommended for predicting outcome and planning treatment for patients with MDS.[21-24]

Patient-related prognostic factors

In MDS, as a group of disorders basically affecting elderly patients, the decision to treat or not to treat, and also the choice of treatment selected, is in many instances highly influenced by age, functional ability (performance status), comorbidity, frailty, and other nutritional, social, economic or personal circumstances.[21-24]

Increasing age is an independent adverse prognostic factor for OS in MDS,[2,4,6,14,25] especially in lower-risk patients.[14,25] ECOG performance status, despite its subjectivity, has also been shown to influence OS.[14] Several comorbidity scores, such as the MDS-Specific Comorbidity Index (MDS-CI)[26] and the Hematopoietic Cell Transplantation Comorbidity Index (HCT-CI),[27] have demonstrated an independent association with OS in MDS. Whereas in lower-risk patients comorbidity directly

Table 3. Revised International Prognostic Scoring System (IPSS-R).[14]

Prognostic characteristic	Points						
	0	0.5	1	1.5	2	3	4
Cytogenetic risk category[a]	Very good		Good		Intermediate	Poor	Very poor
Blasts in bone marrow (%)	≤2		>2-<5		5-10	>10	
Hemoglobin (g/dL)	≥10		8-<10	<8			
Platelet count ($\times10^9$/L)	≥100	50-<100	<50				
Absolute neutrophil count ($\times10^9$/L)	≥0.8	<0.8					

IPSS-R risk group	Score	Median overall survival (years)
Very Low	≤1.5	8.8
Low	>1.5-3	5.3
Intermediate	>3-4.5	3.0
High	>4.5-6	1.6
Very High	>6	0.8

[a]*Very good: –Y and del(11q) as single abnormalities; Good: Normal, del(5q), del(12p), and del(20q) as single abnormalities, double abnormalities including del(5q); Intermediate: del(7q), +8, +19, i(17q), and any other single abnormalities, any other double abnormalities; Poor: -7 and inv(3)/t(3q)/del(3q) as single abnormalities, double abnormalities including -7/del(7q), complex (3 abnormalities); Very poor: > 3 abnormalities.*

increases the risk of non-leukemic death, in higher-risk patients its potential impact is largely overcome by the severity of the disorder. Nonetheless, in the latter patient population comorbidity also influences the final outcome by reducing eligibility for and tolerance of intensive treatment strategies. Thus, the objective assessment of comorbidity and functionality by validated scoring systems is advisable for therapeutic decision-making in both lower- and higher-risk MDS patients.

Treatment-related predictive factors

As will be discussed below, the likelihood of response to a particular treatment modality should be also taken into account for decision-making regarding treatment choice. Characteristics or biomarkers included in this category may be disease- or patient-related and in many instances the underlying biological reasons for their predictive capacity are still undefined.

Definition of lower- and higher-risk patients for therapeutic purposes

Despite the ability of currently available prognostic scoring systems to define 4 or 5 clearly distinct risk groups of patients, from a practical point of view MDS patients have been classically stratified for therapeutic purposes, both in clinical daily practice and for inclusion into clinical trials, only into two risk categories: lower-risk and higher-risk.[22-24] This has been mainly due to the limited treatment armamentarium currently available and the non-specific efficacy of most treatment modalities for a particular subset of MDS patients. Lower-risk patients are defined by IPSS as those having low or intermediate-1 risk scores (IPSS score, 0-1 points), whereas higher-risk patients are those with intermediate-2 or high-risk scores (IPSS score, 1.5-3.5 points).[4] When using the WPSS or IPSS-R, lower-risk patients are those belonging to the very low- or low-risk categories, and higher-risk patients are those categorized as high- or very high-risk patients.[5,14] Remarkably, despite the likely better prognostic segregation achieved by WPSS or IPSS-R over IPSS, those more recent scoring systems create a dilemma for physicians taking care of MDS patients: what do we do with patients in the intermediate-risk category? Median OS for intermediate-risk patients in WPSS and IPSS-R were 40 months and 3 years, respectively.[5,14] The IPSS-R intermediate category appears closer to the initial IPSS intermediate-1 group both in terms of OS and AML risk, but the probability of dying from AML for IPSS-R intermediate-risk category was distinctively greater than for low- or very low-risk categories. Thus, for Greenberg and colleagues it seemed reasonable to suggest placement of IPSS-R intermediate patients into the lower-risk group as far as their potential therapeutic management in daily practice is concerned, but to include them in clinical trials for both lower- and higher-risk clinical trials to substantiate this point.[14] These authors also recommend the use in this risk category of additional differentiating features for better prognostic assessment. To cope with this specific issue, and in the light of available evidence, the Grupo Español de Síndromes Mielodisplásicos (GESMD) has recently recommended that patients categorized as intermediate-risk by IPSS or WPSS, and having high-risk or very high cytogenetics, bone marrow fibrosis (moderate or severe), severe neutropenia (PMN count $<0.5\times10^9$/L) or severe thrombocytopenia (platelet count $<30\times10^9$/L) should be considered as higher-risk patients.[28] One major advantage of this definition of lower- and higher-risk patients is that the only clinical reason that remains for starting treatment in lower-risk MDS patients is the management of symptomatic anemia, except for rarer cases of reiterated infections or bleeding in patients without severe neutropenia or thrombocytopenia, respectively. This feature notably simplifies the development of treatment algorithms for MDS patients.

Another important question is how the discrepancies in risk assignment between the three main prognostic scoring systems (IPSS, WPSS, and IPSS-R) should be resolved. In my opinion, a patient that is categorized as higher-risk by just one of them should be considered as higher-risk for therapeutic purposes.

The GESMD definition of lower/higher-risk MDS will be followed throughout this text except when specifically indicated.

Reasons for starting treatment and treatment aims

The decision to start an active treatment in MDS patients must be based on the individual prognosis and presence of symptomatic cytopenias. The main objective of treatment in higher-risk MDS is to modify the natural history of the disease and to prolong OS. Thus, all patients with higher-risk disease are candidates for active treatment at initial presentation. Furthermore, in those patients treatment should not be substantially delayed. In contrast, as there are no therapies that have undoubtedly demonstrated an increase in OS in lower-risk MDS patients and their life expectancy is longer, treatment for them is intended to improve symptoms and quality of life. For that reason, patients with lower-risk MDS are candidates for active intervention only when a symptomatic cytopenia is present. That is, a watchful-waiting strategy is advisable for asymptomatic lower-risk patients without clinically significant cytopenias. This policy is supported by data showing that the mortality rate of patients with very low risk MDS by WPSS is similar to that of the general population.[5] It must be stressed that the safety of this non-interventional approach in asymptomatic lower-risk patients is dependent on regular monitoring of peripheral blood and bone marrow features to recognize findings of progression that deserve therapeutic intervention at an early stage (L Malcovati *et al*., submitted manuscript, 2013).[24]

Best supportive care

Delivering the best supportive care (BSC) available remains a key element of treatment for every single MDS patient. Very advanced age, comorbidities, frailty, and functional disabilities may disqualify many patients for any active therapy; those patients will exclusively receive BSC. BSC includes RBC and platelet transfusion support, management of iron overload, and antimicrobial therapy.

RBC transfusion support

Chronic anemia is associated with a significant deterioration in the functional status and quality of life of elderly MDS patients and worse OS.[5-7,29] Anemia increases cardiac output, leads to ventricular hypertrophy and cardiac remodeling, and exacerbates coronary symptoms of patients with cardiac comorbidity.[29,30] Remarkably, in some series of lower-risk MDS patients, cardiac failure was the primary cause of non-leukemic death.[6,31] The objectives of RBC transfusions are to improve quality of life by relieving anemia-related symptoms and to avoid ischemic organ damage. No single hemoglobin concentration can be recommended as being the optimal level below which red cell support should be given (L Malcovati *et al*., submitted manuscript, 2013).[21-24] The decision to indicate RBC transfusion support should not be based only on hemoglobin level but mainly on patient's symptoms and presence of comorbidity. A hemoglobin level below 7 g/dL should be avoided. Thus, it seems advisable to always transfuse when the hemoglobin level is lower than 8 g/dL and to increase this threshold up to 10 g/dL in those patients with symptomatic milder anemia or comorbidities (L Malcovati *et al*., submitted manuscript, 2013).[24]

Platelet transfusion support

The incidence of thrombocytopenia is close to 40%,[14] being severe (platelet count <30×10^9/L) in 8-16% of the patients.[11] Several series have shown the negative impact of thrombocytopenia on outcomes[2,4,14,32] and in one study of lower-risk patients severe thrombocytopenia increased the risk of death due to bleeding.[11] There is no specific evidence of criteria for administering platelet transfusions in MDS to prevent major bleeding. In patients who only receive supportive treatment, with chronic long-lasting thrombocytopenia, the use of platelet transfusion should be very restrictive and probably limited to the presence of bleeding to avoid alloimmunization.[9] In contrast, in patients receiving active treatment with transient thrombocytopenia, the use of platelet transfusions should follow the same criteria employed in patients with AML. In those instances, prophylactic administration of platelet transfusions is recommended when the platelet count is lower than 10×10^9/L or lower than 20×10^9/L if any risk factor for bleeding is present (L Malcovati *et al*., submitted manuscript, 2013).[22,24]

Antimicrobial prophylaxis

There are no data to support the use of any prophylactic antimicrobial therapy in neutropenic MDS patients and its use is not generally recommended (L Malcovati *et al*., submitted manuscript, 2013).[24] Patients with MDS receiving active treatment with transient neutropenia could be considered for antibiotic prophylaxis but efficacy of this policy is still unproven.[33]

Iron chelation therapy

Chronic RBC transfusion support invariably results in development of iron overload. The potential role of excess of iron in organ damage in MDS is supported by indirect data showing a markedly higher incidence of different comorbidities in RBC transfusion-dependent patients, and T2* magnetic resonance imaging (MRI) and autopsy data in heavily transfused patients.[31,34,35] Furthermore, several retrospective series in MDS have also demonstrated that RBC transfusion dependency and raised serum ferritin (presumed to be due to iron overload) are independently associated with poorer outcomes both in untreated patients[5,6,8,14] and patients undergoing allo-HSCT.[36-38] This effect seems more remarkable in OS of lower-risk patients,[6,14] but in one large study raised serum ferritin reduced OS and increased AML risk also in higher-risk patients.[8] Whether iron chelation therapy will not only reduce iron overload but also improve OS in MDS patients is still unclear and a subject for debate because no data from a prospective randomized trial with OS as primary efficacy end point are available. Three retrospective comparative studies have suggested that iron chelation therapy would be beneficial in terms of OS in lower-risk MDS with RBC transfusion dependency.[39-41] Most treatment guidelines in MDS, frequently translating the vast information available in thalassemia and considering the preliminary data obtained in MDS, currently recommend starting iron chelation therapy in lower-risk RBC transfusion-dependent patients with iron overload (mainly defined as serum ferritin level > 1000 ng/mL or after receiving 20-25 RBC transfusions) and in candidates for allo-HSCT (L Malcovati *et al*., submitted manuscript, 2013).[22-24] The iron chelators deferoxamine, deferiprone, and deferasirox are all able to induce negative iron balance, reduce or normalize labile plasma iron, and reduce ferritin levels and liver iron content in MDS patients.[42-45] The route of administration, lack of serious hematologic adverse events, and manageable safety profile make deferasirox the preferred first-line treatment for iron overload in some guidelines.[22,24] The most common drug-related adverse events with deferasirox are gastrointestinal disturbances and increased serum creatinine, leading to discontinuation in 25% of the patients.[44,45] Deferasirox has also recently been shown to transiently improve hematologic parameters, including occasionally RBC transfusion independency, in some patients (10%-20%).[45,46] The mechanism responsible for this effect is unclear. The recommended deferasirox starting dose is 10-20 mg/kg/d and maintenance dose is 20-30 mg/kg/d that should be modified according to efficacy and transfusion requirements.[24] Regular monitoring of iron overload by measuring serum ferritin is strongly recommended.[24]

Hematopoietic growth factors

There is a huge amount of data regarding the use of hematopoietic growth factors, especially erythropoiesis-stimulating agents (ESAs), in patients with MDS, including meta-analyses and systematic reviews of the literature, randomized controlled trials and prospective and retrospective non-randomized clinical trials.

Erythropoiesis-stimulating agents

Despite the lack of specific approval in Europe for this indication, ESAs are universally considered as first-line treatment of symptomatic anemia in lower-risk MDS patients (L Malcovati *et al*., submitted manuscript, 2013).[21-24] Multiple prospective, randomized or uncontrolled, and retrospective studies have shown that a substantial proportion of MDS patients have an erythroid response to ESAs treatment.[47-50] Sixteen to 50% of patients failing ESAs respond to the addition of G-CSF.[48-50] A dose

effect seems to be present with higher doses of ESAs (erythropoietin [EPO], 60,000-80,000 IU per week; darbepoetin [DPO], 300 mcg per week-500 mcg every 2 weeks) showing a superior response rate[48,49] and equipotent doses of DPO result in clinical effects similar to those obtained with EPO.[47,48,50] Most responses occur after 8-12 weeks of treatment but very late responses may be occasionally observed.[51] Response rate has widely varied (median 40%-50%; range 12%-71%) depending on the stringency of response definition criteria, differences in incidence of predictive factors for response between series, and ESA dose. Though several characteristics have been associated with response rate to ESAs,[50] the two most important factors are endogenous serum level of EPO and RBC transfusion dependency, and a validated predictive model for response to ESAs plus EPO including these two variables developed by the Nordic MDS study group[52,53] is usually recommended to guide ESA use in lower-risk MDS (L Malcovati *et al*., submitted manuscript, 2013).[22-24] In a large retrospective study of 129 patients treated with EPO and G-CSF median duration of response was 23 months,[54] whereas in a series by the Group Francophone des Myélodysplasies (GFM) on 99 patients treated with DPO and with addition of G-CSF in non-responders median duration of response had not been achieved after 52 months of median follow up,[55] suggesting that response duration with DPO is, at least, not inferior to that achieved with EPO. Remarkably, no single study has reported an increased incidence of cardiovascular events or transformation to AML, and two large retrospective studies comparing ESAs with or without G-CSF and best supportive care have shown a beneficial effect of treatment on OS.[56,57] Thus, current national treatment guidelines recommend that lower-risk MDS patients with symptomatic anemia and with serum EPO level lower than 500 mU/mL and/or RBC transfusion requirement lower than 2 RBC units per month should be considered for therapy with ESAs (L Malcovati *et al*., submitted manuscript, 2013).[21-24] ESAs should be started at higher doses, with dosage regularly adjusted according to hemoglobin level to avoid polycythemia.[24] Response should be evaluated after 8-12 weeks of therapy and G-CSF (300 mcg per week divided in 2-3 doses for 8 additional weeks) added in case of ESA failure.[24] The association of ESAs and G-CSF from the beginning may be considered in patients with refractory anemia with ring sideroblasts. Patients losing their response to ESAs must be fully evaluated for MDS progression and concomitant causes of anemia, including iron deficiency.[24]

Granulocyte colony-stimulating factor

There are no data on efficacy and safety to recommend the use of G-CSF for preventing or treating infections in neutropenic MDS patients.[33] It may be considered in two circumstances: 1) for patients under active treatment in whom neutropenia supposes a limitation or delay of such therapy; and 2) for neutropenic patients with recurrent severe infections.[24] Though G-CSF mobilization does not appear to induce the appearance of monosomy 7 clones in healthy donors of hematopoietic progenitors of peripheral blood,[58] *in vitro* data of the preferential expansion by G-CSF of clones carrying monosomy 7 are of concern,[59] and support restricting the use of this hematopoietic growth factor in MDS patients with chromosome 7 abnormalities. The use of G-CSF in conjunction with ESAs to manage anemia has been discussed above.

Thrombopoietin receptor agonists

Romiplostim and eltrombopag, two thrombopoietin receptor agonists that stimulate platelet production currently approved for use in chronic autoimmune thrombocytopenia, are under investigation in MDS. In a small phase I/II clinical trial in thrombocytopenic patients with lower-risk MDS (IPSS low or intermediate-1 risk) receiving supportive care, romiplostim induced durable platelet responses in 46% of the patients, respectively, with 9% of the patients showing transient increases in marrow blast counts.[60] A large phase III randomized, placebo-controlled trial in the same setting was prematurely terminated due to concern of the data monitoring committee regarding a potential increase of AML risk (6% *vs*. 2%) outweighing the potential benefit in reduction of bleeding in patients receiving romiplostim.[61] However, a recent report with more prolonged follow up has shown a similar incidence of AML (9%) in both arms, and no differences in OS or AML-free survival between patients treated with romiplostim or placebo.[62] In that study, the number of platelet transfusions in patients with severe thrombocytopenia and the number of clinically significant bleeding events in those with moderate thrombocytopenia was significantly lower in patients receiving romiplostim. This drug has also been tested in small series of lower-risk patients treated with azacitidine,[63] decitabine[64] or lenalidomide.[65] In all of these studies there was a reduction, though no statistically significant, in the incidence of clinically significant thrombocytopenic events, number of platelet transfusions or bleeding events in romiplostim-treated patients. Eltrombopag is being evaluated both in lower- and higher-risk MDS. Preliminary data on both cohorts of patients show eltrombopag may be effective in raising platelet counts and reducing bleeding events.[66,67] Thus, available data do not allow us to recommend the use of thrombopoietin receptor agonists, and these should be restricted to clinical trials.

Lenalidomide and other immunomodulatory drugs

By still undefined biological mechanisms, MDS patients with chromosome 5q deletion (del(5q)) have a special sensitivity to lenalidomide, a thalidomide derivative with potent immunomodulatory and antiangiogenic properties but lacking the neurotoxicity of the maternal compound. Lenalidomide induces high rates of RBC transfusion independence (RBC-TI 43%-67%) and cytogenetic response (25%-73%), including complete cytogenetic response (16%-45%), in lower-risk MDS patients (IPSS low or Intermediate-1) with del(5q) and RBC transfusion dependency.[68-70] Rates of RBC-TI and cytogenetic response after lenalidomide seem higher with cycles of 10 mg per day for 21-28 days every 28 days than with 5 mg per day for 28 days[68-70] and, therefore, the former is the recommended dose for starting therapy. Erythroid response is usually seen after only 1-3 cycles of lenalidomide (median time to response 5 weeks) and median duration of RBC-TI is longer than two years.[68-70] The most common grade 3 or 4 adverse events in patients treated

with lenalidomide are neutropenia (55%-75%) and thrombocytopenia (33%-44%), and G-CSF is commonly used to prevent or ameliorate neutropenia.[68-70] The occurrence of deep venous thrombosis has been reported in 1%-6% of the patients.[70] Data on the effect of lenalidomide treatment on OS and progression to AML are limited. Long-term outcomes were not addressed in the earlier single-arm clinical trials of lenalidomide in this subset of patients[68,69] and the cross-over design of the MDS-004 randomized placebo-controlled clinical trial[70] precluded meaningful analysis of the long-term effect of lenalidomide. In that study, the cumulative probability of AML evolution for lenalidomide-treated patients was 17% at two years and 25% at three years from randomization.[70] Thus, lack of data and reports of a possible increase in the risk of progression to AML, especially in non-responders,[71] are of concern.[72] As there is no randomized clinical trial in progress or planned to determine the potential long-term influence of lenalidomide, the only way to address this issue is by means of large retrospective comparative studies that use the most adequate methodology to reduce their limitations. Interestingly, three such studies currently available employing different methodologies did not show any significant impact of lenalidomide treatment on AML risk (J Sánchez-García *et al.*, submitted manuscript, 2013).[73,74] In contrast, the effect of lenalidomide on OS in these retrospective comparative studies differed. In the GFM retrospective series, which used a complex propensity score to try to match as much as possible their untreated control cohort (n=71) to the lenalidomide-treated group (n=95) and accounting for the time-dependent characteristic of treatment with lenalidomide by considering different initial time points for estimating survival in both cohorts (date of diagnosis for untreated and date of starting lenalidomide for treated patients, respectively), OS was similar in both cohorts.[73] The retrospective multinational study, which compared a cohort of 295 lenalidomide-treated patients included in previous clinical trials in RBC transfusion-dependent patients with lower-risk MDS and del(5q)[69,70] with a historical untreated cohort of 125 similar patients and that used left truncation to adjust for study entry differences between cohorts, the lenalidomide-treated group had superior OS.[74] This effect on OS was not evident in univariate analysis (P=0.76) and only become apparent in multivariate analysis (P=0.012), which is surprising because base-line characteristics, except for a higher RBC transfusion burden in lenalidomide-treated patients, were well balanced across cohorts. The GESMD study, which assessed the effect of lenalidomide therapy on outcomes by multivariable time-dependent methodology and included 86 patients receiving lenalidomide with 125 untreated patients, did not reveal any significant impact of lenalidomide treatment in OS either in the overall series of lower-risk MDS patients with del(5q) or in the smaller group of RBC transfusion-dependent patients (J Sánchez-García *et al.*, submitted manuscript, 2013). Nevertheless, in this study those patients who attained RBC-TI or, more especially, those who showed a cytogenetic response with lenalidomide had a substantial survival benefit (J Sanchez-Garcia *et al*., submitted manuscript, 2013). These results are in line with those observed in the MDS-004 trial in which patients showing an RBC-TI response with lenalidomide had longer OS and AML-free survival than RBC-TI non-responders and where there was also a trend for better OS for cytogenetic responders compared with cytogenetic non-responders.[70] Identification of biomarkers of response to lenalidomide is important because benefit of lenalidomide treatment in lower-risk MDS patients with del(5q) seems to be restricted to responding patients and the observation of stem cells carrying del(5q) in most patients in cytogenetic remission, which suggests that lenalidomide alone does not cure MDS with del(5q).[75] A base-line platelet count lower than 150x10^9/L and presence of *TP53* gene mutations, observed in up to 20% of patients,[76-78] have been associated with a lower probability of response and greater risk of AML progression.[70,77]

Data for lenalidomide treatment in other subsets of MDS patients are very limited. In a series of 47 higher-risk patients, 12 became RBC-TI (median RBC-TI duration 6.5 months) and 7 achieved complete remission (CR) that lasted for a median of 11.5 months.[79] Significantly, 6 of 7 CR patients had del(5q) alone and all 7 a platelet count greater than 100×10^9/L. In a phase II study of 214 lower-risk RBC transfusion-dependent MDS patients without del(5q), 26% of patients attained RBC-TI (median duration 41 weeks) and the overall rate of hematologic improvement was 43%; severe neutropenia and thrombocytopenia occurred in 30% and 25% of patients.[80] A phase III clinical trial in this population is ongoing.

Taking all this information into account, lenalidomide should be considered as first-line treatment in lower-risk MDS patients with del(5q) and RBC transfusion dependence with very low probability of response according to the Nordic score[52,53] or who have failed treatment with ESAs (L Malcovati *et al*., submitted manuscript, 2013).[24] Inclusion of these patients in clinical trials or prospective registries is strongly recommended to enhance our knowledge of lenalidomide's mechanism of action and long-term effect. Patients failing to respond or losing response to lenalidomide should be fully evaluated for presence of TP53 mutations or classical features of disease progression. Lenalidomide could be considered as second-line therapy for non-thrombocytopenic higher-risk MDS with del(5q) alone who have failed or who are not candidates to other therapies.[24] In non-del(5q) patients, lenalidomide use should be restricted to clinical trials. Neurological toxicity of thalidomide makes its use inadvisable, despite this agent being able to induce major erythroid responses in a fraction of patients.[81]

Immunosuppressive therapy

Several reports have shown that treatment with anti-thymocytic globulin (ATG) with or without cyclosporine A (CSA) induces hematologic responses in one-third of patients, especially in those with younger age, hypoplastic marrow, lower-risk IPSS without excess of marrow blasts, presence of DR15, and short disease duration.[82-86] However, severe toxicity is frequent and one small series reported a 90% treatment-related mortality (TRM).[87] Although one retrospective comparative study reported a survival benefit for ATG-treated patients in comparison to untreated patients,[85] a recent prospective randomized trial of ATG plus CSA *versus* best supportive care did not detect any significant difference in OS and AML-free survival.[86] Based on these data, immunosuppressive therapy

with ATG should only be offered to younger patients with RBC transfusion dependency, lower-risk disease, and hypoplastic marrow who have failed first-line therapy (L Malcovati *et al*., submitted manuscript, 2013).[24] This treatment should be delivered only in centers with great experience in the use of ATG and under close surveillance.[24]

Hypomethylating drugs

The notable efficacy of hypomethylating agents, azacitidine and decitabine, in higher-risk MDS patients has substantially changed their therapeutic management.

Azacitidine has demonstrated in two prospective randomized trials to be superior to conventional care approaches in terms of overall response, partial remission, and CR rates, time to progression to AML, OS, and quality of life.[88-90] The most frequent toxicity is hematologic, but TRM is low.[88] Survival benefit is apparent in different age, FAB and WHO, and cytogenetic risk categories,[90-92] and in comparison with low-dose cytarabine,[90,93] but azacitidine advantage over intensive AML-type chemotherapy remains unproven.[90] The GFM has shown that OS after azacitidine is poorer in patients with blasts in blood, intermediate or high-risk IPSS cytogenetics, high RBC transfusion dependency, and poor ECOG performance status.[94] Preliminary results of azacitidine in patients in CR after AML chemotherapy,[95] and before[96,97] and after allo-HSCT[98] are promising, but its benefit in comparison to other approaches is uncertain. The approved schedule of azacitidine is 75 mg/m^2/day for seven consecutive days every 28 days subcutaneously, but alternative schedules avoiding weekend administration, such as 75 mg/m^2/day for five days, two days off, and 75 mg/m^2/day for two additional days or 75 mg/m^2/day x five days are commonly used in daily practice. These alternative schedules have shown mainly in lower-risk patients a similar RBC-TI rate to the conventional schedule,[99] and although their long-term efficacy remains unproven, given the strong relationship between achieving RBC-TI and OS after azacitidine,[94] it seems reasonable to use them.[24] As pilot study results with an oral formulation of azacitidine appear similar to those achieved subcutaneously,[100,101] more definitive data are eagerly awaited.

Low-dose decitabine (15 mg/m^2) given intravenously over four hours three times a day for three days in 6-week cycles has also shown in two prospective randomized trials to be beneficial in comparison with supportive care in terms of overall response, partial remission and CR rates, time to AML, and quality of life.[102,103] However, OS was not significantly longer in any of both studies. Alternative dose schedules not requiring hospitalization have similar efficacy,[104,105] and in a retrospective study have demonstrated better OS compared to intensive AML-chemotherapy.[106] In a randomized trial, the best alternative decitabine schedule was 20 mg/m^2 intravenously daily for five days.[104] Despite no formal comparison between azacitidine and decitabine having been performed, CR rate (but not overall response rate) and neutropenic fever seem to be higher with decitabine.

Based on current evidence, azacitidine is preferable to decitabine (L Malcovati *et al*., submitted manuscript, 2013).[24] Azacitidine should be considered as first-line treatment in higher-risk MDS not considered candidates for intensive treatment. Azacitidine should also be considered as first-line treatment in higher-risk patients who are candidates to intensive treatment but lack an appropriate donor for allo-HSCT (L Malcovati *et al*., submitted manuscript, 2013).[24] In this situation, the selection of initial treatment (azacitidine or AML-type chemotherapy) should be based on patient-related (age and comorbidity) and disease-related (chromosomal abnormalities) factors.[24] In patients aged over 65 years or with comorbidity,[24] and in those presenting high-risk cytogenetics (L Malcovati *et al*., submitted manuscript, 2013),[24] azacitidine is the best option. In the remaining cases, the choice is uncertain.

AML-type chemotherapy and autologous HSCT

With remission induction AML-type chemotherapy, the CR, early death and refractory disease rates are close to 50%, 25%, and 25%, respectively.[24,106-111] Long-term results after AML-type intensive chemotherapy are poor with a high relapse rate (75%), short remission (median 8 months) and OS length (median 12 months), and low disease free-survival (DFS) rate (10%-15%).[106-111] The use of newer chemotherapeutic agents and G-CSF has not improved those results.[109,110] Probability of long-term DFS is remote for patients aged over 65 years, with comorbidity or high-risk cytogenetics.[109,111] Although there are no studies formally comparing quality of life after azacitidine and intensive AML-type chemotherapy, hospital stay is much more prolonged with chemotherapy.

There is no evidence to support the use of autologous HSCT in MDS. Relapse rate is high (75%), especially in patients with high-risk cytogenetics (close to 100%), and DFS at four years is 15%.[112,113] Furthermore, a recent randomized trial has been unable to show a significant difference in OS between autologous HSCT and intensive chemotherapy.[114] For the moment there are no reasons to recommend the use of low-dose cytarabine, as this agent is inferior to azacitidine (L Malcovati *et al*., submitted manuscript, 2013).[24,90,93]

Allogeneic HSCT

The only proven curative modality in MDS is allo-HSCT. The probability of DFS at three years after allo-HSCT from an HLA-identical sibling is 40%.[115] Main factors independently associated with outcome include age, comorbidity index, disease status (FAB, WHO morphological subtype, and percentage of blasts at transplantation), cytogenetic risk group according to IPSS, RBC transfusion dependency, iron overload, time from diagnosis to transplantation, and IPSS or WPSS score.[27,38,115-120]

Several relevant questions regarding allo-HSCT remain unclear. One is the optimum time to proceed to transplantation. The Center for International Bone Marrow Transplant Research (CIBMTR), by using a Markov model of decision analysis concluded that the greatest gain in life expectancy was achieved by delaying transplantation until progression in patients with low or intermediate-1 IPSS risk, and with early transplantation in intermediate-2 or high IPSS risk.[121] However, this study did not account for the effect of age, comorbidity or delaying HSCT in transplantation results and did not clearly define progression. Using a more sophisticated decision model, the Italian Group of Bone Marrow Transplantation (Gruppo Italiano di Trapianto di Midollo Osseo, GITMO) has con-

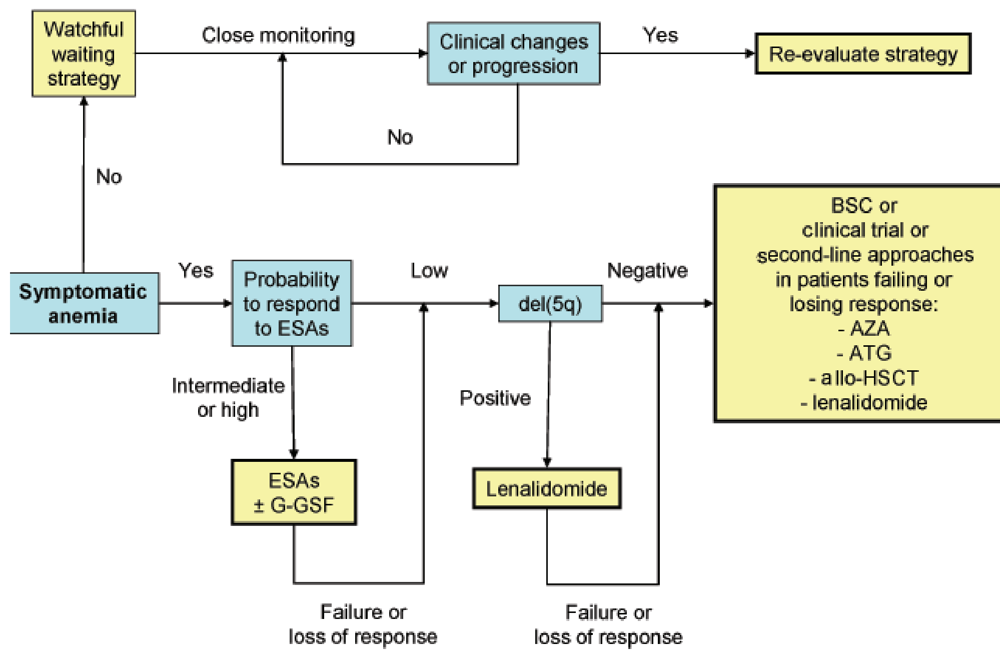

Figure 1. Therapeutic algorithm for lower-risk MDS patients as defined by GESMD (see text). ESAs: erythropoiesis-stimulating agents; G-CSF: granulocyte colony-stimulating factor; del(5q): deletion 5q; BSC: best supportive care; AZA: azacitidine; ATG: anti-thymocytic globulin; Allo-HSCT: allogeneic hematopoietic stem cell transplantation. Probability of response to ESAs according to Hellstrom-Lindberg *et al.*[51,52] (see text).

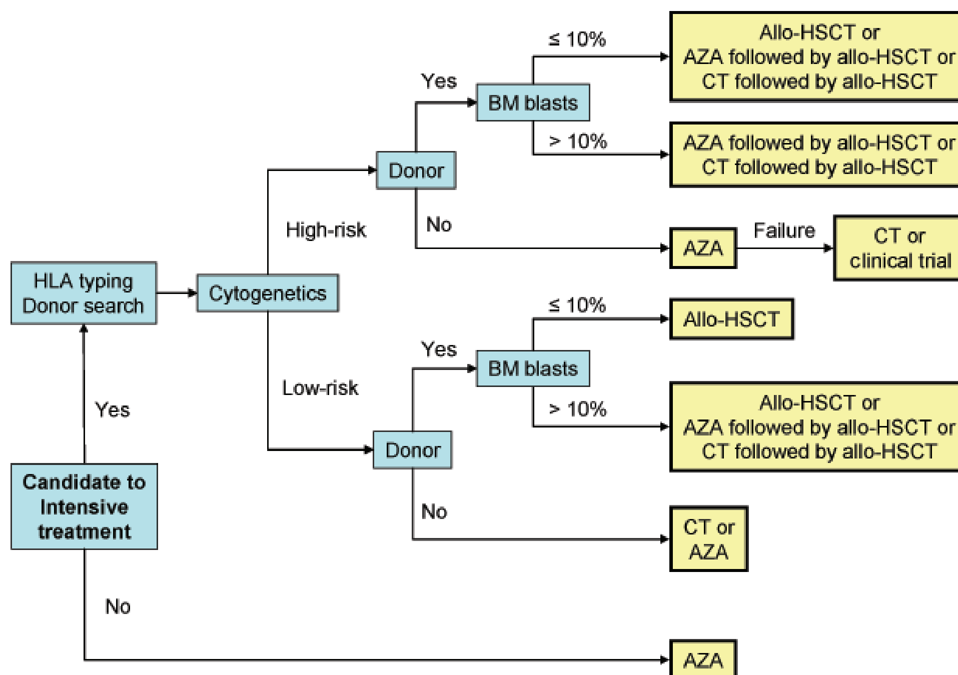

Figure 2. Therapeutic algorithm for higher-risk MDS patients as defined by GESMD (see text). BM: bone marrow; Allo-HSCT: allogeneic hematopoietic stem cell transplantation; CT: AML-type chemotherapy; AZA: azacitidine. High-risk cytogenetics includes -7 and inv(3)/t(3q)/del(3q) as single abnormalities, double abnormalities including -7/del(7q), and complex/very complex (≥3) abnormalities; and low-risk cytogenetics the remaining abnormalities.

firmed the benefit of early transplantation in higher-risk patients, but has suggested that delaying transplant may be harmful in patients with intermediate-1 IPSS risk or intermediate WPSS risk, and particularly more so the younger they are.[122] The preferable source of hematopoietic stem cells has not been determined, although some retrospective series show better results with mobilized peripheral blood,[123,124] and the ideal conditioning regimen has not yet been established.[125] Finally, another important unsolved issue is the convenience to administer AML-type chemotherapy or, more recently, hypomethylating agents before transplantation in patients with advanced disease (i.e. >10% marrow blasts) or high-risk cytogenetics. AML-type chemotherapy is an excellent method for selecting good candidates for transplant (those who attain CR) but prevents the transplant in a substantial number of patients. Hypomethylating drugs, especially azacitidine, are less toxic than AML-type chemotherapy, which make them preferable when a reduced intensity conditioning (RIC) regimen is planned. On the contrary, these drugs are less efficient in terms of CR rate. No randomized prospective trial comparing both strategies has been reported. In a retrospective study from Seattle transplant outcomes were similar.[97] The use of unrelated donors and RIC has greatly expanded the access to transplant. Although the probability of DFS is somewhat lower than after HLA-identical sibling donors,[126,127] the results of allo-HSCT with unrelated donors have improved in recent years thanks to best HLA typing and supportive care.[125] Apart from the degree of HLA compatibility, the characteristics with prognostic influence in this transplant modality are very similar to those after HLA-identical siblings.[125-127] There has not been enough experience with umbilical cord blood transplantation to be able to draw meaningful conclusions.[128,129] Allo-HSCT with RIC is especially attractive in MDS. A large retrospective comparative multicenter study has shown a lower relapse rate with myeloablative conditioning (MAC), lower treatment-related mortality with RIC, and no clear differences in DFS between both types of conditioning, despite the fact that many patients receiving RIC where considered unsuitable for MAC due to advanced age or comorbidity.[130] Data on so-called sequential conditioning transplant are still immature.[131]

Taking all this information into account, allo-HSCT is considered the treatment of choice for higher-risk MDS. Thus, the first thing to consider in higher-risk patients is to ascertain whether the patient is eligible for and accepts such a procedure, and if so, whether a suitable donor is available. As stated above, age comorbidity, performance status, frailty, other personal circumstances, and patient's preference should be taken into account when defining eligibility. The patient should be HLA-typed shortly after diagnosis. If there is no HLA-matched family donor a search for an unrelated donor, including both adults and cord blood units, should be started immediately to be ready to perform the transplant whenever indicated. An RIC could be recommended for patients with advanced age or comorbidity (L Malcovati *et al*., submitted manuscript, 2013).[24]

New agents and combinations

The available information on the use of new drugs, such as histone deacetylase inhibitors (vorinostat, valproic acid), novel nucleosides (clofarabine, sapacitabine), farnesyltransferase inhibitors (tipifarn, lorafarnib), kinase inhibitors (rigosertib, ezatiostat, erlotinib), aminopeptidase inhibitors (tosedostat) and others (siltuximab), is limited to preliminary results of phase I/II trials in a very small number of patients. In most cases, the use of these new agents is being investigated in patients who have failed hypomethylating agents and have a dismal prognosis.[132] Similarly, information about the use of combinations of new agents is still at a very preliminary stage and is, therefore, not able to establish any recommendation on their use.

Risk-adapted treatment algorithm for patients with MDS

Figures 1 and 2 show the suggested risk-adapted treatment algorithm for lower- and higher-risk patients with MDS of the current guidelines for management of patients with MDS of the GESMD.[24]

References

1. Mufti GJ, Stevens JR, Oscier DG, Hamblin TJ, Machin D. Myelodysplastic syndromes: a scoring system with prognostic significance. Br J Haematol. 1985;59:425-33.
2. Sanz GF, Sanz MA, Vallespi T, Canizo MC, Torrabadella M, García S, et al. Two regression models and a scoring system for predicting survival and planning treatment in myelodysplastic syndromes: a multivariate analysis of prognostic factors in 370 patients. Blood. 1989;74:395-408.
3. Morel P, Hebbar M, Lai JL, Duhamel A, Preudhomme C, Wattel E, et al. Cytogenetic analysis has strong independent prognostic value in de novo myelodysplastic syndromes and can be incorporated in a new scoring system: a report on 408 cases. Leukemia. 1993;7:1315-23.
4. Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood. 1997;89(6):2079-88.
5. Malcovati L, Germing U, Kuendgen A, Della Porta MG, Pascutto C, Invernizzi R, et al. Time-dependent prognostic scoring system for predicting survival and leukemic evolution in myelodysplastic syndromes. J Clin Oncol. 2007;25(23):3503-10.
6. Malcovati L, Porta MG, Pascutto C, Invernizzi R, Boni M, Travaglino E, et al. Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: a basis for clinical decision making. J Clin Oncol. 2005;23(30):7594-603.
7. Malcovati L, Della Porta MG, Strupp C, Ambaglio I, Kuendgen A, Nachtkamp K, et al. Impact of the degree of anemia on the outcome of patients with myelodysplastic syndrome and its integration into the WHO classification-based Prognostic Scoring System (WPSS). Haematologica. 2011;96:1433-40.
8. Sanz G, Nomdedeu B, Such E, Bernal T, Belkaid M, Ardanaz MT, et al. Independent impact of iron overload and transfusion dependency on survival and leukemic evolution in patients with myelodysplastic syndrome. ASH Annual Meeting Abstracts. Blood. 2008;112(11):640.
9. Della Porta MG, Malcovati L, Boveri E, Travaglino E, Pietra D, Pascutto C, et al. Clinical relevance of bone marrow fibrosis and CD34-positive cell clusters in primary myelodysplastic syndromes. J Clin Oncol. 2009;27(5):754-62.
10. Córdoba I, González-Porras JR, Such E, Nomdedeu B, Luno E, de Paz R, et al. The degree of neutropenia has a prognostic impact in low risk myelodysplastic syndrome. Leuk Res. 2012;36(3):287-92.
11. González-Porras JR, Cordoba I, Such E, Nomdedeu B, Vallespi T, Carbonell F, et al. Prognostic impact of severe thrombocytopenia in low-risk myelodysplastic syndrome. Cancer. 2011;117(24):5529-37.
12. Schanz J, Steidl C, Fonatsch C, Pfeilstocker M, Nosslinger T,

Tuechler H, et al. Coalesced multicentric analysis of 2,351 patients with myelodysplastic syndromes indicates an underestimation of poor-risk cytogenetics of myelodysplastic syndromes in the international prognostic scoring system. J Clin Oncol. 2011;29(15):1963-70.
13. Schanz J, Tüchler H, Solé F, Mallo M, Luño E, Cervera J, et al. New comprehensive cytogenetic scoring system for primary myelodysplastic syndromes (MDS) and oligoblastic acute myeloid leukemia after MDS derived from an international database merge. J Clin Oncol. 2012;30(8):820-9.
14. Greenberg PL, Tuechler H, Schanz J, Sanz G, Garcia-Manero G, Solé F, et al. Revised international prognostic scoring system for myelodysplastic syndromes. Blood. 2012;120(12):2454-65.
15. Van de Loosdrecht AA, Westers TM, Westra AH, Drager AM, van der Velden VH, Ossenkoppele GJ. Identification of distinct prognostic subgroups in low- and intermediate-1-risk myelodysplastic syndromes by flow cytometry. Blood. 2008;111(3):1067-77.
16. Westers TM, Ireland R, Kern W, Alhan C, Balleisen JS, Bettelheim P, et al. Standardization of flow cytometry in myelodysplastic syndromes: a report from an international consortium and the European LeukemiaNet Working Group. Leukemia. 2012;26(7):1730-41.
17. Bejar R, Stevenson K, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, et al. Clinical effect of point mutations in myelodysplastic syndromes. N Engl J Med. 2011;364:2496-506.
18. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. Nature. 2011;478(7367):64-9.
19. Malcovati L, Papaemmanuil E, Bowen DT, Boultwood J, Della Porta MG, Pascutto C, et al. Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms. Blood. 2011;118:6239-46.
20. Bejar R, Stevenson KE, Caughey BA, Abdel-Wahab O, Steensma DP, Galili N, et al. Validation of a prognostic model and the impact of mutations in patients with lower-risk myelodysplastic syndromes. J Clin Oncol. 2012;30(27):3376-82.
21. Bowen D, Culligan D, Jowitt S, Kelsey S, Mufti G, Oscier D, et al. Guidelines for the diagnosis and therapy of adult myelodysplastic syndromes. Br J Haematol. 2003;120(2):187-200.
22. Santini V, Alessandrino PE, Angelucci E, Barosi G, Billio A, Di Maio M, et al. Clinical management of myelodysplastic syndromes: update of SIE, SIES, GITMO practice guidelines. Leuk Res. 2010;34(12):1576-88.
23. Greenberg PL, Attar E, Bennett JM, Bloomfield CD, De Castro CM, et al. NCCN Clinical Practice Guidelines in Oncology: myelodysplastic syndromes. J Natl Compr Canc Netw. 2011;9(1):30-56.
24. Grupo Español de Síndromes Mielodisplásicos. Guías de diagnóstico y tratamiento de los síndromes mielodisplásicos y la leucemia mielomonocítica crónica. Haematologica. (ed. española) 2012;97(5 Suppl):1-58.
25. Morel P, Declercq C, Hebbar M, Bauters F, Fenaux P. Prognostic factors in myelodysplastic syndromes: critical analysis of the impact of age and gender and failure to identify a very-low-risk group using standard mortality ratio techniques. Br J Haematol. 1996;94:116-9.
26. Della Porta MG, Malcovati L, Strupp C, Ambaglio I, Kuendgen A, Zipperer E, et al. Risk stratification based on both disease status and extra-hematologic comorbidities in patients with myelodysplastic syndrome. Haematologica. 2011;96:441-9.
27. Sorror ML, Sandmaier BM, Storer BE, Maris MB, Baron F, Maloney DG, et al. Comorbidity and disease status based risk stratification of outcomes among patients with acute myeloid leukemia or myelodysplasia receiving allogeneic hematopoietic cell transplantation. J Clin Oncol. 2007;25(27):4246-54.
28. Sanz G, Solé F, Vallespí T, Benavente C, Cervera J, Díez-Campelo M, et al. [Estratificación pronóstica de los síndromes mielodisplásicos.] Haematologica. (ed. española). 2012;97(Suppl 5):11-4.
29. Hellström-Lindberg E, Malcovati L. Supportive care, growth factors, and new therapies in myelodysplastic syndromes. Blood Rev. 2008;22(2):75-91.
30. Oliva EN, Dimitrov BD, Benedetto F, D'Angelo A, Nobile F. Hemoglobin level threshold for cardiac remodelling and quality of life in myelodysplastic syndrome. Leuk Res. 2005;29(10):1217-9.
31. Goldberg SL, Chen E, Corral M, Guo A, Mody-Patel N, Pecora AL, et al. Incidence and clinical complications of myelodysplastic syndromes among United States Medicare beneficiaries. J Clin Oncol. 2010;28(17):2847-52.
32. Kantarjian H, O'Brien S, Ravandi F, Cortés C, Shan J, Bennett JM, et al. Proposal for a new risk model in myelodysplastic syndrome that account for events not considered in the original International Prognostic Scoring System. Cancer. 2008;113:1351-61.
33. Toma A, Fenaux P, Dreyfus F, Cordonnier C. Infections in myelodysplastic syndromes. Haematologica. 2012;97(10):1459-70.
34. Roy NB, Myerson S, Schuh AH, Bignell P, Patel R, Wainscoat JS, et al. Cardiac iron overload in transfusion-dependent patients with myelodysplastic syndromes. Br J Haematol. 2011;154:521-4.
35. Schafer AI, Cheron RG, Dluhy R, et al. Clinical consequences of acquired transfusional iron overload in adults. N Engl J Med. 1981;304:319-24.
36. Altès A, Remacha AF, Sardá P, Baiget M, Sureda A, Martino R, et al. Early clinical impact of iron overload in stem cell transplantation. A prospective study. Ann Hematol. 2007;86(6):443-7.
37. Armand P, Kim HT, Cutler CS, Ho VT, Koreth J, Alyea EP, et al. Prognostic impact of elevated pre-transplant serum ferritin in patients undergoing myeloablative stem cell transplantation. Blood. 2007;109:4586-8.
38. Alessandrino EP, Della Porta MG, Bacigalupo A, Van Lint MT, Falda M, Onida F, et al. WHO classification and WPSS predict posttransplantation outcome in patients with myelodysplastic syndrome: a study from the Gruppo Italiano Trapianto di Midollo Osseo (GITMO). Blood. 2008;112(3):895-902.
39. Rose C, Brechignac S, Vassilief D, Pascal L, Stamatoullas A, Guerci A, et al. Does iron chelation therapy improve survival in lower risk MDS patients? A multicenter study by the GFM. Leuk Res. 2010;34(7):864-70.
40. Leitch HA, Chan C, Leger CS, Foltz LM, Ramadan KM, Vickars LM. Improved survival with iron chelation therapy for red blood cell transfusion dependent lower IPSS risk MDS may be more significant in patients with a non-RARS diagnosis. Leuk Res. 2012;36(11):1380-6.
41. Neukirchen J, Fox F, Kündgen A, Nachtkamp K, Strupp C, Haas R, et al. Improved survival in MDS patients receiving iron chelation therapy - a matched pair analysis of 188 patients from the Düsseldorf MDS registry. Leuk Res. 2012;36(8):1067-70.
42. Jensen PD, Jensen FT, Christensen T, Nielsen JL, Ellegaard J. Relationship between hepatocellular injury and transfusional iron overload prior to and during iron chelation with desferrioxamine: a study in adult patients with acquired anemias. Blood. 2003;101:91-96.
43. Cermak J, Jonasova A, Vondrakova J, et al. Efficacy and safety of administration of oral iron chelator deferiprone in patients with early myelodysplastic syndrome. Hemoglobin. 2011;35(3):217-27.
44. Gattermann N, Finelli C, Porta MD, Fenaux P, Ganser A, Guerci-Bresler A, et al. Deferasirox in iron-overloaded patients with transfusion-dependent myelodysplastic syndromes: Results from the large 1-year EPIC study. Leuk Res. 2010;34:1143-50.
45. List AF, Baer MR, Steensma DP, Raza A, Esposito J, Martinez-Lopez N, et al. Deferasirox reduces serum ferritin and labile plasma iron in RBC transfusion-dependent patients with myelodysplastic syndrome. J Clin Oncol. 2012;30:2134-9.
46. Gattermann N, Finelli C, Della Porta M, Fenaux P, Stadler M, Guerci-Bresler A, et al. Hematologic responses with deferasirox therapy in transfusion-dependent myelodysplastic syndromes patients. Haematologica. 2012;97(9):1364-71.
47. Ross SD, Allen IE, Probst CA, Sercus B, Crean SM, Ranganathan G. Efficacy and safety of erythropoiesis-stimulating proteins in myelodysplastic syndrome: a systematic review and meta-analysis. Oncologist. 2007;12(10):1264-73.
48. Moyo V, Lefebvre P, Duh MS, Yektashenas B, Mundle S. Erythropoiesis-stimulating agents in the treatment of anemia in myelodysplastic syndromes: a meta-analysis. Ann Hematol. 2008;87:527-36.
49. Mundle S, Lefebvre P, Vekeman F, Duh MS, Rastogi R, Moyo V. An assessment of erythroid response to epoetin alpha as a single agent versus in combination with granulocyte- or granulocyte-macrophage-colony-stimulating factor in myelodysplastic syndromes using a meta-analysis approach. Cancer. 2009;115(4):706-15.
50. Santini V. Clinical use of erythropoietic stimulating agents in

myelodysplastic syndromes. Oncologist. 2011;16(3 Suppl): 35-42.

51. Terpos E, Mougiou A, Kouraklis A, Chatzivassili A, Michalis E, Giannakoulas N, et al. Prolonged administration of erythropoietin increases erythroid response rate in myelodysplastic syndromes: a phase II trial in 281 patients. Br J Haematol. 2002;118(1):174-80.
52. Hellström-Lindberg E, Negrin R, Stein R, Krantz S, Lindberg G, Vardiman J, et al. Erythroid response to treatment with G-CSF plus erythropoietin for the anaemia of patients with myelodysplastic syndromes: proposal for a predictive model. Br J Haematol. 1997;99(2):344-51.
53. Hellström-Lindberg E, Gulbrandsen N, Lindberg G, Ahlgren T, Dahl IM, Dybedal I, et al. A validated decision model for treating the anaemia of myelodysplastic syndromes with erythropoietin + granulocyte colony-stimulating factor: significant effects on quality of life. Br J Haematol. 2003;120(6):1037-46.
54. Jadersten M, Montgomery SM, Dybedal I, Porwit-MacDonald A, Hellström-Lindberg E. Long-term outcome of treatment of anemia in MDS with erythropoietin and GCSF. Blood. 2005;106(3):803-11.
55. Kelaidi C, Beyne-Rauzy O, Braun T, Sapena R, Cougoul P, Adès L, et al. High response rate and improved exercise capacity and quality of life with a new regimen of darbepoetin alfa with or without filgrastim in lower-risk myelodysplastic syndromes: a phase II study by the GFM. Ann Hematol. 2013;92(5):621-31.
56. Park S, Grabar S, Kelaidi C, Beyne-Rauzy O, Picard F, Bardet V, et al. Predictive factors of response and survival in myelodysplastic syndrome treated with erythropoietin and G-CSF: the GFM experience. Blood. 2008;111:574-82.
57. Jadersten M, Malcovati L, Dybedal I, Della Porta MG, Invernizzi R, Montgomery SM, et al. Erythropoietin and granulocyte-colony stimulating factor treatment associated with improved survival in myelodysplastic syndrome. J Clin Oncol. 2008;26(21):3607-13.
58. Olnes MJ, Poon A, Miranda SJ, Pfannes L, Tucker Z, Loeliger K, et al. Effects of granulocyte-colony-stimulating factor on monosomy 7 aneuploidy in healthy hematopoietic stem cell and granulocyte donors. Transfusion. 2012;52(3):537-41.
59. Sloand EM, Yong AS, Ramkissoon S, Solomou E, Bruno TC, Kim S, et al. Granulocyte colony-stimulating factor preferentially stimulates proliferation of monosomy 7 cells bearing the isoform IV receptor. Proc Natl Acad Sci USA. 2006;103(39):14483-8.
60. Kantarjian H, Fenaux P, Sekeres MA, Becker PS, Boruchov A, Bowen D, et al. Safety and efficacy of romiplostim in patients with lower-risk myelodysplastic syndrome and thrombocytopenia. J Clin Oncol. 2010;28:437-44.
61. Giagounidis A, Mufti GJ, Kantarjian HM, Fenaux P, Sekeres M, Szer J, et al. Treatment with the thrombopoietin (TPO)-receptor agonist romiplostim in thrombocytopenic patients (pts) with low or intermediate-1 (int-1) risk myelodysplastic syndrome (MDS): Results of a randomized, double-blind, placebo(PBO)-controlled study. ASH Annual Meeting Abstracts. Blood. 2011;118:117.
62. Kantarjian HM, Mufti GJ, Fenaux P, Sekeres MA, Szer J, Platzbecker U, et al. Treatment with the thrombopoietin (TPO)-receptor agonist romiplostim in thrombocytopenic patients (pts) with low or intermediate-1 (int-1) risk myelodysplastic syndrome (MDS): Follow-up AML and survival results of a randomized, double-blind, placebo (PBO)-controlled study. ASH Annual Meeting Abstracts. Blood. 2012;120:421.
63. Kantarjian HM, Giles FJ, Greenberg PL, Paquette RL, Wang ES, Gabrilove JL, et al. Phase 2 study of romiplostim in patients with low- or intermediate-risk myelodysplastic syndrome receiving azacitidine therapy. Blood. 2010;116:3163-70.
64. Greenberg PL, Garcia-Manero G, Moore M, Damon L, Roboz G, Hu K, et al. A randomized controlled trial of romiplostim in patients with low- or intermediate-risk myelodysplastic syndrome receiving decitabine. Leuk Lymphoma. 2013;54(2):321-8.
65. Wang ES, Lyons RM, Larson RA, Gandhi S, Liu D, Matei C, et al. A randomized, double-blind, placebo-controlled phase 2 study evaluating the efficacy and safety of romiplostim treatment of patients with low or intermediate-1 risk myelodysplastic syndrome receiving lenalidomide. J Hematol Oncol. 2012 Nov 29. [Epub ahead of print]
66. Oliva EN, Santini V, Zini G, et al. Efficacy and safety of eltrombopag for the treatment of thrombocytopenia of low and intermediate-1 IPSS risk myelodysplastic syndromes: Interim analysis of a prospective, randomized, single-blind, placebo-controlled trial (EQoL-MDS). ASH Annual Meeting Abstracts. Blood. 2012;120:923.
67. Mittelman M, Assouline S, Briasoulis E, et al. Eltrombopag treatment of thrombocytopenia in advanced myelodysplastic syndromes and acute myeloid leukemia: results of the 8-week open-label part of an ongoing study. ASH Annual Meeting Abstracts. Blood. 2012;120:3822.
68. List A, Kurtin S, Roe DJ, Buresh A, Mahadevan D, Fuchs D, et al. Efficacy of lenalidomide in myelodysplastic syndromes. N Engl J Med. 2005;352(6):549-57.
69. List A, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. N Engl J Med. 2006;355(14):1456-65.
70. Fenaux P, Giagounidis A, Selleslag D, Beyne-Rauzy O, Mufti G, Mittelman M, et al. A randomized phase 3 study of lenalidomide versus placebo in RBC transfusion-dependent patients with Low-/Intermediate-1-risk myelodysplastic syndromes with del5q. Blood. 2011;118(14):3765-76.
71. Göhring G, Giagounidis A, Büsche G, Kreipe HH, Zimmermann M, Hellström-Lindberg E, et al. Patients with del(5q) MDS who fail to achieve sustained erythroid or cytogenetic remission after treatment with lenalidomide have an increased risk for clonal evolution and AML progression. Ann Hematol. 2010;89(4):365-74.
72. European Medicines Agency. Questions and answers on recommendation for the refusal of the marketing authorisation for Lenalidomide Celgene Europe. [Internet] Available from: www.emea.europa.eu/pdfs/human/opinion/Lenalidomide_27128808en.pdf - 2008-05-30
73. Ades L, Lebras F, Sebert M, Kelaidi C, Lamy T, Dreyfus F, et al. Treatment with lenalidomide does not appear to increase the risk of leukemia progression in lower risk myelodysplastic syndrome with 5q deletion. A comparative analysis by the GFM. Haematologica. 2012;97(2):213-8.
74. Kuendgen A, Lauseker M, List AF, Fenaux P, Giagounidis AA, Brandenburg NA, et al. Lenalidomide does not increase AML progression risk in RBC transfusion-dependent patients with low-/intermediate-1-risk MDS with del(5q): a comparative analysis. Leukemia. 2013 Feb 1. [Epub ahead of print]
75. Tehranchi R, Woll PS, Anderson K, et al. Persistent malignant stem cells in del(5q) myelodysplasia in remission. New Engl J Med. 2010;363(11):1025-37.
76. Jädersten M, Saft L, Pellagatti A, Göhring G, Wainscoat JS, Boultwood J, et al. Clonal heterogeneity in the 5q- syndrome: p53 expressing progenitors prevail during lenalidomide treatment and expand at disease progression. Haematologica. 2009;94(12):1762-66.
77. Jadersten M, Saft L, Smith A, Kulasekararaj A, Pomplun S, Göhring G, et al. TP53 mutations in low-risk myelodysplastic syndromes with del(5q) predict disease progression. J Clin Oncol. 2011;29(15):1971-9.
78. Sebaa A, Ades L, Baran-Marzack F, Mozziconacci MJ, Penther D, Dobbelstein S, et al. Incidence of 17p deletions and TP53 mutation in myelodysplastic syndrome and acute myeloid leukemia with 5q deletion. Genes Chromosomes Cancer. 2012;51(12):1086-92.
79. Ades L, Boehrer S, Prebet T, Beyne-Rauzy O, Legros L, Ravoet C, et al. Efficacy and safety of lenalidomide in intermediate-2 or high-risk myelodysplastic syndromes with 5q deletion: results of a phase 2 study. Blood. 2009;113(17):3947-52.
80. Raza A, Reeves JA, Feldman EJ, Dewald GW, Bennett JM, Deeg HJ, et al. Phase 2 study of lenalidomide in transfusion-dependent, low-risk, and intermediate-1 risk myelodysplastic syndromes with karyotypes other than deletion 5q. Blood. 2008;111(1):86-93.
81. Raza A, Meyer P, Dutt D, Zorat F, Lisak L, Nascimben F, et al. Thalidomide produces transfusion independence in long-standing refractory anemias of patients with myelodysplastic syndromes. Blood. 2001;98:958-65.
82. Sauntharajah Y, Nakamura R, Nam JM, Wang QJ, Barrett AJ. HLA-DR15 (DR2) is overrepresented in myelodysplastic syndrome and aplastic anemia and predicts a response to immunosuppression in myelodysplastic syndrome. Blood. 2002;100:1570-4.
83. Stadler M, Germing U, Kliche KO, et al. A prospective, randomised, phase II study of horse antithymocyte globulin vs rabbit antithymocyte globulin as immune-modulating therapy in patients with low-risk myelodysplastic syndromes. Leukemia. 2004;18:460-5.
84. Lim ZY, Killick S, Germing U, Cavenagh J, Culligan D, Bacigalupo A, et al. Low IPSS score and bone marrow hypocellularity in MDS patients predict hematological

responses to antithymocyte globulin. Leukemia. 2007;21(7):1436-41.
85. Sloand EM, Wu CO, Greenberg P, Young N, Barrett J. Factors affecting response and survival in patients with myelodysplasia treated with immunosuppressive therapy. J Clin Oncol. 2008;26:2505-11.
86. Passweg JR, Giagounidis AA, Simcock M, Aul C, Dobbelstein C, Stadler M, et al. Immunosuppressive therapy for patients with myelodysplastic syndrome: a prospective randomized multicenter phase III trial comparing antithymocyte globulin plus cyclosporine with best supportive care – SAKK 33/99. J Clin Oncol. 2011;29(3):303-9.
87. Steensma DP, Dispenzieri A, Moore SB, Schroeder G, Tefferi A. Antithymocyte globulin has limited efficacy and substantial toxicity in unselected anemic patients with myelodysplastic syndrome. Blood. 2003;101:2156-8.
88. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol. 2002;20(10):2429-40.
89. Kornblith AB, Herndon JE 2nd, Silverman LR, Demakos EP, Odchimar-Reissig R, Holland JF, et al. Impact of azacytidine on the quality of life of patients with myelodysplastic syndrome treated in a randomized phase III trial: a Cancer and Leukemia Group B study. J Clin Oncol. 2002;20(10):2441-52.
90. Fenaux P, Mufti GJ, Hellström-Lindberg E, Santini V, Finelli C, Giagounidis A, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. Lancet Oncol. 2009;10(3):223-32.
91. Fenaux P, Mufti GJ, Hellström-Lindberg E, Santini V, Gattermann N, Germing U, et al. Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. J Clin Oncol. 2010;28(4):562-9.
92. Seymour JF, Fenaux P, Silverman LR, Mufti GJ, Hellström-Lindberg E, Santini V, et al. Effects of azacitidine compared with conventional care regimens in elderly (≥ 75 years) patients with higher-risk myelodysplastic syndromes. Crit Rev Oncol Hematol. 2010;76(3):218-27.
93. Fenaux P, Gattermann N, Seymour JF, Hellström-Lindberg E, Mufti GJ, Duehrsen U, et al. Prolonged survival with improved tolerability in higher-risk myelodysplastic syndromes: azacitidine compared with low dose ara-C. Br J Haematol. 2010;149(2):244-9.
94. Itzykson R, Thépot S, Quesnel B, Dreyfus F, Beyne-Rauzy O, Turlure P, et al. Prognostic factors for response and overall survival in 282 patients with higher-risk myelodysplastic syndromes treated with azacitidine. Blood. 2011;117:403-11.
95. Grovdal M, Karimi M, Khan R, Aggerholm A, Antunovic P, Astermark J, et al. Maintenance treatment with azacytidine for patients with high-risk myelodysplastic syndromes (MDS) or acute myeloid leukaemia following MDS in complete remission after induction chemotherapy. Br J Haematol. 2010;150(3):293-302.
96. Field T, Perkins J, Huang Y, Kharfan-Dabaja MA, Alsina M, Ayala E, et al. 5-Azacitidine for myelodysplasia before allogeneic hematopoietic cell transplantation. Bone Marrow Transplant. 2010;45(2):255-60.
97. Gerds AT, Gooley TA, Estey EH, Appelbaum FR, Deeg HJ, Scott BL. Pretransplantation therapy with azacitidine vs induction chemotherapy and posttransplantation outcome in patients with MDS. Biol Blood Marrow Transplant. 2012;18(8):1211-8.
98. Platzbecker U, Wermke M, Radke J, Oelschlaegel U, Seltmann F, Kiani A, et al. Azacitidine for treatment of imminent relapse in MDS or AML patients after allogeneic HSCT: results of the RELAZA trial. Leukemia. 2012;26(3):381-9.
99. Lyons RM, Cosgriff TM, Modi SS, Gersh RH, Hainsworth JD, Cohn AL, et al. Hematologic response to three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes. J Clin Oncol. 2009;27(11):1850-6.
100. Garcia-Manero G, Gore SD, Cogle C, Ward R, Shi T, Macbeth KJ, et al. Phase I study of oral azacitidine in myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia. J Clin Oncol. 2011;29:2521-7.
101. Garcia-Manero G, Gore SD, Kambhampati S, Scott BL, Cogle CR, Tefferi A, et al. Safety and efficacy of oral azacitidine (CC-486) administered in extended treatment schedules to patients with lower-risk myelodysplastic syndromes. ASH Annual Meeting Abstracts. Blood. 2012;120:424.
102. Kantarjian H, Issa JP, Rosenfeld CS, Bennett JM, Albitar M, DiPersio J, et al. Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer. 2006;106(8):1794-803.
103. Lubbert M, Suciu S, Baila L, Ruter BH, Platzbecker U, Giagounidis A, et al. Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: final results of the randomized phase III study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group. J Clin Oncol. 2011;29(15):1987-96.
104. Kantarjian H, Oki Y, García-Manero G, Huang X, O'Brien S, Cortes J, et al. Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. Blood. 2007;109(1):52-7.
105. Kantarjian HM, O'Brien S, Shan J, Aribi A, García-Manero G, Jabbour E, et al. Update of the decitabine experience in higher risk myelodysplastic syndrome and analysis of prognostic factors associated with outcome. Cancer. 2007;109(2):265-73.
106. Kantarjian HM, O'Brien S, Huang X, García-Manero G, Ravandi F, Cortes J, et al. Survival advantage with decitabine versus intensive chemotherapy in patients with higher risk myelodysplastic syndrome: comparison with historical experience. Cancer. 2007;109(6):1133-7.
107. Beran M, Shen Y, Kantarjian H, O'Brien S, Koller CA, Giles FJ, et al. High-dose chemotherapy in high-risk myelodysplastic syndrome: covariate-adjusted comparison of five regimens. Cancer. 2001;92(8):1999-2015.
108. Estey EH, Thall PF, Cortes JE, Giles FJ, O'Brien S, Pierce SA, et al. Comparison of idarubicin + ara-C-, fludarabine + ara-C-, and topotecan + ara-C-based regimens in treatment of newly diagnosed acute myeloid leukemia, refractory anemia with excess blasts in transformation, or refractory anemia with excess blasts. Blood. 2001;98(13):3575-83.
109. Kantarjian H, Beran M, Cortes J, O'Brien S, Giles F, Pierce S, et al. Long-term follow-up results of the combination of topotecan and cytarabine and other intensive chemotherapy regimens in myelodysplastic syndrome. Cancer. 2006;106(5):1099-109.
110. Ossenkoppele GJ, van der Holt B, Verhoef GE, Daenen SM, Verdonck LF, Sonneveld P, et al. A randomized study of granulocyte colony-stimulating factor applied during and after chemotherapy in patients with poor risk myelodysplastic syndromes: a report from the HOVON Cooperative Group. Dutch-Belgian Hemato-Oncology Cooperative Group. Leukemia. 1999;13(8):1207-13.
111. Knipp S, Hildebrand B, Kundgen A, Giagounidis A, Kobbe G, Haas R, et al. Intensive chemotherapy is not recommended for patients aged >60 years who have myelodysplastic syndromes or acute myeloid leukemia with high-risk karyotypes. Cancer. 2007;110:345-52.
112. Ducastelle S, Ades L, Gardin C, Dombret H, Prebet T, Deconinck E, et al. Long-term follow-up of autologous stem cell transplantation after intensive chemotherapy in patients with myelodysplastic syndrome or secondary acute myeloid leukemia. Haematologica. 2006;91(3):373-6.
113. Sanz GF, Mena-Duran AV, Ribera JM, Bernal T, Palomera L, del Canizo MC, et al. Autologous stem cell transplantation after FLAG-IDA chemotherapy for high-risk myelodysplastic syndromes (MDS) and acute myeloid leukemias secondary to MDS (sAML) does not improve outcome: A PETHEMA experience in 103 patients. ASH Annual Meeting Abstracts. Blood 2005;106(11):793.
114. de Witte T, Hagemeijer A, Suciu S, Belhabri A, Delforge M, Kobbe G, et al. Value of allogeneic versus autologous stem cell transplantation and chemotherapy in patients with myelodysplastic syndromes and secondary acute myeloid leukemia. Final results of a prospective randomized European Intergroup Trial. Haematologica. 2010;95:1754-61.
115. Sierra J, Pérez WS, Rozman C, Carreras E, Klein JP, Rizzo JD, et al. Bone marrow transplantation from HLA-identical siblings as treatment for myelodysplasia. Blood. 2002;100(6):1997-2004.
116. Appelbaum FR, Barrall J, Storb R, Fisher LD, Schoch G, Ramberg RE, et al. Bone marrow transplantation for patients with myelodysplasia. Pretreatment variables and outcome. Ann Intern Med. 1990;112(8):590-7.
117. Anderson JE, Appelbaum FR, Schoch G, Gooley T, Anasetti C, Bensinger WI, et al. Allogeneic marrow transplantation for refractory anemia: a comparison of two preparative regimens and analysis of prognostic factors. Blood. 1996;87(1):51-8.
118. Sutton L, Chastang C, Ribaud P, Jouet JP, Kuentz M, Attal M, et al. Factors influencing outcome in de novo myelodysplastic syndromes treated by allogeneic bone marrow transplantation:

a long-term study of 71 patients Societe Francaise de Greffe de Moelle. Blood. 1996;88(1):358-65.

119. Nevill TJ, Fung HC, Shepherd JD, Horsman DE, Nantel SH, Klingemann HG, et al. Cytogenetic abnormalities in primary myelodysplastic syndrome are highly predictive of outcome after allogeneic bone marrow transplant. Blood. 1998;92(6):1910-7.
120. de Witte T, Hermans J, Vossen J, Bacigalupo A, Meloni G, Jacobsen N, et al. Haematopoietic stem cell transplantation for patients with myelo-dysplastic syndromes and secondary acute myeloid leukaemias: a report on behalf of the Chronic Leukaemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT). Br J Haematol. 2000;110(3):620-30.
121. Cutler CS, Lee SJ, Greenberg P, Deeg HJ, Pérez WS, Anasetti C, et al. A decision analysis of allogeneic bone marrow transplantation for the myelodysplastic syndromes: delayed transplantation for low-risk myelodysplasia is associated with improved outcome. Blood. 2004;104:579-85.
122. Alessandrino EP, Porta MGD, Malcovati L, Jackson CH, Pascutto C, Bacigalupo A, et al. Decision analysis of allogeneic stem cell transplantation in patients with myelodysplastic syndrome stratified according to the WHO classification-based prognostic scoring system (WPSS). ASH Annual Meeting Abstracts. Blood. 2011;118(21):116.
123. Guardiola P, Runde V, Bacigalupo A, Ruutu T, Locatelli F, Boogaerts MA, et al. Retrospective comparison of bone marrow and granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells for allogeneic stem cell transplantation using HLA identical sibling donors in myelodysplastic syndromes. Blood. 2002;99(12):4370-8.
124. del Canizo MC, Martínez C, Conde E, Vallejo C, Brunet S, Sanz G, et al. Peripheral blood is safer than bone marrow as a source of hematopoietic progenitors in patients with myelodysplastic syndromes who receive an allogeneic transplantation. Results from the Spanish registry. Bone Marrow Transplant. 2003;32(10):987-92.
125. Parmar S, de Lima M, Deeg HJ, Champlin R. Hematopoietic stem cell transplantation for myelodysplastic syndrome: a review. Semin Oncol. 2011;38(5):693-704.
126. Arnold R, de Witte T, van Biezen A, Hermans J, Jacobsen N, Runde V, et al. Unrelated bone marrow transplantation in patients with myelodysplastic syndromes and secondary acute myeloid leukemia: an EBMT survey. European Blood and Marrow Transplantation Group. Bone Marrow Transplant. 1998;21(12):1213-6.
127. Castro-Malaspina H, Harris RE, Gajewski J, Ramsay N, Collins R, Dharan B, et al. Unrelated donor marrow transplantation for myelodysplastic syndromes: outcome analysis in 510 transplants facilitated by the National Marrow Donor Program. Blood. 2002;99(6):1943-51.
128. Sato A, Ooi J, Takahashi S, Tsukada N, Kato S, Kawakita T, et al. Unrelated cord blood transplantation after myeloablative conditioning in adults with advanced myelodysplastic syndromes. Bone Marrow Transplant. 2011;46(2):257-61.
129. Robin M, Sanz GF, Ionescu I, Rio B, Sirvent A, Renaud M, et al. Unrelated cord blood transplantation in adults with myelodysplasia or secondary acute myeloblastic leukemia: a survey on behalf of Eurocord and CLWP of EBMT. Leukemia. 2011;25(1):75-81.
130. Martino R, Iacobelli S, Brand R, Jansen T, van Biezen A, Finke J, et al. Retrospective comparison of reduced-intensity conditioning and conventional high-dose conditioning for allogeneic hematopoietic stem cell transplantation using HLA-identical sibling donors in myelodysplastic syndromes. Blood. 2006;108(3):836-46.
131. Saure C, Schroeder T, Zohren F, Groten A, Bruns I, Czibere A, et al. Upfront allogeneic blood stem cell transplantation for patients with high-risk myelodysplastic syndrome or secondary acute myeloid leukemia using a FLAMSA-based high-dose sequential conditioning regimen. Biol Blood Marrow Transplant. 2012;18(3):466-72.
132. Prebet T, Gore SD, Esterni B, Gardin C, Itzykson R, Thepot S, et al. Outcome of high-risk myelodysplastic syndrome after azacitidine treatment failure. J Clin Oncol. 2011;29(24):3322-7.

The biology of myeloproliferative neoplasms

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A B S T R A C T

The last eight years have seen major advances in the understanding of the molecular pathogenesis of the chronic myeloproliferative neoplasms (MPNs), beginning with the identification of the *JAK2V617F* mutation. This review discusses recent evidence regarding the mechanisms through which *JAK2V617F* causes disease, including the key signaling pathways implicated and how they may be perturbed by other specific mutations in MPNs. However, it is also increasingly apparent that *JAK2V617F* and similar signaling mutations, whilst contributing to MPN phenotype, may require co-operating mutations to drive disease. In particular, mutations in genes for epigenetic modifiers have an important role in MPNs, including those positive and negative for *JAK2V617F*, as well as in other malignant myeloid disorders. We, therefore, also summarize the mechanisms through which mutations in key regulators, including *TET2, IDH1/2, DNMT3A, ASXL1* and PRC2 components, may alter DNA and histone modifications to affect hematopoietic stem cell function. Lastly, we review recent evidence that MPNs are disorders of surprising clonal complexity. This complexity must be unraveled in order to understand how different mutations co-operate or act independently to determine the phenotype of chronic-phase MPNs and to drive disease transformation.

Learning goals

At the conclusion of this activity, participants should know that:

- the *JAK2V617F* mutation results in activation of JAK2 and of multiple downstream signaling pathways; for example, STAT5 activation appears to be particularly important in the phenotype of polycythemia vera;
- non-canonical effects of JAK2V617F in the nucleus may be mediated through direct phosphorylation of histones by JAK2 and indirectly through modification of histone methylation;
- mutations in *TET2, IDH1/2, DNMT3A, EZH2* and *ASXL1* occur recurrently in myeloproliferative neoplasms but are also common in other myeloid malignancies, and are likely to influence hematopoietic stem cell function by affecting DNA methylation or histone modifications directly;
- studies of clonal hematopoietic populations have shown that the structure of clonal hierarchies in MPN patients may be complex, and that dynamic changes in these structures may influence disease phenotype or drive disease transformation.

Introduction

In 1951, William Dameshek speculated that the conditions now known as Philadelphia-negative myeloproliferative neoplasms (MPNs) (polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF)) were pathogenetically related.[1] This hypothesis was confirmed in 2005 by the identification of the *V617F* mutation in the *JAK2* gene in over 95% of patients with PV and 50-60% of those with ET and PMF.[2-5] Several lines of evidence have confirmed that *JAK2V617F* contributes to MPN pathogenesis, and have started to outline the biochemical mechanisms responsible. In parallel, other recurrent mutations have been identified in smaller proportions of MPN patients: some affect similar signaling pathways to *JAK2V617F* (*e.g.* mutations in *JAK2* exon 12 and *MPL*), whilst a growing group affects epigenetic modifiers that regulate chromatin structure and function. In this review, we discuss the biochemical and signaling abnormalities induced by *JAK2V617F* and mutations in similar pathways, followed by the novel epigenetic mechanisms implicated in MPN pathogenesis. Finally, we consider the increasing evidence that MPNs are disorders of significant clonal complexity, both in chronic phase ET and PV, and in the more 'accelerated' phases of myelofibrosis and secondary acute myeloid leukemia.

Signaling abnormalities in MPNs

Canonical signaling abnormalities downstream of JAK2V617F

JAK2 is a cytoplasmic tyrosine kinase that mediates signal transduction from several cytokine receptors including those for erythropoietin and thrombopoietin.[6] Binding of ligand to the receptor drives autophosphorylation of JAK2,[7] receptor transphosphorylation and

binding of STAT proteins, which are phosphorylated and translocate to the nucleus as homodimers or heterodimers to affect gene transcription. The V617F mutation is located within the JH2 or 'pseudokinase' domain of JAK2. This region appears to have an auto-inhibitory function on the catalytically active JH1 domain: JH2 deletion increases JAK2 phosphorylation and the activity of downstream signaling pathways,[8] and when expressed in bacterial cells the JH2 domain negatively regulates JAK2 activity.[9] The V617F mutation has an activating effect on JAK2 activity in cell line-based assays of cytokine sensitivity, cytokine-dependent survival and signaling activity, and it was proposed that this could reflect interference with the JH2 auto-inhibitory function.[2-5] Recent work has suggested one mechanism for this: the JH2 domain itself has serine and tyrosine kinase activity and phosphorylates specific residues that regulate JH1 activity (Figure 1A),[10] and this JH2 kinase activity is impaired in the presence of V617F. Moreover, it appears that V617F co-operates with other specific residues within JH2 to actively promote JH1 kinase activity.[11,12] A greater understanding of the biochemical effects of the V617F mutation has been supported by a recent report of the JH2 domain crystal structure (wild-type and V617F), an advance that may also permit design of more rational targeted drug therapies.[12]

Downstream of JAK2V617F and its cognate receptors, a number of signaling abnormalities (Figure 1B) have been shown using various experimental systems. A role for STAT5 activation in human MPNs is supported by the observation that constitutively active STAT5 in erythroid progenitors can induce the formation of erythropoietin (Epo)-independent endogenous erythroid colonies, a cardinal feature of PV.[13] Increased STAT5 phosphorylation is found in *JAK2V617F*-expressing cell lines[2,4,5] retroviral bone marrow transplantation mouse models,[14,15] erythroblasts cultured *ex vivo* from CD34+ cells of PV patients compared to normal individuals,[16] and in bone marrow trephine biopsies from patients with *JAK2V617F*-positive MPNs compared to normal individuals or *JAK2V617F*-negative MPNs.[17] Activation of phosphatidylinositol-3-kinase (PI(3)K)/Akt and MAPK/ERK pathways have been shown in similar systems in some studies.[2,5,15-17]

The signaling and phenotypic consequences of *JAK2V617F* have also been studied in mouse models. Retroviral bone marrow transplantation models typically developed erythrocytosis, leukocytosis, splenomegaly and, in some cases, bone marrow fibrosis and/or late anemia,[14,15,18] and transgenic models developed variable phenotypes depending on the construct (*e.g.* use of human or murine *JAK2V617F*) and expression levels.[19-21] These models confirmed the ability of *JAK2V617F* to drive a myeloproliferative phenotype, but more recently the development of four knock-in mouse models has allowed study of the mutation when expressed at physiological levels in hematopoietic cells.[22-25] Three knock-in models with a heterozygous murine mutant *Jak2* gene showed a phenotype resembling PV with erythrocytosis, leukocytosis, splenomegaly, variable thrombocytosis and increased megakaryocyte-erythroid progenitors,[23-25] whilst a fourth, with a heterozygous human *JAK2V617F* construct, showed thrombocytosis with only mild erythrocytosis, similar to human ET.[22] The explanation for the phenotypic differences between the heterozygous models is not yet clear, but may include technical issues reflecting the different targeting strategies, or inherent differences between mutant human and mouse proteins.

All four knock-in models showed increased activation of STAT5, either at baseline or following Epo stimulation.[22-25] In one model for which *Jak2V167F*-homozygous mice were generated, STAT5 activation was more pronounced compared to heterozygous mice, together with increased Akt and ERK1/2 activation, and this was associated with more marked neutrophilia, thrombocytosis, splenomegaly and marrow fibrosis.[25] Moreover, the critical importance of STAT5 in *JAK2V617F*-driven PV has been demonstrated by observations that *Stat5* deletion in a *Jak2V617F* knock-in mouse[26] or retroviral bone marrow transplantation[27] model abrogates the erythrocytosis. Interestingly, in the latter model the deletion of *Stat5* did not prevent the development of myelofibrosis,[27] highlighting that different pathways downstream of JAK2 may account for different aspects of MPN phenotype. Recent data also show that expression of *Jak2V617F* in the erythroid lineage is sufficient to drive a PV phenotype, since erythrocytosis develops in murine models when the mutation is activated only in cells expressing the Epo receptor,[28,29] although in one model this phenotype was attenuated compared to expression from the hematopoietic stem cell (HSC) level.[28]

Recent reports have also indicated the importance of crosstalk between JAK2V617F and cytokine pathways other than Epo. The mutation is associated with downregulation of the thrombopoietin receptor (TpoR) by proteosomal degradation in hematopoietic cells, and this has been suggested to prevent anti-proliferative signaling through the TpoR at high levels of JAK2 activation, thus permitting clonal expansion.[30] The presence of JAK2V617F may also directly influence the microenvironment through upregulation of TNFα, a cytokine that inhibits growth of normal hematopoietic progenitors but does not inhibit, or may even stimulate, growth of *JAK2V617F*-mutant cells.[31] Levels of TNFα and other inflammatory cytokines are increased in MPN patients and it will be important to assess the contribution of related molecules to disease pathogenesis.

Non-canonical effects of JAK2V617F

Whilst activation of signaling pathways such as STAT5 can be considered canonical effects of JAK2, a number of non-canonical effects have also been recognized and may contribute to MPN biology (Figure 1C). In 2009, it was reported that JAK2 was present in the nucleus and could phosphorylate histone H3 at Tyr41 (Y41).[32] This phosphorylation prevents the heterochromatin protein HP1α binding to H3 and has direct effects on expression of genes such as *Lmo2*. Analysis of the distribution of phosphorylated H3Y41 by chromatin immunoprecipitation coupled to massively parallel DNA sequencing (ChIP-Seq) has revealed three main locations for this chromatin mark: at certain active promoters; at putative enhancers within non-coding regions of JAK-STAT target genes, where STAT5 also binds; and 'blanketing' the entire coding regions of some active hematopoietic genes.[33] These data are starting to throw light on the mechanisms by which JAK2-induced chromatin modification directly regulates gene expression, both alone and in combination with activated STATs. It also appears that JAK2V617F may modulate chromatin by a second mechanism: the mutant protein was found to

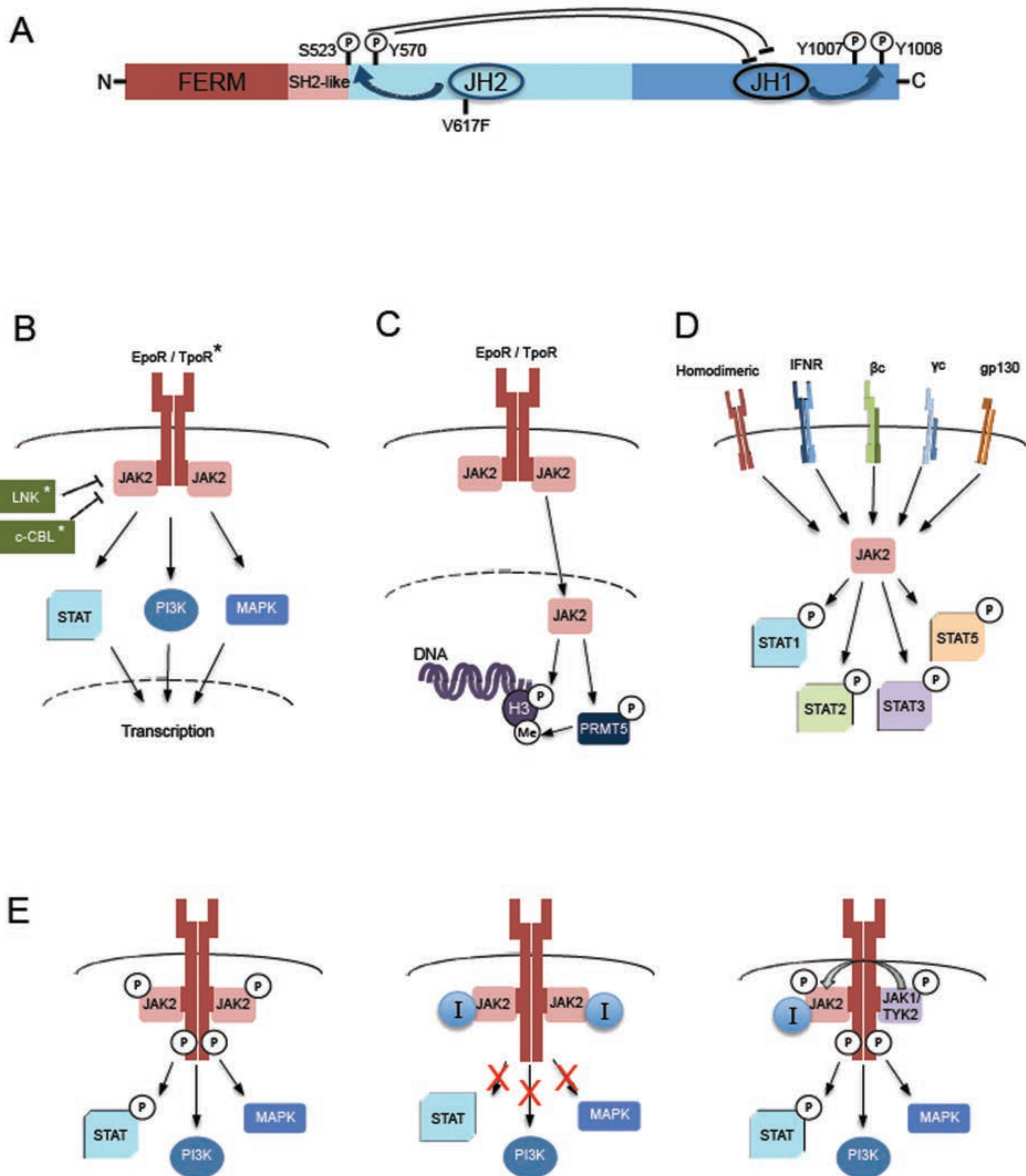


Figure 1. Molecular effects of JAK2V617F. (A) Domain structure of the JAK2 molecule. Recent evidence indicates that the JH2 domain has kinase activity, resulting in phosphorylation of S523 and Y570, which negatively regulate the activity of the JH1 catalytic domain.[10] The JH1 domain usually phosphorylates additional residues including Y1007 and Y1008, of which Y1007 is particularly important in JAK2 activation.[7] The position of V617F in the JH2 domain is also shown. (B) Canonical signaling effects of JAK2V617F. *Molecules other than JAK2 that are affected by recurrent mutations in MPN patients. Note that two mutant JAK2 molecules are shown in association with the receptor, which would occur in cells with *JAK2V617F* homozygosity and also for a minority of receptors in *JAK2V617F*-heterozygous cells. Crosstalk with other pathways such as TNFα are not shown since the pathways responsible are not fully clarified. (C) Nuclear effects of JAK2V617F (see text for details). (D) Complexity of signaling molecules interacting with JAK2 (see text for details). (E) Model for signaling alterations in cells that persist in the presence of JAK2 inhibitors.[35] JAK2 signaling in inhibitor-naïve cells is associated with phosphorylation of the cytokine receptor, JAK2, STATs and activation of downstream pathways (left). On acute treatment with inhibitor (left), all of these processes are inhibited (center). In ‘persistent’ cells, levels of JAK2 are increased and the tyrosine kinases JAK1 and TYK2 can transphosphorylate JAK2 (right). This leads to resumption of downstream signaling, which may in part result from the inhibited JAK2 molecules acting as a scaffold for these processes. P: phosphorylated protein; Me: methylated protein.

bind and phosphorylate the arginine methyltransferase PRMT5 more strongly than wild-type JAK2, thus impairing its histone methylation activity.[34] Given that *PRMT5* knockdown in human CD34+ cells increased formation of hematopoietic colonies and promoted erythroid differentiation, these effects seem likely to contribute to MPN phenotype.

Although the effects of JAK2V617F on STAT5, PI3K/Akt and MAPK/ERK pathways are well documented, it should also be noted that signaling consequences of mutant and wild-type JAK2 have additional layers of complexity. JAK2 not only associates with homodimeric cytokine receptors such as EpoR and TpoR, but also with the interferon-gamma receptor and receptors containing βc, γc and gp130 subunits (*e.g.* GM-CSF, IL-2 and IL-6 receptors, respectively)[6] (Figure 1D). STAT1, STAT2, and STAT3 may be activated downstream in addition to STAT5. Moreover, JAK2 has recently been reported to heterodimerize with the related tyrosine kinases JAK1 and TYK2, and trans-phosphorylation of JAK2 by these kinases may represent a mechanism for persistence of *JAK2*-mutant clones in the face of chronic pharmacological JAK2 inhibition[35] (Figure 1E). Therefore, the consequences of a *JAK2* mutation will depend on the panoply of receptors expressed by a given cell type and also the precise downstream signaling targets (*e.g.* STAT family members) present in the cell.

Other signaling abnormalities in MPNs

Although some studies have investigated signaling abnormalities in human MPN cells, these have frequently required an *ex vivo* culture system which may not faithfully reproduce the most important changes *in vivo*. A recent study used intracellular flow cytometry to analyze signaling in CD34+ and more differentiated cells directly from MPN bone marrow. A notable result was that patterns of pSTAT3, pSTAT5, pERK1/2 and pAkt correlated best with disease subtype rather than with the presence of *JAK2V617F* or its allele burden.[36] For example, pSTAT5 levels in CD34- cells were higher in PV than ET, and pSTAT3 levels were higher in myelofibrosis than PV. These findings highlight that MPN patients with the same mutation (*JAK2V61F*) may show significant heterogeneity in signaling abnormalities, which may contribute to disease phenotype, and this seems likely to reflect the spectrum of other genetic lesions found in these patients.

Conversely in patients with *JAK2V617F*-negative MPNs, alternative molecular mechanisms must account for activation of similar signaling pathways to those associated with *JAK2V617F* (Figure 1B). Important examples include: 1) *JAK2* exon 12 mutations, found in most patients with PV who lack *JAK2V617F*, which are associated with more marked activation of JAK2 than *V617F;*[37] 2) *MPL* mutations, found in 4-9% of patients with PMF and 1-11% of those with ET, which result in increased signaling from TpoR and activation of JAK2, STAT3, STAT5, Akt and ERK;[38-40] 3) *LNK* mutations, identified in a small proportion of patients with ET, myelofibrosis, idiopathic erythrocytosis and blast-phase MPNs, and which are likely to abrogate the negative regulatory effect of LNK on Tpo- and Epo-dependent JAK-STAT signaling;[41-43] and 4) *c-CBL* mutations, found in approximately 6% of patients with myelofibrosis and in other myeloid malignancies, which may impair the negative effects of c-CBL on signaling by cytokine receptors through JAK-STAT pathways and also by receptor tyrosine kinases such as Flt3.[44,45]

Epigenetic abnormalities in MPNs

Mutations in the *TET2* gene were first reported in MPNs in 2009,[46] and an increasing group of epigenetic modifiers has since been implicated in MPN biology. These mutations are predicted to alter several different DNA and histone modifications through a variety of mechanisms (Figure 2). With the exception of *TET2*, mutations in these genes have mostly been identified in 5% or less of patients with PV or ET (Table 1), but are found at higher frequencies in myelofibrosis and/or blast-phase MPNs and have also been identified in other disorders including myelodysplasias (MDS) and acute myeloid leukemia (AML). Most have also been identified in at least some patients who also carry *JAK2V617F* or other classical MPN signaling mutations, suggesting that mutations in signaling molecules and those in epigenetic regulators may have complementary functions in pathogenesis.

Mutations altering DNA methylation: TET2, IDH1/2 and DNMT3A

TET2 is the most frequently mutated gene in chronic MPNs apart from *JAK2*[47,48] (Table 1). TET proteins convert 5-methylcytosine (5mC) in DNA to 5-hydroxymethylcytosine (5hmC), thought to be an intermediate in DNA demethylation, and can generate other products from 5mC including 5-formylcytosine and 5-carboxylcytosine.[59] *TET2* mutations in myeloid malignancies cause loss of function and are associated with reduced levels of genomic 5hmC in patient bone marrow samples.[60] *TET2* knockdown in human CD34+ cells leads to skewed differentiation towards the granulomonocytic lineage, and especially an increase in monocytic cells.[61] Consistent with this, several groups have found that *TET2* disruption in mice causes a chronic myelomonocytic leukemia (CMML)-like disease with leukocytosis, neutrophilia, monocytosis, splenomegaly, extramedullary hematopoiesis and, in some cases, anemia and/or thrombocytopenia.[62-65] A consistent finding in these models was an increase in HSC numbers, with increased HSC self-renewal and colony-forming progenitors. These phenotypes contrast with the PV or ET-like phenotypes observed in

Table 1. Mutations in epigenetic regulators and their frequencies in human MPNs. References have been restricted owing to space limitations. NF: mutations not found in small cohorts of patients; NK: frequency of mutations not known.

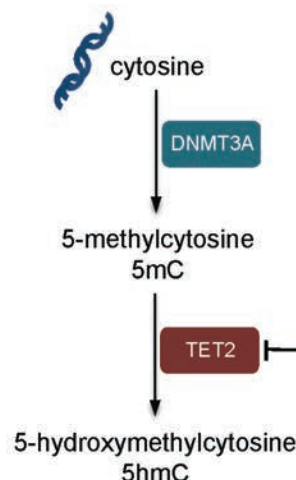
Gene	PV (%)	ET (%)	MF (%)	Blast phase (%)
TET2[46-48]	10-16	4-5	7-17	17-32
IDH1/2[49,50]	2	1	4	9-22
DNMT3A[51-53]	3-7	NF	2-15	14-17
EZH2[54,55]	3	NF	7-13	NK
ASXL1[53,56-58]	2-7	0-3	13-32	18-33

JAK2V617F mouse models, and highlight that *TET2* mutations probably act in MPNs by conferring a selective advantage to HSCs, rather than driving erythroid or megakaryocytic differentiation. This concept is also consistent with recent data that *TET2* mutations can be identified in a significant proportion of elderly individuals who show skewed X-inactivation in blood cells, in association with altered DNA methylation but no hematologic abnormalities.[66]

Mutations in the isocitrate dehydrogenase 1 and 2 (*IDH1/2*) genes the most common in PMF and blast-phase MPNs and confer inferior overall and leukemia-free survival in chronic-phase PMF and inferior overall survival in blast-phase.[49,50,67] IDH1 and IDH2 catalyze oxidative carboxylation of isocitrate to α-ketoglutarate, and the specific mutations found in myeloid neoplasms not only impair this reaction, but also cause a neomorphic catalytic activity converting α-ketoglutarate to 2-hydroxyglutarate.[68] Levels of 2-hydroxyglutarate are raised in myeloid malignancies with *IDH1* and *IDH2* mutations[68,69] and have been suggested to inhibit conversion of 5-methylcytosine to 5-hydroxymethylcytosine by TET2, with subsequent impaired DNA demethylation.[70] *IDH* mutations may, therefore, share pathogenetic mechanisms with *TET2* mutations, and this is consistent with a report that these are mutually exclusive in AML.[71]

A more recent report suggests that *IDH* mutations are also associated with impaired histone demethylation, with hypermethylation particularly at repressive H3K9 marks.[72] A knock-in mouse expressing the most common *IDH1* mutation (*R132H*) in hematopoietic cells shows anemia, splenomegaly, extramedullary hematopoiesis and increased lineage-restricted progenitors, together with increased 2-hydroxyglutarate levels and DNA and histone hypermethylation, supporting the importance of these mechanisms.[73] Interestingly, *IDH* mutations in PMF have recently been found to show a positive association with mutations in *SRSF2*, a spliceosome component gene mutated in approximately 17% of PMF patients.[74] Whilst the pathogenetic mechanisms for spliceosome mutations remain unclear, these data hint at co-operation between these two abnormalities.

Mutations in the DNA methyltransferase gene *DNMT3A*, originally identified in AML,[75] have also been found in patients with PMF, blast-phase MPNs and a small number with PV[51-53] (Table 1), and in other myeloid disorders. DNMT enzymes catalyze methylation of DNA at CpG dinucleotides, and although one residue is particularly affected by missense mutations (R882), the finding of nonsense mutations and deletions in other patients suggests that the pathogenetic mechanisms reflect dominant-negative or loss-of-function effects.[75] Ablation of *DNMT3A* in murine HSCs, followed by serial transplantation, has been reported to cause an increase in HSC numbers, impaired differentiation and altered DNA methylation, consistent with the concept that loss of DNMT3A function may promote HSC self-renewal in myeloid malignancies.[76] It is interesting to note that whilst *TET2* and *IDH* mutations would be predicted to cause increased DNA methylation, *DNMT3A* mutations should impair DNA methylation. These different predictions suggest that the different mutations may result in site-specific methylation changes and that these may be more important in disease pathogenesis than global hyper- or hypomethylation.



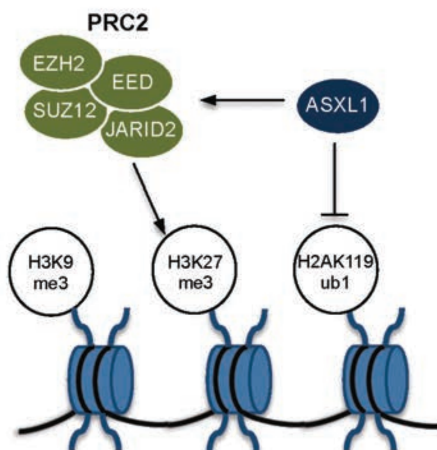


Figure 2. Pathogenetic mechanisms of mutations in epigenetic regulators in MPNs. See text for details of individual mutations. Note that mutations such as *IDH1/2* and *ASXL1* may have multiple points of action. 2-HG: 2-hydroxyglutarate; K: lysine; me3: trimethylated histone; ub1: monoubiquitinated histone.

Mutations altering histone methylation: EZH2, ASXL1 and PRC2 components

EZH2 is a histone methyltransferase that forms part of the Polycomb Repressive Complex-2 (PRC2), and catalyzes methylation of histone H3 at lysine 27 (H3K27), leading to transcriptional repression through recruitment of DNA methyltransferases.[77] *EZH2* mutations were found in myeloid disorders following the identification of acquired uniparental disomy at chromosome 7q.[54] Within the MPNs these mutations are most often present in myelofibrosis (Table 1),[54,55] in which they are associated with higher white cell counts, larger spleen size at diagnosis and reduced leukemia-free and overall survival.[55] *EZH2* mutations in myeloid malignancies are typically nonsense or missense, may be mono- or bi-allelic, and cause loss of histone methyltransferase activity.[54] Interestingly, these characteristics contrast with the heterozygous *EZH2* mutations found in B-cell lymphomas, which affect a single residue (Y641), appear to have a gain-of-function effect, and are associated with increased H3K27 trimethylation.[78] It, therefore, seems that *EZH2* may have either a tumor suppressor or pro-oncogenic function depending on the disease context. However, increased *EZH2* expression in a murine model has also been reported to cause a myeloproliferative disorder with leukocytosis, splenomegaly, and increased HSCs and granulocyte-monocyte progenitors.[79] Given that EZH2 inhibition has been suggested as a therapeutic strategy for *EZH2*-mutant lymphomas,[80] it will be particularly important to understand its role in normal and clonal myelopoiesis, and to establish how normal hematopoiesis may be affected by such pharmacological interventions.

Mutations in *ASXL1* (*additional sex combs like 1*), a member of the Enhancer of Trithorax and Polycomb (ETP) gene family, have been identified in patients with myelofibrosis, MDS, AML, and occasionally in PV or ET,[53,56-58] and may confer an adverse prognosis in myelofibrosis.[81] The mutations are predominantly nonsense, leading to C-terminal truncation and loss of protein expression.[82] *ASXL1* is the mammalian homolog of the Drosophila gene *Asx*, which forms part of a complex (Polycomb repressive deubiquitinase) with the protein BAP1. This complex removes monoubiquitin from histone H2A, and this property has been confirmed *in vitro* for the mammalian complex.[83] However, more recent data suggest that the function of *ASXL1* mutations in myeloid disorders is BAP1-independent, and rather depends on loss of H3K27 trimethylation.[82] This is likely to be mediated through an effect on the PRC2 complex, since *ASXL1* knockdown led to reduced EZH2 occupancy at target genes, and co-immunoprecipitation experiments confirmed that ASXL1 interacts with PRC2 components.[82] Interestingly, an *ASXL1* homozygous loss-of-function mouse model, expressing a truncated protein similar to the predicted products of mutations in myeloid disease, did not display any myeloid malignancy or abnormalities of blood counts, HSC numbers or function.[84] The explanation for this discrepancy with human MPN phenotypes is unclear.

The potential importance of PRC2 in disease is also highlighted by the identification of mutations or deletions in other components of the complex, including *SUZ12*, *EED* and *JARID2*, in smaller proportions of patients with myeloid malignancies.[53,85,86] Most reports are of patients with MDS, MDS/MPN or blast phase disease, but there are isolated reports in MPNs including *SUZ12* mutations in secondary myelofibrosis and PV.[53,86]

Clonal complexity in MPNs

An important message that has emerged from the study of molecular abnormalities in MPNs is that even in apparently simple disorders such as PV and ET, which often remain clinically stable for many years, there may be significant clonal complexity. Indeed, a major strength of the study of MPNs is that analysis of clonal hematopoietic populations, such as through hematopoietic colony assays, allows the reconstruction of clonal hierarchies to better understand disease biology. The first suggestion of this complexity was raised by studies in which patients with *JAK2V617F*-positive ET or PV were found to have transformed to AML in which the leukemic blast cells were negative for the mutation.[87] Overall, AML transformation from *JAK2V617F*-positive MPNs is associated with loss of *JAK2V617F* in approximately 60% of patients studied.[87-89] Additional experiments showed that this did not reflect reversion of the mutant *JAK2* allele back to wild-type, suggesting either that the AML arose in a pre-*JAK2V617F* stem cell within the MPN clone, or in a clonally unrelated, normal stem cell.[87-89] A lower frequency of *JAK2V617F* loss (9%) has been reported in one study, although this patient group was notable in that a high proportion had transformed to AML from *JAK2V617F*-positive myelofibrosis rather than from PV or ET.[90]

A detailed study of 16 patients who had undergone AML transformation from a *JAK2*-mutant MPN, including 9 who developed *JAK2*-wild-type AML, showed that a variety of molecular lesions were acquired around the time of transformation (*e.g. TP53*, *RUNX1*) and that these did not show specificity for *JAK2*-mutant or wild-type AML.[89] For a number of patients, clonal analyses of these additional mutations did not confirm a shared clone of origin for the *JAK2*-mutant MPN and *JAK2*-wild-type AML. Although a *TET2* mutation could be identified as a shared founder lesion in a patient with chronic-phase MPN, in whom *JAK2* and *MPL* mutations were found in separate daughter clones, there was no evidence that a *TET2* mutation preceded *JAK2V617F* in 2 other patients with *JAK2V617F*-wild-type, *TET2*-mutant AML.[89] It, therefore, remains possible that *JAK2*-wild-type AML arises from a clonally unrelated, independent HSC, or from a shared pre-*JAK2* founder clone that has not been identified with current methods. The ability of deep-sequencing technologies, including whole-genome sequencing, to identify large numbers of mutations will provide an important tool in resolving the clonal evolution events that occur in these AML transformations.

A number of studies have highlighted complexity within chronic-phase MPNs, by analyzing the clonal hierarchy of multiple molecular lesions. Studies of patients carrying both *JAK2V617F* and chromosome 20q deletions demonstrated that these lesions could co-exist in the same clone, that either lesion could occur first within an individual patient, and that both del(20q) and 9p loss of heterozygosity (LOH, causing *JAK2V617F* homozygosity) could occur twice in the same patient.[91,92] Similarly, in an individual patient a *TET2* mutation may occur before a *JAK2* mutation, or *vice versa*, or the mutations may occur in different clones.[89,93] These studies suggest that co-operation between *JAK2V617F* and other lesions in driving disease does not require a particular temporal order of mutation.

By contrast to patients with co-existent *JAK2* mutations and cytogenetic abnormalities, studies of patients with two tyrosine kinase mutations (*e.g. MPL* and *JAK2*) demonstrated that in all cases the two mutations were in separate clones. Moreover, in 2 cases X-chromosome inactivation studies demonstrated that these had independent origins and did not arise from a shared founder clone.[92] It, therefore, appears that the addition of a second tyrosine kinase mutation may not confer a selective advantage to a particular clone, but separate clones with independent mutations may arise and co-exist persistently in chronic-phase MPNs. The recurrent acquisition of pathogenetic mutations in such patients, together with the identification of multiple acquisitions of *JAK2V617F* in other patients,[94] also raises the question of whether these individuals are somehow predisposed to developing such mutant clones. It is increasingly recognized that germline factors may have roles in genetic predispositions to MPNs,[95] although the underlying mechanisms are frequently unclear.

It is also apparent that differences in clonal substructure may be important in distinguishing between disease phenotypes, such as between PV and ET. A number of lines of circumstantial evidence have linked the development of a PV phenotype to acquisition of homozygosity for *JAK2V617F*: *JAK2V617F*-homozygous erythroid colonies were initially identified in most patients with PV but not with ET;[96] a higher *JAK2V617F* allele burden in granulocyte DNA correlates with more extreme hematologic features in PV patients;[97] higher *JAK2V617F* expression levels have been associated with a PV rather than ET phenotype in transgenic mice;[21] *JAK2* exon 12 mutations have stronger signaling consequences than *JAK2V617F* and are exclusively associated with PV rather than ET;[37] and, conversely, a germline *JAK2V617I* mutation, with more limited effects on JAK2 activation, was only associated with thrombocytosis.[98] However, a recent genotyping study of numerous erythroid colonies grown at low erythropoietin concentrations showed that small *JAK2V617F*-homozygous clones can, in fact, be identified in approximately half the patients with ET.[99] Homozygous-mutant clones in PV were larger than those in ET and present in 80% of patients. Moreover, recurrent acquisition of *JAK2V617F* homozygosity was shown to be a frequent occurrence in patients with PV, and also occurred in ET. PV and ET were, therefore, not distinguished by the absolute presence or recurrent acquisition of *JAK2V617F* homozygosity, but PV patients all showed a dominant homozygous subclone, which was much larger than other homozygous subclones in the same patient. These data suggest a model whereby loss of heterozygosity for chromosome 9p, resulting in *JAK2V617F* homozygosity, is a frequent event in MPNs but does not necessarily confer a significant selective advantage to these clones. Instead, it is likely that additional genetic or epigenetic lesions are required to permit expansion of a particular clone, which, in the case of *JAK2V617F*-homozygous clones, may lead to the development of a PV phenotype (Figure 3).

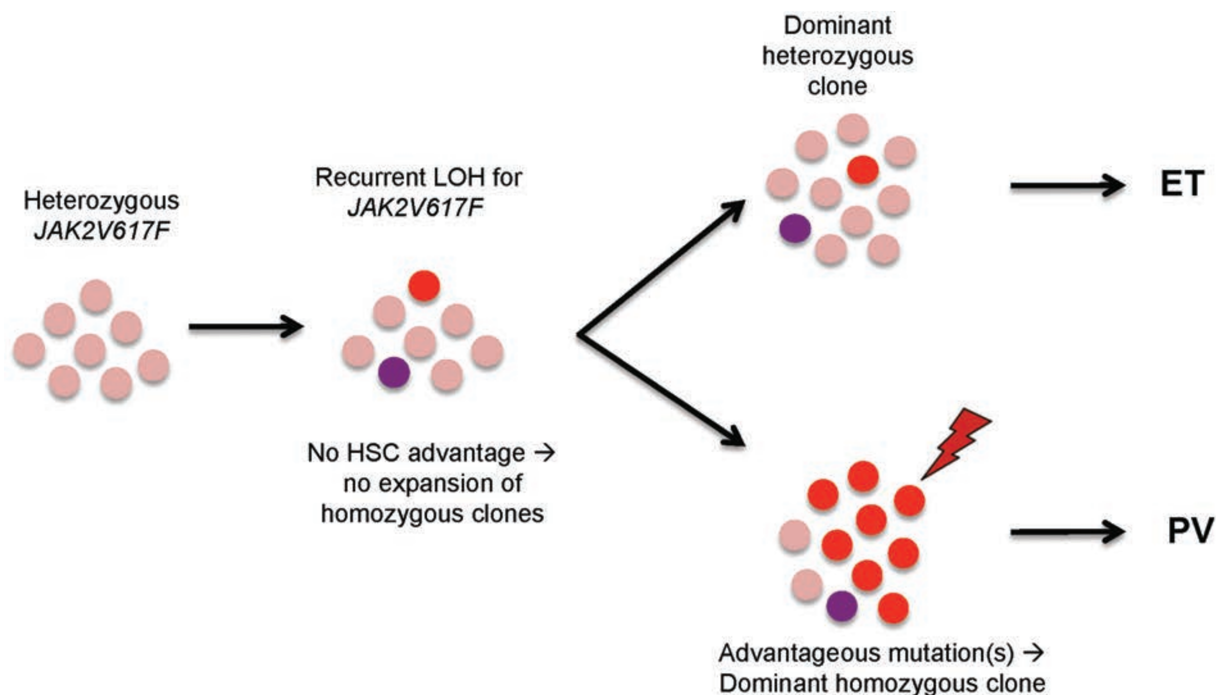


Figure 3. Model for mechanisms distinguishing *JAK2V617F*-positive PV and ET. In this model, *JAK2V617F-homozygous precursors* arise frequently, and may occur recurrently, in patients with both PV and ET through loss of heterozygosity (LOH) at chromosome 9p. However, these homozygous-mutant cells do not necessarily have a selective advantage at the HSC level. Patients with PV are distinguished by the expansion of a dominant homozygous subclone which is likely to reflect a selective advantage imparted by additional genetic and/or epigenetic lesions.[99] Note that other mechanisms must account for the development of PV in approximately 20% of patients who lack evidence of *JAK2V617F* homozygosity. Pink circles represent *JAK2V617F*-heterozygous precursors; red and purple circles represent independent *JAK2V617F*-homozygous subclones.

Conclusions and future perspectives

The identification of the *JAK2V617F* mutation demonstrated the first molecular link between PV, ET and PMF, confirming the close relationship between the pathogenesis of the three disorders. A series of advances has not only identified the complex ways in which *JAK2V617F* can contribute to MPN pathogenesis, but also the increasing spectrum of mutations implicated in driving disease both independent of and in co-operation with *JAK2V617F*. Here we have discussed the epigenetic modifiers mutated in myeloid malignancies, but other groups of regulators have recently been recognized to be mutated in myeloid malignancies. For example, spliceosome component mutations were originally identified in MDS but also occur in MPNs, and of these, *SRSF2* mutations have recently been associated with leukemic transformation of MPNs and with adverse prognosis.[100] Mutational heterogeneity probably contributes significantly to the phenotypic heterogeneity of the MPNs, especially between the different chronic-phase diseases and in driving the transformation events that occur in a proportion of patients. In the future, large-scale whole exome and genome sequencing projects are likely to identify additional mutations that contribute to MPN pathogenesis. It will be particularly important to learn how these mutations co-operate or interact at a molecular level to drive the clonal expansion and distinct phenotypes seen within MPNs and other myeloid disorders.

References

1. Dameshek W. Some speculations on the myeloproliferative syndromes. Blood. 1951;6:372-5.
2. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434:1144-8.
3. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005;365:1054-61.
4. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352:1779-90.
5. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005;7:387-97.
6. Rane SG, Reddy EP. Janus kinases: components of multiple signaling pathways. Oncogene. 2000;19:5662-79.
7. Feng J, Witthuhn BA, Matsuda T, Kohlhuber F, Kerr IM, Ihle JN. Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. Mol Cell Biol. 1997;17:2497-501.
8. Saharinen P, Silvennoinen O. The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. J Biol Chem. 2002;277:47954-63.
9. Saharinen P, Vihinen M, Silvennoinen O. Autoinhibition of Jak2 tyrosine kinase is dependent on specific regions in its pseudokinase domain. Mol Biol Cell. 2003;14:1448-59.
10. Ungureanu D, Wu J, Pekkala T, et al. The pseudokinase domain of JAK2 is a dual-specificity protein kinase that negatively regulates cytokine signaling. Nat Struct Mol Biol. 2011; 18:971-6.
11. Dusa A, Mouton C, Pecquet C, Herman M, Constantinescu SN. JAK2 V617F constitutive activation requires JH2 residue F595: a pseudokinase domain target for specific inhibitors. PLoS One. 2010;5:e11157.
12. Bandaranayake RM, Ungureanu D, Shan Y, Shaw DE, Silvennoinen O, Hubbard SR. Crystal structures of the JAK2 pseudokinase domain and the pathogenic mutant V617F. Nat Struct Mol Biol. 2012;19:754-9.
13. Garcon L, Rivat C, James C, et al. Constitutive activation of STAT5 and Bcl-xL overexpression can induce endogenous erythroid colony formation in human primary cells. Blood. 2006;108:1551-4.
14. Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood. 2006;107:4274-81.
15. Zaleskas VM, Krause DS, Lazarides K, et al. Molecular Pathogenesis and Therapy of Polycythemia Induced in Mice by JAK2 V617F. PLoS One. 2006;1:e18.
16. Laubach JP, Fu P, Jiang X, Salter KH, Potti A, Arcasoy MO. Polycythemia vera erythroid precursors exhibit increased proliferation and apoptosis resistance associated with abnormal RAS and PI3K pathway activation. Exp Hematol. 2009;37: 1411-22.
17. Grimwade LF, Happerfield L, Tristram C, et al. Phospho-STAT5 and phospho-Akt expression in chronic myeloproliferative neoplasms. Br J Haematol. 2009;147:495-506.
18. Lacout C, Pisani DF, Tulliez M, Gachelin FM, Vainchenker W, Villeval JL. JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. Blood. 2006;108:1652-60.
19. Shide K, Shimoda HK, Kumano T, et al. Development of ET, primary myelofibrosis and PV in mice expressing JAK2 V617F. Leukemia. 2008;22:87-95.
20. Xing S, Wanting TH, Zhao W, et al. Transgenic expression of JAK2V617F causes myeloproliferative disorders in mice. Blood. 2008;111:5109-17.
21. Tiedt R, Hao-Shen H, Sobas MA, et al. Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. Blood. 2008;111:3931-40.
22. Li J, Spensberger D, Ahn JS, et al. JAK2 V617F impairs hematopoietic stem cell function in a conditional knock-in mouse model of JAK2 V617F-positive essential thrombocythemia. Blood. 2010;116:1528-38.
23. Marty C, Lacout C, Martin A, et al. Myeloproliferative neoplasm induced by constitutive expression of JAK2V617F in knock-in mice. Blood. 2010;116:783-7.
24. Mullally A, Lane SW, Ball B, et al. Physiological Jak2V617F expression causes a lethal myeloproliferative neoplasm with differential effects on hematopoietic stem and progenitor cells. Cancer Cell. 2010;17:584-96.
25. Akada H, Yan D, Zou H, Fiering S, Hutchison RE, Mohi MG. Conditional expression of heterozygous or homozygous Jak2V617F from its endogenous promoter induces a polycythemia vera-like disease. Blood. 2010;115:3589-97.
26. Yan D, Hutchison RE, Mohi G. Critical requirement for Stat5 in a mouse model of polycythemia vera. Blood. 2012;119: 3539-49.
27. Walz C, Ahmed W, Lazarides K, et al. Essential role for Stat5a/b in myeloproliferative neoplasms induced by BCR-ABL1 and JAK2(V617F) in mice. Blood. 2012;119:3550-60.
28. Mullally A, Poveromo L, Schneider RK, Al-Shahrour F, Lane SW, Ebert BL. Distinct roles for long-term hematopoietic stem cells and erythroid precursor cells in a murine model of Jak2V617F-mediated polycythemia vera. Blood. 2012;120: 166-72.
29. Akada H, Akada S, Hutchison RE, Mohi G. Erythroid lineage-restricted expression of Jak2V617F is sufficient to induce a myeloproliferative disease in mice. Haematologica. 2012;97:1389-93.
30. Pecquet C, Diaconu CC, Staerk J, et al. Thrombopoietin receptor down-modulation by JAK2 V617F: restoration of receptor levels by inhibitors of pathologic JAK2 signaling and of proteasomes. Blood. 2012;119:4625-35.
31. Fleischman AG, Aichberger KJ, Luty SB, et al. TNFalpha facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. Blood. 2011;118:6392-8.
32. Dawson MA, Bannister AJ, Gottgens B, et al. JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature. 2009;461:819-22.
33. Dawson MA, Foster SD, Bannister AJ, et al. Three distinct patterns of histone H3Y41 phosphorylation mark active genes. Cell Rep. 2012;2:470-7.
34. Liu F, Zhao X, Perna F, et al. JAK2V617F-mediated phosphorylation of PRMT5 downregulates its methyltransferase activity and promotes myeloproliferation. Cancer Cell. 2011;19: 283-94.
35. Koppikar P, Bhagwat N, Kilpivaara O, et al. Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. Nature. 2012;489:155-9.

36. Anand S, Stedham F, Gudgin E, et al. Increased basal intracellular signaling patterns do not correlate with JAK2 genotype in human myeloproliferative neoplasms. Blood. 2011;118: 1610-21.
37. Scott LM, Tong W, Levine RL, et al. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med. 2007;356:459-68.
38. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PLoS Med. 2006;3:e270.
39. Beer PA, Campbell PJ, Scott LM, et al. MPL mutations in myeloproliferative disorders: analysis of the PT-1 cohort. Blood. 2008;112:141-9.
40. Boyd EM, Bench AJ, Goday-Fernandez A, et al. Clinical utility of routine MPL exon 10 analysis in the diagnosis of essential thrombocythaemia and primary myelofibrosis. Br J Haematol. 2010;149:250-7.
41. Oh ST, Simonds EF, Jones C, et al. Novel mutations in the inhibitory adaptor protein LNK drive JAK-STAT signaling in patients with myeloproliferative neoplasms. Blood. 2010;116:988-92.
42. Lasho TL, Pardanani A, Tefferi A. LNK mutations in JAK2 mutation-negative erythrocytosis. N Engl J Med. 2010;363: 1189-90.
43. Pardanani A, Lasho T, Finke C, Oh ST, Gotlib J, Tefferi A. LNK mutation studies in blast-phase myeloproliferative neoplasms, and in chronic-phase disease with TET2, IDH, JAK2 or MPL mutations. Leukemia. 2010;24:1713-8.
44. Grand FH, Hidalgo-Curtis CE, Ernst T, et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood. 2009;113:6182-92.
45. Sanada M, Suzuki T, Shih L-Y, et al. Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. Nature. 2009;460:904-8.
46. Delhommeau F, Dupont S, Della Valle V, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009;360:2289-301.
47. Tefferi A, Pardanani A, Lim KH, et al. TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia. 2009;23:905-11.
48. Abdel-Wahab O, Mullally A, Hedvat C, et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood. 2009;114:144-7.
49. Tefferi A, Lasho TL, Abdel-Wahab O, et al. IDH1 and IDH2 mutation studies in 1473 patients with chronic-, fibrotic- or blast-phase essential thrombocythemia, polycythemia vera or myelofibrosis. Leukemia. 2010;24:1302-9.
50. Pardanani A, Lasho TL, Finke CM, Mai M, McClure RF, Tefferi A. IDH1 and IDH2 mutation analysis in chronic- and blast-phase myeloproliferative neoplasms. Leukemia. 2010;24:1146-51.
51. Stegelmann F, Bullinger L, Schlenk RF, et al. DNMT3A mutations in myeloproliferative neoplasms. Leukemia. 2011;25:1217-9.
52. Abdel-Wahab O, Pardanani A, Rampal R, Lasho TL, Levine RL, Tefferi A. DNMT3A mutational analysis in primary myelofibrosis, chronic myelomonocytic leukemia and advanced phases of myeloproliferative neoplasms. Leukemia. 2011;25:1219-20.
53. Brecqueville M, Rey J, Bertucci F, et al. Mutation analysis of ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12, and TET2 in myeloproliferative neoplasms. Genes Chromosomes Cancer. 2012;51:743-55.
54. Ernst T, Chase AJ, Score J, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet. 2010;42:722-6.
55. Guglielmelli P, Biamonte F, Score J, et al. EZH2 mutational status predicts poor survival in myelofibrosis. Blood. 2011;118:5227-34.
56. Carbuccia N, Murati A, Trouplin V, et al. Mutations of ASXL1 gene in myeloproliferative neoplasms. Leukemia. 2009;23:2183-6.
57. Abdel-Wahab O, Pardanani A, Patel J, et al. Concomitant analysis of EZH2 and ASXL1 mutations in myelofibrosis, chronic myelomonocytic leukemia and blast-phase myeloproliferative neoplasms. Leukemia. 2011;25:1200-2.
58. Stein BL, Williams DM, O'Keefe C, et al. Disruption of the ASXL1 gene is frequent in primary, post-essential thrombocytosis and post-polycythemia vera myelofibrosis, but not essential thrombocytosis or polycythemia vera: analysis of molecular genetics and clinical phenotypes. Haematologica. 2011;96:1462-9.
59. Ito S, Shen L, Dai Q, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science. 2011;333:1300-3.
60. Ko M, Huang Y, Jankowska AM, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature. 2010;468:839-43.
61. Pronier E, Almire C, Mokrani H, et al. Inhibition of TET2-mediated conversion of 5-methylcytosine to 5-hydroxymethylcytosine disturbs erythroid and granulomonocytic differentiation of human hematopoietic progenitors. Blood. 2011;118:2551-5.
62. Quivoron C, Couronne L, Della Valle V, et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell. 2011;20:25-38.
63. Ko M, Bandukwala HS, An J, et al. Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. Proc Natl Acad Sci USA. 2011;108:14566-71.
64. Li Z, Cai X, Cai C-L, et al. Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. Blood. 2011;118:4509-18.
65. Moran-Crusio K, Reavie L, Shih A, et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell. 2011;20:11-24.
66. Busque L, Patel JP, Figueroa ME, et al. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. Nat Genet. 2012;44:1179-81.
67. Tefferi A, Jimma T, Sulai NH, et al. IDH mutations in primary myelofibrosis predict leukemic transformation and shortened survival: clinical evidence for leukemogenic collaboration with JAK2V617F. Leukemia. 2012;26:475-80.
68. Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 2009;462:739-44.
69. Ward PS, Patel J, Wise DR, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell. 2010;17:225-34.
70. Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell. 2010;18:553-67.
71. Chou W-C, Chou S-C, Liu C-Y, et al. TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. Blood. 2011;118:3803-10.
72. Lu C, Ward PS, Kapoor GS, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. Nature. 2012;483:474-8.
73. Sasaki M, Knobbe CB, Munger JC, et al. IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. Nature. 2012;488:656-9.
74. Lasho TL, Jimma T, Finke CM, et al. SRSF2 mutations in primary myelofibrosis: significant clustering with IDH mutations and independent association with inferior overall and leukemia-free survival. Blood. 2012;120:4168-71.
75. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010;363:2424-33.
76. Challen GA, Sun D, Jeong M, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet. 2012;44:23-31.
77. Vire E, Brenner C, Deplus R, et al. The Polycomb group protein EZH2 directly controls DNA methylation. Nature. 2006;439:871-4.
78. Yap DB, Chu J, Berg T, et al. Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. Blood. 2011;117:2451-9.
79. Herrera-Merchan A, Arranz L, Ligos JM, de Molina A, Dominguez O, Gonzalez S. Ectopic expression of the histone methyltransferase Ezh2 in haematopoietic stem cells causes myeloproliferative disease. Nat Commun. 2012;3:623.
80. McCabe MT, Ott HM, Ganji G, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature. 2012;492:108-12.
81. Lasho TL, Gangat N, Finke C, et al. Prognostic Interactions Between SRSF2, ASXL1, and IDH Mutations in Primary Myelofibrosis and Determination of Added Value to Cytogenetic Risk Stratification and DIPSS-Plus. ASH meeting. Atlanta, USA, 2012.
82. Abdel-Wahab O, Adli M, LaFave LM, et al. ASXL1 mutations promote myeloid transformation through loss of PRC2-mediated gene repression. Cancer Cell. 2012;22:180-93.
83. Scheuermann JC, de Ayala Alonso AG, Oktaba K, et al. Histone H2A deubiquitinase activity of the Polycomb repressive complex PR-DUB. Nature. 2010;465:243-7.

84. Fisher CL, Pineault N, Brookes C, et al. Loss-of-function Additional sex combs like 1 mutations disrupt hematopoiesis but do not cause severe myelodysplasia or leukemia. Blood. 2010;115:38-46.
85. Score J, Hidalgo-Curtis C, Jones AV, et al. Inactivation of polycomb repressive complex 2 components in myeloproliferative and myelodysplastic/myeloproliferative neoplasms. Blood. 2012;119:1208-13.
86. Puda A, Milosevic JD, Berg T, et al. Frequent deletions of JARID2 in leukemic transformation of chronic myeloid malignancies. Am J Hematol. 2012;87:245-50.
87. Campbell PJ, Baxter EJ, Beer PA, et al. Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation. Blood. 2006;108:3548-55.
88. Theocharides A, Boissinot M, Girodon F, et al. Leukemic blasts in transformed JAK2-V617F-positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation. Blood. 2007;110:375-9.
89. Beer PA, Delhommeau F, LeCouedic JP, et al. Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. Blood. 2010;115:2891-900.
90. Rinaldi CR, Rinaldi P, Gemei M, et al. JAK2V617F mutation persists in blasts and mature cells of transformed JAK2V617F-positive-myeloproliferative neoplasia: a European Leukemia Net (ENL) study. Am J Hematol. 2010; 85:383-6.
91. Schaub FX, Jager R, Looser R, et al. Clonal analysis of deletions on chromosome 20q and JAK2-V617F in MPD suggests that del20q acts independently and is not one of the pre-disposing mutations for JAK2-V617F. Blood. 2009;113:2022-7.
92. Beer PA, Jones AV, Bench AJ, et al. Clonal diversity in the myeloproliferative neoplasms: independent origins of genetically distinct clones. Br J Haematol. 2009;144:904-8.
93. Schaub FX, Looser R, Li S, et al. Clonal analysis of TET2 and JAK2 mutations suggests that TET2 can be a late event in the progression of myeloproliferative neoplasms. Blood. 2010;115:2003-7.
94. Beer PA, Ortmann CA, Campbell PJ, Green AR. Independently acquired biallelic JAK2 mutations are present in a minority of patients with essential thrombocythemia. Blood. 2010;116:1013-4.
95. Harutyunyan AS, Kralovics R. Role of germline genetic factors in MPN pathogenesis. Hematol Oncol Clin North Am. 2012;26:1037-51.
96. Scott LM, Scott MA, Campbell PJ, Green AR. Progenitors homozygous for the V617F mutation occur in most patients with polycythemia vera, but not essential thrombocythemia. Blood. 2006;108:2435-7.
97. Passamonti F, Rumi E, Pietra D, et al. A prospective study of 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease transformation and vascular complications. Leukemia. 2010;24:1574-9.
98. Mead AJ, Rugless MJ, Jacobsen SE, Schuh A. Germline JAK2 mutation in a family with hereditary thrombocytosis. N Engl J Med. 2012;366:967-9.
99. Godfrey AL, Chen E, Pagano F, et al. JAK2V617F homozygosity arises commonly and recurrently in PV and ET, but PV is characterized by expansion of a dominant homozygous subclone. Blood. 2012;120:2704-7.
100. Zhang SJ, Rampal R, Manshouri T, et al. Genetic analysis of patients with leukemic transformation of myeloproliferative neoplasms shows recurrent SRSF2 mutations that are associated with adverse outcome. Blood. 2012;119:4480-5.

Mast cell neoplasms

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A B S T R A C T

Mast cell (MC) neoplasms comprise a heterogeneous group of clonal disorders characterized by abnormal expansion and accumulation of tissue MC in one or multiple organs. In most adult patients, systemic mastocytosis (SM) is diagnosed. Based on histopathological findings and organ damage, SM is divided into indolent SM (ISM), smoldering SM (SSM), SM with an associated hematologic non-MC-lineage disease (SM-AHNMD), aggressive SM (ASM), and MC leukemia (MCL). The clinical course and prognosis vary greatly among these patients. In all SM-variants and most patients, neoplastic cells display the disease-related *KIT* mutation D816V, suggesting that additional, KIT-independent, molecular lesions or other factors are responsible for disease progression. Indeed, additional mutations, including *RAS* and *TET2* mutations, have recently been identified in advanced SM. In SM-AHNMD, such additional lesions are often detectable in the "AHNMD-component" of the disease. Clinically relevant symptoms of SM result from malignant MC infiltration and the subsequent organ damage seen in advanced SM and/or the release of pro-inflammatory and vasoactive mediators from MC. Therapy of SM has to be adapted to the individual situation in each case. In ISM, the aim is to control mediator-release and mediator-effects. In advanced SM, a major goal is to control MC proliferation by conventional or targeted anti-neoplastic drugs. In rapidly progressing ASM, MCL and drug-resistant AHNMD, stem cell transplantation should be considered.

Learning goals

At the conclusion of this activity, participants should know:
- the correct classification and diagnosis of SM;
- the correct application of diagnostic tests and algorithms;
- the delineation between SM variants and various differential diagnoses;
- about the establishment of treatment plans in indolent and advanced SM.

Introduction

Mastocytosis is a term used for a heterogeneous group of clonal hematopoietic disorders characterized by abnormal expansion and accumulation of tissue mast cells (MC) in the skin and/or in visceral organs.[1-10] Depending on the affected organ system(s), mastocytosis can be divided into cutaneous mastocytosis (CM), systemic mastocytosis (SM), and localized MC tumors.[1-15] The classification of the World Health Organization (WHO) discriminates between several different categories of CM and SM.[11-15] The clinical course and prognosis vary substantially among these patients.[11-18] In addition, patients with mastocytosis may suffer from symptoms caused by various MC-derived mediators, especially when an allergic disease is also present.[19-24] Mediator-related symptoms may be mild, severe, or even life-threatening.[19-24] In some of these patients, an overt MC activation syndrome (MCAS) is diagnosed.[22-24] Patients with SM may also suffer from osteoporosis, gastrointestinal symptoms, neurological or psychiatric symptoms or/and symptoms related to skin lesions, such as pruritus or flushing (Table 1).[19-21,25-28] In advanced mastocytosis, additional problems, such as cytopenia, ascites, malabsorption, lymphadenopathy, splenomegaly, hepatopathy, or osteolysis, may develop (Table 1).[11-18] Whereas the prognosis in CM and indolent SM (ISM) is excellent, the prognosis and life-expectancy in aggressive SM (ASM) and MC leukemia (MCL) are poor.[15-18] The current article provides an overview on the biology, molecular features, diagnosis, classification, and treatment of patients with mastocytosis.

Biology, history and classification

Mast cells (MC) are myeloid cells that express histamine and other pro-inflammatory mediators as well as high-affinity binding-sites for IgE.[29,30] Similar to other leukocytes, MC are constantly replenished from a pool of pluripotent and committed hematopoietic progenitor cells.[29-31] MC progenitor cells express the tyrosine kinase receptor KIT.[29-32] The ligand of this oncogenic receptor, stem cell factor (SCF), induces MC development in uncommitted and MC-committed progenitor cells.[31-33] However, in patients with mastocytosis, SCF-independent differentiation and accumulation of MC is seen.[6-10] Historically, mastocytosis

was first described as a skin disease, named urticaria pigmentosa (UP).[34] Indeed, most patients with mastocytosis present with typical skin lesions. However, absence of skin lesions does not exclude the presence of SM. The classification of mastocytosis stems back to 1949, when a first case of mastocytosis with internal organ involvement was described in an autopsy.[35] Between 1950 and 1975, a number of different disease variants, including MCL, were described. A first comprehensive classification proposal was introduced by the Kiel group with Karl Lennert in 1979.[1] Later, in 1991, a similar classification was proposed by Dean Metcalfe and his colleagues in the US.[2] Between 1990 and 2000, a number of clinical, histomorphological, immunological, and biochemical markers of CM and SM were developed and were in part validated.[36-42] In the Year 2000 Working Conference on Mastocytosis, these disease-related parameters were discussed and formulated into criteria to define mastocytosis and to classify CM and SM variants.[11] The resulting consensus proposal was adopted by the WHO as official classification of mastocytosis in 2001.[12] Later, in 2008, this classification was reconfirmed by the WHO.[14] Based on the WHO classification, the following disease variants are defined: ISM, SM with an associated hematologic non-MC-lineage disease (SM-AHNMD), aggressive SM (ASM), and MCL. As mentioned above, the clinical course and prognosis vary greatly among these patients. The smoldering subtype of SM (SSM) was initially described as a subvariant of ISM.[11] However, later, the EU-US consensus group described this entity as a distinct variant of SM.[21] The currently proposed classification of mast cell disorders is shown in Table 2.

Between 2002 and 2013, the consensus group continued to work on markers, criteria, and standards, in order to improve diagnosis, staging and prognostication in CM and SM, and to formulate treatment response criteria.[15,21-23,43] In 2002, the European Competence Network on Mastocytosis (ECNM) was inaugurated.[44,45] The main aim of this academic platform is to provide doctors and patients with all available information and to improve diagnosis and therapy in patients with MC disorders.[44,45]

Diagnostic criteria

Minimal diagnostic criteria of CM and SM, proposed by the consensus group and the WHO, are widely used and generally accepted. CM is defined by typical skin lesions detected by inspection (macroscopy), a "positive" histology, and absence of criteria sufficient to diagnose SM.[11-14,21] It is important to know that a minimal infiltration of the bone marrow by neoplastic MC often remains "subdiagnostic" regarding SM, so that the final diagnosis is CM in these cases.[11-14,21] Even in patients in whom two minor SM criteria are fulfilled, the diagnosis remains CM.[11-14] The major SM criterion is a histologically confirmed infiltration of MC in one or more extracutaneous (visceral) organs. In most cases, the bone marrow (BM) is examined. The recommended two stains for detection and enumeration of MC and MC infiltrates in the BM (and all other organs) are KIT (CD117) and tryptase.[11-14,40] In typical cases of SM, smaller or/and larger compact infiltrates of spindle-shaped MC are found in KIT- and tryptase-stained BM sections.[11-14,40] Minor SM criteria include: i) an atypical morphology of MC; ii) expression of CD2 or/and CD25 in MC; iii) the presence of KIT D816V in the BM or another extracutaneous organ; and iv) a basal serum tryptase level exceeding 20 ng/mL.[11-14] If at least one major and one minor or at least three minor SM criteria are fulfilled, the diagnosis SM is established (Table 3). With regard to diagnostic algorithms, assays and standards used in daily practice, we refer to the available literature.[11-14,21,22] An important aspect is that most patients with CM are children, whereas in most adult patients, SM is diagnosed. Therefore, in children, no BM biopsy is required unless clear signs for advanced SM or an AHNMD are found.[21] By contrast, in adults, a BM biopsy is always required to establish the final diagnosis.[11-14,21] In adult patients who present with skin lesions but refuse a BM biopsy, the provisional diagnosis of "mastocytosis in the skin" (MIS) is appropriate,[21] whereas the traditional way to diagnose CM in such cases is obsolete and should be avoided.

Table 1. Recurrent findings and symptoms in patients with systemic mastocytosis (SM).

	Typically seen in patient with	
Findings/symptoms	indolent SM	advanced SM*
Skin involvement (MIS)	+/-**	-/+ **
Leukocytosis	-	+/-
Eosinophilia	+/-	+
Circulating mast cells	-	-/+***
Marked cytopenia	-	+
Bone marrow (BM) fibrosis	-/+	+/-
Marked BM dysplasia	-	+/-
Mast cells in BM smears >5%	-	+/-
Serum tryptase > 200 ng/mL	+/-	+
Splenomegaly	-/+	+
Lymphadenopathy (abdominal)	-/+	+/-
Hepatopathy with ascites	-	+
Elevated alkaline phosphatase	-	+
Large osteolysis	-	-/+
Osteoporosis	+/-	-/+
Recurrent severe anaphylaxis	+/-	-/+
Hypotension and tachycardia	+/-	+/-
Fever and night sweats	-	-/+
Fatigue	+/-	-/+
Psychiatric or neurological symptoms	+/-	-/+
Gastrointestinal (GI) cramps	+	+/-
Loose stools or diarrhea	+	+/-
Ulcerative GI tract disease	+/-	+/-
Malabsorption	-	-/+
Weight loss	-	+

*Advanced SM includes aggressive SM (ASM) and mast cell leukemia (MCL). **In most adult patients with indolent SM (ISM), urticaria pigmentosa-like skin lesions are found. Those who have indolent SM but do not exhibit skin lesions are classified as (isolated) bone marrow mastocytosis (BMM). In these cases, it is important to exclude advanced SM, a condition that typically presents without skin lesions (>50% of cases). ***Circualting mast cells are typically found in patients with classical MCL. MIS: mastocytosis in the skin; BM: bone marrow. + frequently seen; +/- found in a subset of patients; -/+ rarely seen; - not found.*

Molecular features and target antigens

Mastocytosis is a group of clonal myeloid neoplasms defined by factor-independent expansion of neoplastic MC. The key molecular lesions recurrently detected in patients with mastocytosis, are activating *KIT* mutations that may explain the autonomous growth and expansion of neoplastic MC.[10,37,38,42,46-48] In pediatric patients with CM, a number of different *KIT* mutations, including *KIT* D816V, have been identified.[10,46-48] By contrast, in most adult patients suffering from SM, the *KIT* mutation D816V is detected, independent of the variant of SM.[10-14,37,38,42] It is remarkable that in all these patients, including cases with ISM, who have a (near) normal life-expectancy, neoplastic MC display KIT D816V. This points to additional mechanisms and molecular defects responsible for disease progression in ASM, MCL, and SM-AHNMD. In other words, manifestation of an AHNMD, ASM or MCL cannot be explained by KIT D816V alone. Indeed, recent data suggest that a number of additional lesions are detectable in patients with SM-AHNMD, ASM and MCL. These lesions include *RAS* mutations, *TET2* mutations, mutations in *IgE receptor* genes, and other mutations.[49-52] A summary of molecular lesions typically found in advanced SM is shown in Table 4. A special condition is SM-AHNMD. Based on molecular and functional studies, the AHNMD-component of the disease is considered to develop in distinct (yet monoclonal) subclones that may derive from an early (KIT D816V$^-$) or later (already KIT D816V$^+$) common neoplastic stem cell. A number of different AHNMD variants and related molecular lesions have been identified.[56,58-61] In most patients, an associated myeloid malignancy is detected.[56,58-61] By contrast, lymphoid variants of AHNMD are rarely diagnosed. In some cases, hypereosinophilia occurs. In these patients, chronic eosinophilic leukemia (CEL) may be diagnosed. In rare cases, the *FIP1L1/PDGFRA* fusion gene is detectable.[53-55] However, in these patients, the SM component is usually small and neoplastic cells usually lack KIT D816V. Moreoever, in most cases of *FIP1L1/PDGFRA*$^+$ CEL, the criteria for SM are not fulfilled even if MC are spindle-shaped cells expressing CD25.[54,55] The delineation between *FIP1L1/PDGFRA*$^+$ CEL and KIT D816V$^+$ advanced SM with eosinophilia has important clinical implications, as only patients with typical CEL with a rearranged PDGFR, but not those with advanced SM exhibiting KIT D816V, respond to treatment with imatinib.

Diagnostic algorithm and staging in patients with suspected SM

In adult patients with histologically confirmed mastocytosis in the skin (MIS), a BM biopsy is recommended, regardless of the serum tryptase level.[6,11,12,21] In adult patients without skin lesions who are suffering from typical mediator-related symptoms, the basal serum tryptase level is an important "pre-invasive" screen parameter. In patients who have a clearly elevated basal serum tryptase level, a BM biopsy should be performed.[21] It is of great importance to know that the serum tryptase increases transiently during an anaphylactic episode.[8,21,22,62,63] In these patients, serum samples for basal tryptase measurements should be collected at least 48 h after complete resolution of all symptoms.[21] Another useful screen approach is to examine peripheral blood cells for the presence of KIT D816V by a highly sensitive test. The presence of KIT D816V is highly indicative for the presence of SM in such cases. In patients with known SM, a number of different staging investigations need to be performed. BM investigations include BM smears (Wright-Giemsa staining), histology and immunohistochemistry, cytogenetics, PCR to detect KIT D816V, and flow cytometry if available.[6,11-14,21,39] Flow cytometry should be performed in order to document expression of CD2 and/or CD25 on neoplastic MC.[21,39] However, today, expression of CD25 in BM MC can also be demonstrated easily by immunohistochemistry (IHC).[64,65] In both staining methods (flow and IHC), CD25 is the more sensitive and more specific diagnostic stain.[65] Peripheral blood investigations include a complete blood count with (microscopic) differential counts, blood chemistry, including serum tryptase,

Table 2. Classification of mast cell disorders (mastocytosis).

Variant	Abbreviation	Subvariant(s)
Cutaneous mastocytosis	CM	- Urticaria pigmentosa (UP) = - Maculopapular CM (MPCM) - Diffuse CM (DCM) - Mastocytoma of skin
Indolent systemic mastocytosis	ISM	- (Isolated) bone marrow mastocytosis (BMM)
Smoldering systemic mastocytosis	SSM	
Systemic mastocytosis with an associated clonal hematologic non-mast cell lineage disease	SM-AHNMD - - - -	- SM-AML - SM-MDS - SM-MPN - SM-CMML* - SM-CEL** - SM-NHL
Aggressive systemic mastocytosis	ASM	- Lymphadenopathic SM with eosinophilia
Mast cell leukemia	MCL	- Typical MCL - Aleukemic variant of MCL
Mast cell sarcoma	MCS	
Extracutaneous mastocytoma		
Myelomastocytic leukemia	MML	- Aleukemic variant of MML
Mast cell activation syndrome	MCAS	- Primary MCAS - Secondary MCAS - Idiopathic MCAS
Mast cell hyperplasia		

**SM-CMML is the most frequent form of SM-AHNMD. **In a subset of patients with SM-CEL, FIP1L1/PDGFRA, but no KIT D816V, is found. In each case of SM-AHNMD, both the SM variant and the AHNMD variant of the disease has to be established by using WHO criteria. AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; MPN: myeloproliferative neoplasm; CMML: chronic myelomonocytic leukemia; CEL: chronic eosinophilic leukemia; NHL: non-Hodgkin s lymphoma.*

Table 3. Diagnostic criteria for systemic mastocytosis (SM).

Major:*	Multifocal dense infiltrates of MC in bone marrow or other extracutaneous organ(s) (>15 MCs in aggregate)
Minor:*	i. MC in bone marrow or other extracutaneous organ(s) show an abnormal (spindle-shaped) morphology (>25%) ii. KIT mutation at codon 816** in extracutaneous organ(s) iii. MC in bone marrow express CD2 and/or CD25 iv. Serum total tryptase >20 ng/mL (does not count in patients who have AHNMD-type disease)

**When at least one major and one minor or at least three minor criteria are present, the diagnosis SM is established. **Activating mutations at codon 816 of KIT; in most cases, KIT D816V is found. MC: mast cell(s); AHNMD: associated clonal hematologic non-mast cell lineage disease.*

calcium, alkaline phosphatase, coagulation parameters, total IgE, and allergy-diagnostics. Further staging examinations include an osteodensitometry (T Score by Dexa-Scan), bone X-ray, X-ray of thorax, and an abdomen ultrasound.[11-14,21] In those patients who have a decreased T Score, a yearly Dexa-Scan is recommended. In select cases, additional investigations, such as a CT scan, may be required. It is important to note that these investigations are appropriate in adult mastocytosis, whereas in children, most of these staging investigations are usually not required. Notably, in most children with MIS, only the peripheral blood and spleen size are examined, whereas all other staging investigations are usually not performed as significant systemic involvement is rarely seen.[21]

Differential diagnoses

A number of differential diagnoses have to be considered in patients with suspected SM, especially when typical skin lesions (MIS) are not present. In fact, mediator-related symptoms are also recorded in patients with allergies, atopic patients or patients who are intolerant against certain drugs, food, plants or metals. In addition, a number of different internal disorders, neurological or psychiatric diseases, and other conditions, can mimic MC-mediator-induced symptoms. A summary of relevant 'hematologic' differential diagnoses are shown in Table 5.

In patients with cytopenia(s) and an elevated serum tryptase

Table 4. Molecular somatic lesions and abnormalities found in patients with mastocytosis.

Molecular abnormality	Reported in patients with	Estimated frequency in patients with SM	Reference#
KIT D816V	all SM variants, rarely in CM	>80%	37,38,42
KIT D816Y	CM, ISM, SM-AHNMD	<5%	10,47,48
KIT D816F	CM	<5%	10,47,48
KIT D816H	MCL, ASM, SM-AHNMD	<5%	10,47
KIT D820G	ASM	<5%	10,46
KIT V560G	ISM	<5%	10,46
KIT F522C	ISM	<5%	10,47
KIT E839K	CM	<5%	10,47,48
KIT V530I	SM-AHNMD	<5%	10,47
KIT K509I	SM (familial type)	<5%	10,47,48
Other *KIT* mutations	CM and/or SM variants	<5%	10,47,48
FIP1L1/PDGFRA	SM-CEL	<5%	53,54,55
AML1/ETO	SM-AML with t(8;21)	<5%	56
JAK2 V617F	SM-PMF	<5%	57
RAS mutations	ASM, SM-AHNMD	<5%	51
TET2 mutations	SM-AHNMD, ISM, ASM	<5%	49,50
DNMT3A mutations	ISM, SM-AHNMD	<5%	50
ASXL1 mutations	SM-AHNMD	<5%	50
CBL mutations	SM-AHNMD	<5%	50

CM: cutaneous mastocytosis; SM: systemic mastocytosis; SM-AHNMD: SM with an associated hematologic clonal non-mast cell lineage disease.

Table 5. Hematologic disorders as major differential diagnoses of SM.

Clinical findings/features	Major differential diagnoses
Cytopenia + elevated tryptase*	Myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML)
Thrombocytosis and/or splenomegaly + elevated rryptase*	Primary myelofibrosis (PMF), essential thrombocythemia (ET), RARS-T
Leukocytosis + eosinophilia + elevated tryptase*	CEL, CML, AML-M4eo, PDGFR- or FGFR-rearranged neoplasms
Leukocytosis with an increase in blast cells + elevated tryptase*	Tryptase+ AML, CML blast phase (BP)
Increase in circulating metachromatic cells	Chronic myeloid leukemia CP/AP Myelomastocytic leukemia (MML) Chronic basophilic leukemia
Lymphadenopathy and Hepato/splenomegaly	Malignant lymphoma (NHL) and Hodgkin s lymphoma**
Huge osteolysis with bone fractures + osteoporosis + elevated tryptase***	Multiple myeloma***

SM: systemic mastocytosis; RARS-T: refractory anemia with ring sideroblasts and thrombocytosis; CEL: chronic eosinophilic leukemia; CP: chronic phase; AP: accelerated phase; NHL: Non-Hodgkin s Lymphoma.
**A serum tryptase level exceeding 20 ng/mL is usually found in patients with a myeloid neoplasm. In some patients with AML, serum tryptase levels may increase to >500 ng/mL. **Neoplastic cells in advanced SM usually express CD30 (Ki-1), a marker that is otherwise specifically expressed in lymphoma cells in patients with Morbus Hodgkin's and anaplastic large cell lymphoma. ***In some patients with SM, a paraproteinemia may be detected, and in a very few patients, an overt multiple myeloma (MM) develops (SM-MM); however, osteopathy in SM usually develops independent of paraproteinemia..*

level, a number of hematologic neoplasms have to be considered. These neoplasms include myelodysplastic syndromes (MDS), primary myelofibrosis (PMF), and acute myeloid leukemia (AML).[66-69] In those patients with eosinophilia, the presence of CEL has to be considered. An increase in immature metachromatic cells in the peripheral blood may be a diagnostic challenge. In these patients, acute or chronic basophilic leukemia has to be excluded. Chronic myeloid leukemia (CML) typically presents with basophilia. In advanced CML, massive basophilia, including immature forms, may be detected. It is of importance to note that contrasting the morphology of mature cells, immature basophils are mononuclear cells, whereas immature mast cells often exhibit bi- or multi-lobed nuclei (so-called "promastocytes").[41] In cases presenting with very immature metachromatic cells (metachromatic blasts) it is usually impossible to differentiate between mast cells and basophils. In these patients, immunophenotyping and electron microscopy is required to define the type (lineage) of the affected cell.[67,70,71] A classification of metachromatic cells detectable in patients with MC disorders is shown in Table 6. One important differential diagnosis to MCL is myelomastocytic leukemia (MML).[70-72] In these patients, metachromatic blasts and promastocytes are detectable and often represent the predominant population of cells.

Table 6. Morphologically defined subsets of mast cells found in bone marrow smears in patients with mastocytosis.*

Cell type	Morphological features/criteria*
Metachromatic blast	Blast cell with a few metachromatic granules
Promastocyte = atypical MC type II	Mature or immature mast cells with bi- or multi-lobed nuclei, often hypogranulated
Atypical MC type I	Mast cells exhibiting 2 or 3 of the following three morphological criteria: i) cytoplasmic extensions (spindle shape) ii) hypogranulated cytoplasm iii) oval decentralized nucleus
Mature MC= typical tissue MC	Round cell with round central nucleus and well granulated cytoplasm

*MC: mast cell(s). *See Sperr et al. for details of morphologies and typical examples (images) of various stages of mast cell development.[41]*

Treatment options in indolent SM

In many patients with SM, no relevant symptoms occur, even when observed over years. However, because of the risk of unexpected severe anaphylaxis, prophylactic histamine receptor antagonists are usually recommended.[6,11,21] The basis of therapy in SM is a combination of an H1- and H2 histamine receptor antagonist.[6-11,21] In case of severe GI-tract symptoms, a proton pump-inhibitor (PPI) should be added.[11,21] However, such PPI should not be used without an H2 histamine receptor blocker in these patients. In patients with anaphylaxis or other severe mediator-related symptoms despite antihistamines, additional glucocorticosteroids may be required. In addition, MC-stabilizing agents are sometimes used to treat mediator-related symptoms in these patients. Some of the novel TKI, such as dasatinib and midostaurin, have been described to block IgE-dependent histamine release.[73,74] It is not known whether these effects have clinical relevance.

Osteopathy is another important clinical feature in ISM that needs attention and often requires therapy. Especially those patients who are treated with glucocorticosteroids have a rather high risk of developing osteoporosis. Repeated Dexa-Scan studies (evaluation of T score) is recommended for all patients with SM. In those in whom the T score is below -2, bisphosphonates should be considered.[21,75] Overt osteoporosis (T score <-2.5) is a major challenge in the management of SM. In many cases, pathological fractures are found despite continuous treatment with bisphosphonates. Additional treatment with low-dose interferon-alpha has been proposed for these patients, but responses have only been seen in a subgroup of patients.

A major clinical challenge in SM are co-existing allergic diseases. Notably, in patients with SM, the risk for severe life-threatening anaphylaxis is very high.[76-79] Therefore, all patients with SM are advised to avoid all known (and potential) triggers, and to carry an epi-pen self-injector.[5,8,21] Certain allergies seem to correlate with severe anaphylaxis in patients with SM. The most famous example is allergy to bee and wasp venom.[76-81] Therefore, all patients with documented allergy against hymenoptera venom should undergo specific desensitization.[82-84] Depletion of IgE has also been discussed as a potential therapeutic maneuver in SM with severe anaphylaxis, but the value of this approach remains questionable. All patients with SM who suffer from a co-existing allergy should be managed and treated in an allergy-center if possible.

Treatment options in advanced SM

Advanced SM is a term used to denote the following categories of SM: SSM, SM-AHNMD, ASM, and MCL.[11-14] These entities differ substantially from each other in terms of course and prognosis. Therefore, it is of great importance to establish the correct final diagnosis before establishing a treatment plan. In most patients with SSM, no therapy is required. However, these patients may suffer from mild anemia or other signs of incipient ASM. In addition, SSM patients may suffer from severe repeated (life-threatening) anaphylaxis. In these cases, the high burden of MC may be a decisive factor, and cytoreductive therapy may be required to reduce the risk of repeated life-threatening anaphylactic events. A number of case reports and smaller case series suggest that treatment with cladribine (2CdA) is followed by a substantial and long-lasting decrease in the MC burden (and of serum tryptase levels) in patients with SSM, and that this therapy lowers the risk of fatal anaphylaxis in these patients.[85-87] However, not all patients with SSM may respond to 2CdA.[85,86]

In patients with SM-AHNMD, the prognosis and course is usually determined by the AHNMD component of the disease, even if ASM is diagnosed (ASM-AHNMD).[5-8,16-18,56] In each case it is important to classify both the SM component and the AHNMD type according to WHO criteria in order to establish a robust treatment plan for these patients.[5-8,10-14,21,56] In general, the SM component of the disease should be treated as if no AHNMD was diagnosed and the AHNMD should be treated as if no SM was present.[11-14,21] However, there are a number of pitfalls and aspects one should consider when treating a patient with SM-AHNMD using cytoreductive

agents. Likewise, in SM-AML, the leukemia must be regarded as secondary AML, and the prognosis of these patients is unfavorable and comparable to that of other patients with secondary AML.[88] In these patients, more intensive therapy (with or without stem cell transplantation) has to be considered (Table 7). It is also important to mention that in most patients suffering from a so-called "AML with KIT D816V", a concomitant SM is detectable if a thorough histological investigation is performed (otherwise SM is just overlooked).[88,89] Another important condition is SM with associated eosinophilia (SM-eo). In these patients, a thorough molecular investigation is required.[53-55] In some of these patients, a rearranged *PDGFRA* but no *KIT D816V* is detectable.[53-55] These patients often respond to imatinib, whereas patients with KIT D816V$^+$ SM with eosinophilia show no response to imatinib because the KIT mutant confers resistance.

In patients with ASM, cytoreductive therapy is almost always required. In those who have a slowly progressing type of ASM, interferon-alpha plus prednisolone or cladribine (2CdA) is recommended.[11,21,90-92] However, only a subset of these patients show a long-lasting response.[90-92] In patients who show or develop resistance or who are suffering from rapidly progressing ASM, chemotherapy is required. In young patients who are fit and have a suitable donor, allogeneic stem cell transplantation should be considered (Table 7). In elderly patients and those who refuse a stem cell transplant, induction and repeated consolidation cycles of chemotherapy should be applied. The regimens are the same as those used to treat secondary (high risk) AML. One frequently used protocol is the FLAG (fludarabin + ARA-C + G-CSF) regimen. An alternative option is to use experimental drugs such as PKC412 (midostaurin), and hydroxyurea is commonly used as a palliative drug to control MC expansion in advanced SM.

In patients with MCL, the same strategy is followed as in ASM. However, most cases with MCL show rapid progression. Without chemotherapy, the life expectancy in MCL is less than one year. In those who have a suitable donor, allogeneic stem cell transplantation should be considered. A special condition is MC sarcoma (MCS). Most of these patients progress to MCL within a relatively short time period. Radiation and chemotherapy is usually recommended. However, despite intensive therapy, most patients die after several weeks or months.

Table 7. Cytoreductive treatment and targeted drugs in patients with SM.

Disease variant	Treatment options
Indolent systemic mastocytosis (ISM)	No cytoreductive treatment is required
Smoldering systemic mastocytosis (SSM)	Watch-and-wait in most cases. In select cases: IFN-α2b+glucocorticosteroids or 2CdA.
SM-AHNMD	Treat AHNMD as if no SM was diagnosed and treat the SM-component of the disease as if no AHNMD was found.
Examples:	
1) ISM-CEL with FIP1L1/PDGFRA	Imatinib (low dose: 100 mg per day) to control the AHNMD-type of disease (CEL)
2) ISM-AML	Chemotherapy followed by allogeneic stem cell transplantation if possible.
Aggressive systemic mastocytosis (ASM) with slow progression	IFN-α2b+glucocorticosteroids, 2CdA, if resistant: experimenal TKI (midostaurin/PKC412) or other experimental drugs/chemotherapy or hydroxyurea.
ASM with rapid progression and patients who do not respond to IFN and 2CdA	Polychemotherapy (CT), consider allogeneic stem cell transplantation in responding patients. If CT does not work: experimental therapy with a TKI (PKC412), 2CdA or other cytoreductive drugs. Hydroxyurea.
Mast cell leukemia (MCL)	Polychemotherapy followed by allogeneic stem cell transplantation (SCT) if possible. If CT and SCT can not be performed: 2CdA or experimental TKI, such as PKC412. Hydroxyurea as palliative drug.

IFN-α2b: interferon-alpha-2b; SM-AHNMD: systemic mastocytosis with an associated hematologic clonal non-mast cell lineage disease; TKI: tyrosine kinase inhibitor.

Conclusion

Mastocytosis is a heterogeneous disease defined by pathological expansion and accumulation of clonal MC in various organ systems. In most adult patients, the systemic form of the disease is diagnosed. Whereas the serum tryptase level and KIT D816V in the peripheral blood are useful screen parameters, a bone marrow examination is always required to establish the final diagnosis and subvariant in these patients. Subsequent staging and correct classification of the disease are further important steps in the evaluation of patients. Notably, the course and prognosis as well as therapy options vary greatly among disease variants. The final treatment plan has to be adapted to the individual situation in each case, and takes the presence of comorbidities and the presence of molecular targets into account. Diagnosis and therapy are thus based on a multidisciplinary approach in all patients. In complicated cases, a Center of Excellence of the ECNM should be contacted. In the past few years, a number of new treatment approaches have been developed for indolent and advanced SM. There is hope that in the future these new concepts can be translated into clinical practice, since for the moment, mastocytosis remains an incurable and often resistant disease.

References

1. Lennert K, Parwaresch MR. Mast cells and mast cell neoplasia: a review. Histopathology. 1979;3:349-65.
2. Metcalfe DD. Classification and diagnosis of mastocytosis: current status. J Invest Dermatol. 1991;96:2S-4S.
3. Valent P. Biology, classification and treatment of human mastocytosis. Wien Klin Wochenschr. 1996;108:385-97.
4. Hartmann K, Henz BM. Mastocytosis: recent advances in defining the disease. Br J Dermatol. 2001;144:682-95.
5. Escribano L, Akin C, Castells M, Orfao A, Metcalfe DD. Mastocytosis: current concepts in diagnosis and treatment. Ann Hematol. 2002;81:677-90.
6. Valent P, Akin C, Sperr WR, Horny HP, Arock M, Lechner K, et al. Diagnosis and treatment of systemic mastocytosis: state of the art. Br J Haematol. 2003;122:1-23.
7. Akin C, Metcalfe DD. Systemic mastocytosis. Annu Rev Med. 2004;55:419-32.
8. Valent P, Sperr WR, Schwartz LB, Horny HP. Diagnosis and classification of mast cell proliferative disorders: delineation from immunologic diseases and non-mast cell hematopoietic neoplasms. J Allergy Clin Immunol. 2004;114:3-11.
9. Horny HP, Sotlar K, Valent P. Mastocytosis: state of the art. Pathobiology. 2007;74:121-32.
10. Arock M, Valent P. Pathogenesis, classification and treatment of mastocytosis: state of the art in 2010 and future perspectives. Expert Rev Hematol. 2010;3:497-516.
11. Valent P, Horny H-P, Escribano L, Longley JB, Li CY, Schwartz LB, et al. Diagnostic criteria and classification of mastocytosis:

a consensus proposal. Report of "Year 2000 Working Conference on Mastocytosis". Leuk Res. 2001;25:603-25.

12. Valent P, Horny H-P, Li CY, Longley JB, Metcalfe DD, Parwaresch RM, et al. Mastocytosis. World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues. (eds.) Jaffe ES, Harris NL, Stein H, Vardiman JW. IARC Press Lyon, France, 2001, pp 291-302.
13. Valent P, Akin C, Sperr WR, Horny HP, Metcalfe DD. Mast cell proliferative disorders: current view on variants recognized by the World Health Organization. Hematol Oncol Clin North Am. 2003;17:1227-41.
14. Horny HP, Akin C, Metcalfe DD, Bain BJ, Akin C, Escribano L, et al. Mastocytosis (mast cell disease). In: World Health Organization (WHO) Classification of Tumours. Pathology & Genetics. Tumours of Haematopoietic and Lymphoid Tissues. (eds.) Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW. IARC Press, Lyon, France, 2008, pp 54-63.
15. Valent P, Akin C, Sperr WR, Escribano L, Arock M, Horny HP, et al. Aggressive systemic mastocytosis and related mast cell disorders: current treatment options and proposed response criteria. Leuk Res. 2003;27:635-41.
16. Lim KH, Tefferi A, Lasho TL, Finke C, Patnaik M, Butterfield JH, et al. Systemic mastocytosis in 342 consecutive adults: survival studies and prognostic factors. Blood. 2009;113: 5727-36.
17. Pardanani A, Lim KH, Lasho TL, Finke CM, McClure RF, Li CY, et al. WHO subvariants of indolent mastocytosis: clinical details and prognostic evaluation in 159 consecutive adults. Blood. 2010;115:150-1.
18. Sperr WR, Valent P. Diagnosis, progression patterns and prognostication in mastocytosis. Expert Rev Hematol. 2012;5:261-74.
19. Brockow K, Jofer C, Behrendt H, Ring J. Anaphylaxis in patients with mastocytosis: a study on history, clinical features and risk factors in 120 patients. Allergy. 2008;63:226-32.
20. Bonadonna P, Perbellini O, Passalacqua G, Caruso B, Colarossi S, Dal Fior D, et al. Clonal mast cell disorders in patients with systemic reactions to Hymenoptera stings and increased serum tryptase levels. J Allergy Clin Immunol. 2009;123:680-6.
21. Valent P, Akin C, Escribano L, Födinger M, Hartmann K, Brockow K, et al. Standards and standardization in mastocytosis: consensus statements on diagnostics, treatment recommendations and response criteria. Eur J Clin Invest. 2007; 37:435-53.
22. Valent P, Akin C, Arock M, Brockow K, Butterfield JH, Carter MC, et al. Definitions, criteria and global classification of mast cell disorders with special reference to mast cell activation syndromes: a consensus proposal. Int Arch Allergy Immunol. 2012;157:215-25.
23. Akin C, Valent P, Metcalfe DD. Mast cell activation syndrome: Proposed diagnostic criteria. J Allergy Clin Immunol. 2010;126:1099-104.e4.
24. Valent P: Definition and classification of mast cell activation syndromes. Allergy. 2013;68:417-24.
25. Johannson C, Roupe G, Linstedt G, Melistrom D. Bone density, bone markers, and bone radiological features in mastocytosis. Age Ageing. 1996;25:1-7.
26. Delsignore JL, Dvoretsky PM, Hicks DG, O'Keefe RJ, Rosier RN. Mastocytosis presenting as a skeletal disorder. Iowa Orthop J. 1996;16:126-34.
27. Hartmann K, Henz BM. Cutaneous mastocytosis - clinical heterogeneity. Int Arch Allergy Immunol. 2002;127:143-6.
28. Sokol H, Georgin-Lavialle S, Grandpeix-Guyodo C, Canioni D, Barete S, Dubreuil P, et al. Gastrointestinal involvement and manifestations in systemic mastocytosis. Inflamm Bowel Dis. 2010;16:1247-53.
29. Valent P, Bettelheim P. Cell surface structures on human basophils and mast cells: biochemical and functional characterization. Adv Immunol. 1992;52:333-423.
30. Metcalfe DD. Mast cells and mastocytosis. Blood. 2008; 112:946-56.
31. Valent P. The riddle of the mast cell: kit(CD117)-ligand as the missing link? Immunol Today. 1994;15:111-4.
32. Valent P, Spanblöchl E, Sperr WR, Sillaber C, Zsebo KM, Agis H, et al. Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. Blood. 1992;80:2237-45.
33. Galli SJ, Tsai M, Wershil BK. The c-kit receptor, stem cell factor, and mast cells. What each is teaching us about the others. Am J Pathol. 1993;142:965-74.
34. Nettleship E, Tay W. Rare forms of urticaria. Brit Med J. 1869;2:323-30.
35. Ellis JM. Urticaria pigmentosa. A report of a case with autopsy. AMA Arch Pathol. 1949;48:426-9.
36. Schwartz LB, Sakai K, Bradford TR, Ren S, Zweiman B, Worobec AS, et al. The alpha form of human tryptase is the predominant type present in blood at baseline in normal subjects and is elevated in those with systemic mastocytosis. J Clin Invest. 1995;96:2702-10.
37. Nagata H, Worobec AS, Oh CK, Chowdhury BA, Tannenbaum S, Suzuki Y, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. Proc Natl Acad Sci USA. 1995;92:10560-4.
38. Longley BJ, Tyrrell L, Lu SZ, Ma YS, Langley K, Ding TG, et al. Somatic c-KIT activating mutation in urticaria pigmentosa and aggressive mastocytosis: establishment of clonality in a human mast cell neoplasm. Nat Genet. 1996;12:312-4.
39. Escribano L, Orfao A, Díaz-Agustin B, Villarrubia J, Cerveró C, López A, et al. Indolent systemic mast cell disease in adults: immunophenotypic characterization of bone marrow mast cells and its diagnostic implications. Blood. 1998;91:2731-6.
40. Horny HP, Sillaber C, Menke D, Kaiserling E, Wehrmann M, Stehberger B, et al. Diagnostic value of immunostaining for tryptase in patients with mastocytosis. Am J Surg Pathol. 1998;22:1132-40.
41. Sperr WR, Escribano L, Jordan JH, Schernthaner GH, Kundi M, Horny HP, et al. Morphologic properties of neoplastic mast cells: delineation of stages of maturation and implication for cytological grading of mastocytosis. Leuk Res. 2001;25:529-36.
42. Fritsche-Polanz R, Jordan JH, Feix A, Sperr WR, Sunder-Plassmann G, Valent P, et al. Mutation analysis of C-KIT in patients with myelodysplastic syndromes without mastocytosis and cases of systemic mastocytosis. Br J Haematol. 2001;113:357-64.
43. Gotlib J, Pardanani A, Akin C, Reiter A, George T, Hermine O, et al. Response Criteria for Advanced Systemic Mastocytosis: An International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) and European Competence Network on Mastocytosis (ECNM) consensus proposal. Blood. 2013;121:2393-401.
44. Valent P, Arock M, Bischoff SC, Bühring HJ, Brockow K, Escribano L, et al. The European Competence Network on Mastocytosis (ECNM). Wien Klin Wochenschr. 2004;116: 647-51.
45. Valent P, Arock M, Brockow K, Elde-Elberink H, Escribano L, Gleixner KV, et al. European Competence Network on Mastocytosis (ECNM): 10 year jubilee, update, and future perspectives. Wien Klin Wochenschr. 2012;124:807-14.
46. Longley BJ, Reguera MJ, Ma Y. Classes of c-KIT activating mutations: proposed mechanisms of action and implications for disease classification and therapy. Leuk Res. 2001;25:571-6.
47. Féger F, Ribadeau Dumas A, Leriche L, Valent P, Arock M. Kit and c-kit mutations in mastocytosis: a short overview with special reference to novel molecular and diagnostic concepts. Int Arch Allergy Immunol. 2002;127:110-4.
48. Bodemer C, Hermine O, Palmérini F, Yang Y, Grandpeix-Guyodo C, Leventhal PS, et al. Pediatric mastocytosis is a clonal disease associated with D816V and other activating c-KIT mutations. J Invest Dermatol. 2010;130:804-15.
49. Tefferi A, Levine RL, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, et al. Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia. 2009;23:900-4
50. Traina F, Visconte V, Jankowska AM, Makishima H, O'Keefe CL, Elson P, et al. Single nucleotide polymorphism array lesions, TET2, DNMT3A, ASXL1 and CBL mutations are present in systemic mastocytosis. PLoS One. 2012;7:e43090.
51. Wilson TM, Maric I, Simakova O, Bai Y, Chan EC, Olivares N, et al. Clonal analysis of NRAS activating mutations in KIT-D816V systemic mastocytosis. Haematologica. 2011;96:459-63.
52. Spector MS, Iossifov I, Kritharis A, He C, Kolitz JE, Lowe SW, et al. Mast-cell leukemia exome sequencing reveals a mutation in the IgE mast-cell receptor β chain and KIT V654A. Leukemia. 2012;26:1422-5.
53. Pardanani A, Ketterling RP, Brockman SR, Flynn HC, Paternoster SF, Shearer BM, et al. CHIC2 deletion, a surrogate for FIP1L1-PDGFRA fusion, occurs in systemic mastocytosis associated with eosinophilia and predicts response to imatinib mesylate therapy. Blood. 2003;102:3093-6.
54. Pardanani A, Brockman SR, Paternoster SF, Flynn HC, Ketterling RP, Lasho TL, et al. FIP1L1-PDGFRA fusion: prevalence and clinicopathologic correlates in 89 consecutive patients with moderate to severe eosinophilia. Blood. 2004;104:3038-45.

55. Florian S, Esterbauer H, Binder T, Müllauer L, Haas OA, Sperr WR, et al. Systemic mastocytosis (SM) associated with chronic eosinophilic leukemia (SM-CEL): detection of FIP1L1/PDGFRalpha, classification by WHO criteria, and response to therapy with imatinib. Leuk Res. 2006;30:1201-5.
56. Sperr WR, Horny HP, Lechner K, Valent P. Clinical and biologic diversity of leukemias occurring in patients with mastocytosis. Leuk Lymphoma. 2000;37:473-86.
57. Sotlar K, Bache A, Stellmacher F, Bültmann B, Valent P, Horny HP. Systemic mastocytosis associated with chronic idiopathic myelofibrosis: a distinct subtype of systemic mastocytosis associated with a clonal hematological non-mast cell lineage disorder carrying the activating point mutations KITD816V and JAK2V617F. J Mol Diagn. 2008;10:58-66.
58. Stellmacher F, Sotlar K, Balleisen L, Valent P, Horny HP. Bone marrow mastocytosis associated with IgM kappa plasma cell myeloma. Leuk Lymphoma. 2004;45:801-5.
59. Sotlar K, Bache A, Stellmacher F, Bültmann B, Valent P, Horny HP. Systemic mastocytosis associated with chronic idiopathic myelofibrosis: a distinct subtype of systemic mastocytosis associated with a clonal hematological non-mast cell lineage disorder carrying the activating point mutations KITD816V and JAK2V617F. J Mol Diagn. 2008;10:58-66.
60. Pardanani A, Lim KH, Lasho TL, Finke C, McClure RF, Li CY, et al. Prognostically relevant breakdown of 123 patients with systemic mastocytosis associated with other myeloid malignancies. Blood. 2009;114:3769-72.
61. Wang SA, Hutchinson L, Tang G, Chen SS, Miron PP, Huh YO, et al. Systemic mastocytosis with associated clonal hematological non-mast cell lineage disease: Clinical significance and comparison of chomosomal abnormalities in SM and AHNMD components. Am J Hematol. 2013;88:219-24.
62. Schwartz LB, Irani AM. Serum tryptase and the laboratory diagnosis of systemic mastocytosis. Hematol Oncol Clin North Am. 2000;14:641-57.
63. Schwartz LB. Diagnostic value of tryptase in anaphylaxis and mastocytosis. Immunol Allergy Clin North Am. 2006;26:451-63.
64. Sotlar K, Horny HP, Simonitsch I, Krokowski M, Aichberger KJ, Mayerhofer M, et al. CD25 indicates the neoplastic phenotype of mast cells: a novel immunohistochemical marker for the diagnosis of systemic mastocytosis (SM) in routinely processed bone marrow biopsy specimens. Am J Surg Pathol. 2004;28:1319-25.
65. Baumgartner C, Sonneck K, Krauth MT, Kneidinger M, Födinger M, Hauswirth AW, et al. Immunohistochemical assessment of CD25 is equally sensitive and diagnostic in mastocytosis compared to flow cytometry. Eur J Clin Invest. 2008;38:326-35.
66. Sperr WR, Jordan JH, Baghestanian M, Kiener HP, Samorapoompichit P, Semper H, et al. Expression of mast cell tryptase by myeloblasts in a group of patients with acute myeloid leukemia. Blood. 2001;98:2200-9.
67. Samorapoompichit P, Kiener HP, Schernthaner GH, Jordan JH, Agis H, Wimazal F, et al. Detection of tryptase in cytoplasmic granules of basophils in patients with chronic myeloid leukemia and other myeloid neoplasms. Blood. 2001;98:2580-3.
68. Sperr WR, Stehberger B, Wimazal F, Baghestanian M, Schwartz LB, Kundi M, et al. Serum tryptase measurements in patients with myelodysplastic syndromes. Leuk Lymphoma. 2002;43:1097-105.
69. Sperr WR, Hauswirth AW, Valent P. Tryptase a novel biochemical marker of acute myeloid leukemia. Leuk Lymphoma. 2002;43:2257-61.
70. Valent P, Sperr WR, Samorapoompichit P, Geissler K, Lechner K, Horny HP, Bennett JM. Myelomastocytic overlap syndromes: biology, criteria, and relationship to mastocytosis. Leuk Res. 2001;25:595-602.
71. Valent P, Samorapoompichit P, Sperr WR, Horny HP, Lechner K. Myelomastocytic leukemia: myeloid neoplasm characterized by partial differentiation of mast cell-lineage cells. Hematol J. 2002;3:90-4.
72. Arredondo AR, Gotlib J, Shier L, Medeiros B, Wong K, Cherry A, et al. Myelomastocytic leukemia versus mast cell leukemia versus systemic mastocytosis associated with acute myeloid leukemia: a diagnostic challenge. Am J Hematol. 2010;85:600-6.
73. Kneidinger M, Schmidt U, Rix U, Gleixner KV, Vales A, Baumgartner C, et al. The effects of dasatinib on IgE receptor-dependent activation and histamine release in human basophils. Blood. 2008;111:3097-107.
74. Krauth MT, Mirkina I, Herrmann H, Baumgartner C, Kneidinger M, Valent P. Midostaurin (PKC412) inhibits immunoglobulin E-dependent activation and mediator release in human blood basophils and mast cells. Clin Exp Allergy. 2009;39:1711-20.
75. Lim AY, Ostor AJ, Love S, Crisp AJ. Systemic mastocytosis: a rare cause of osteoporosis and its response to bisphosphonate treatment. Ann Rheum Dis. 2005;6:965-6.
76. Bonadonna P, Perbellini O, Passalacqua G, Caruso B, Colarossi S, Dal Fior D, et al. Clonal mast cell disorders in patients with systemic reactions to Hymenoptera stings and increased serum tryptase levels. J Allergy Clin Immunol. 2009;123:680-6.
77. Wimazal F, Geissler P, Shnawa P, Sperr WR, Valent P. Severe life-threatening or disabling anaphylaxis in patients with systemic mastocytosis: a single-center experience. Int Arch Allergy Immunol. 2012;157:399-405.
78. Alvarez-Twose I, Bonadonna P, Matito A, Zanotti R, González-de-Olano D, Sánchez-Muñoz L, et al. Systemic mastocytosis as a risk factor for severe Hymenoptera sting-induced anaphylaxis. J Allergy Clin Immunol. 2013;131:614-5.
79. Bonadonna P, Zanotti R, Müller U. Mastocytosis and insect venom allergy. Curr Opin Allergy Clin Immunol. 2010;10: 347-53.
80. Florian S, Krauth MT, Simonitsch-Klupp I, Sperr WR, Fritsche-Polanz R, Sonneck K, et al. Indolent systemic mastocytosis with elevated serum tryptase, absence of skin lesions, and recurrent severe anaphylactoid episodes. Int Arch Allergy Immunol. 2005;136:273-80.
81. Ruëff F, Placzek M, Przybilla B. Mastocytosis and Hymenoptera venom allergy. Curr Opin Allergy Clin Immunol. 2006;6:284-8.
82. Bonadonna P, Zanotti R, Caruso B, Castellani L, Perbellini O, Colarossi S, et al. Allergen specific immunotherapy is safe and effective in patients with systemic mastocytosis and Hymenoptera allergy. J Allergy Clin Immunol. 2008;121:256-7.
83. Niedoszytko M, de Monchy J, van Doormaal JJ, Jassem E, Oude Elberink JN. Mastocytosis and insect venom allergy: diagnosis, safety and efficacy of venom immunotherapy. Allergy. 2009;64:1237-45.
84. González-de-Olano D, Alvarez-Twose I, Vega A, Orfao A, Escribano L. Venom immunotherapy in patients with mastocytosis and hymenoptera venom anaphylaxis. Immunotherapy. 2011;3:637-51.
85. Böhm A, Sonneck K, Gleixner KV, Schuch K, Pickl WF, Blatt K, et al. In vitro and in vivo growth-inhibitory effects of cladribine on neoplastic mast cells exhibiting the imatinib-resistant KIT mutation D816V. Exp Hematol. 2010;38:744-55.
86. Kluin-Nelemans HC, Oldhoff JM, Van Doormaal JJ, Van 't Wout JW, Verhoef G, Gerrits WB, et al. Cladribine therapy for systemic mastocytosis. Blood. 2003;102:4270-6.
87. Schleyer V, Meyer S, Landthaler M, Szeimies RM. Smoldering systemic mastocytosis. Successful therapy with cladribine. Hautarzt. 2004;55:658-62.
88. Fritsche-Polanz R, Fritz M, Huber A, Sotlar K, Sperr WR, Mannhalter C, et al. High frequency of concomitant mastocytosis in patients with acute myeloid leukemia exhibiting the transforming KIT mutation D816V. Mol Oncol. 2010;4:335-46.
89. Schnittger S, Kohl TM, Haferlach T, Kern W, Hiddemann W, Spiekermann K, Schoch C. KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. Blood. 2006;107:1791-9.
90. Kluin-Nelemans HC, Jansen JH, Breukelman H, Wolthers BG, Kluin PM, Kroon HM, et al. Response to interferon alfa-2b in a patient with systemic mastocytosis. N Engl J Med. 1992;326:619-23.
91. Casassus P, Caillat-Vigneron N, Martin A, Simon J, Gallais V, Beaudry P, et al. Treatment of adult systemic mastocytosis with interferon-alpha: results of a multicentre phase II trial on 20 patients. Br J Haematol. 2002;119:1090-7.
92. Hauswirth AW, Simonitsch-Klupp I, Uffmann M, Koller E, Sperr WR, Lechner K, et al. Response to therapy with interferon alpha-2b and prednisolone in aggressive systemic mastocytosis: report of five cases and review of the literature. Leuk Res. 2004;28:249-57.

Management of myelofibrosis

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A B S T R A C T

Recent years have seen an important increase in our knowledge of the molecular biology and prognostic assessment of myelofibrosis (MF). Conventional therapy has a limited impact on survival and is adjusted to the clinical manifestations in each patient. It includes a 'wait-and-see' approach for asymptomatic patients, androgens, erythropoiesis-stimulating agents or immunomodulatory drugs for anemia, cytoreductive drugs such as hydroxyurea for splenomegaly and constitutional symptoms, and splenectomy or radiotherapy in selected patients. Allogeneic stem cell transplantation remains the only curative therapy for MF. While reduced intensity conditioning regimens have made this procedure available to more patients, transplant is still associated with substantial morbidity and mortality; therefore, it is generally recommended for patients with high-risk disease. Discovery of the *JAK2* mutation has paved the way for molecular targeted therapy of MF. Clinical trials with *JAK2* inhibitors are ongoing and ruxolitinib has been approved for the treatment of splenomegaly and constitutional symptoms of MF. These agents, however, usually accentuate the anemia and do not have a meaningful effect on the *JAK2* allele burden, whereas its impact on survival still needs to be confirmed. Combinations of the *JAK2* inhibitors with other agents are being tested and newer drugs are being investigated.

Learning goals

At the conclusion of this activity, participants should know that:

- while a 'pre-fibrotic' form of primary myelofibrosis is recognized in the current WHO classification, the management of these patients should be guided by their clinical profile and evolution rather than by histological features;
- in the absence of an effective treatment able to cover all clinical manifestations of MF, choice of therapy should be primarily guided by the patient's symptom type and burden. Prognostic assessment is also important, especially in decision-making concerning transplantation, since this procedure is usually restricted to patients with high-risk disease;
- the JAK inhibitors are mostly effective in splenomegaly and the constitutional symptoms of MF, and their efficacy is independent of the patient's *JAK2* mutational status. However, for the time being, there is no clear indication that these drugs may substantially modify the natural history of MF.

Introduction

Myelofibrosis (MF), formerly known as idiopathic myelofibrosis or agnogenic myeloid metaplasia, is one of the classical Philadelphia-negative chronic myeloproliferative neoplasms (MPNs).[1] The disease can appear *de novo* (primary myelofibrosis or PMF) or follow a previously known polycythemia vera (PV) or essential thrombocythemia (ET) (post-PV or post-ET MF),[2] but its clinical and histological characteristics and prognosis are essentially the same. MF is a clonal proliferation of a pluripotent hematopoietic stem cell[3,4] in which the abnormal cell population releases several cytokines and growth factors in the bone marrow. This leads to the appearance of marrow fibrosis and stroma changes, and colonizes extramedullary organs such as the spleen and the liver.[5] In its established form, MF is characterized by marrow fibrosis, extramedullary hematopoiesis with splenomegaly, and leukoerythroblastosis in blood.[5] The discovery of the V617F mutation of the *JAK*2 gene[6] represented an important step forward in our understanding of the pathogenesis of MF. Other mutations have subsequently been described.[7,8] However, the genetic trigger of MF remains unknown.

MF is a heterogeneous disease, with a clinical course that can be complicated by progressive bone marrow failure, symptomatic splenomegaly, constitutional symptoms, consumption, and manifestations of extramedullary hematopoiesis.[9] In 15-20% of patients, there is evolution to acute leukemia.[10,11] Conventional treatment is merely palliative. Allogeneic stem-cell transplantation (allo-SCT) is the only curative therapy for MF but, due to its limitations, it is not frequently performed.[12,13] The discovery of the *JAK2* mutation has paved the way for molecular targeted therapy of MF: the so-called *JAK2* inhibitors. Other drugs are currently being investigated. The present article summarizes current and future strategies for the treatment of MF.

Diagnosis and prognostic assessment

The diagnostic criteria of PMF were updated in 2008 and now incorporate the new molecular findings of the disease.[1] Of note, these criteria consider the possibility of diagnosing PMF in patients without bone marrow fibrosis and who lack the clinical-hematologic features typical of the disease. In these cases, the presence of clusters of highly dysplastic megakaryocytes in the bone marrow is the main finding to support PMF diagnosis. However, the recognition of this 'pre-fibrotic' form of the disease is controversial[14,15] as its differential diagnosis with ET is subjective and involves issues of reproducibility. In a recent collaborative study[16] in which 1104 ET patients were retrospectively analyzed, 16% were reclassified as having 'pre-fibrotic' PMF; they more frequently evolved to myelofibrosis or leukemia and had shorter survival. It is likely that a minority of ET patients actually have this histological entity. However, their management should be guided by the patient's clinical profile and evolution rather than by histological features. In turn, the diagnosis of post-PV and post-ET MF requires demonstration of marrow fibrosis in subjects with an antecedent PV or ET, in the presence of several of the typical features of MF, such as anemia, splenomegaly, constitutional symptoms, leukoerythroblastosis, and increased serum LDH.[17]

Median survival of PMF has increased over time and currently approaches seven years.[13] Main causes of death are disease progression leading to consumption, transformation into acute leukemia, infection, bleeding, portal hypertension or hepatic failure secondary to hepatic/splenoportal vein thrombosis or liver myeloid metaplasia, thromboses in other sites, heart failure, and secondary neoplasias.[13] The prognosis is heterogeneous, with a few patients living for more than 20 years and others dying within one or two years of diagnosis. In recent years, important progress has been made in assessing the prognosis of PMF. The most important unfavorable prognostic factors are anemia (Hb <10 g/dL), age over 65 years, constitutional symptoms, leukocytes higher than 25 x10^9/L, and blood blasts 1% or over. These five factors have been integrated into an International Prognostic Scoring System (IPSS). This is used at disease diagnosis and recognizes four prognostic groups: low, intermediate-1, intermediate-2, and high risk. Median survival is approximately 11, eight, four and two years, respectively.[11] The IPSS has been complemented by a dynamic IPSS (DIPSS) that can be useful at any time during the disease course.[18] This has been further refined in a DIPSS-*plus* model, also including thrombocytopenia, transfusion need and karyotypic information.[19] Table 1 summarizes current prognostic models of MF.

Planning therapy in myelofibrosis

When planning therapy in an MF patient, two main factors must be considered: the patient's prognostic group, and the type and burden of symptoms. The prognostic group is especially important for patients with high-risk disease, whose median survival is around two years.[11] Therefore, allo-SCT should be prioritized in these patients. However, quite often, allo-SCT is not a real option, due to the patient's age or the lack of a suitable donor and, consequently, investigational drugs or best palliative therapy are the therapeutic alternatives in these cases. The other extreme of the spectrum are low-risk patients, with a median survival approaching 12 years,[11] most of whom are asymptomatic. For these patients, a

Table 1. Summary of current prognostic models for myelofibrosis.

Variable	IPSS	DIPSS	DIPSS-Plus
Age > 65 years	+	+	+
Constitutional symptoms	+	+	+
Hb <10 g/dL	+	+	+
Leukocytes >25x10^9/L	+	+	+
Blood blasts >1%	+	+	+
Platelets < 100x10^9/L			+
RBC transfusion need			+
Unfavorable karyotype:			
+8, -7/7q-, -5/5q-, i17q, 12p-, 11q23 rearr.			+
	1 point each	1 point each (Hb: 2 points)	DIPSS high: 3 points DIPSS int-2: 2 points DIPPS int-1: 1 point Platelets < 100x10^9/L, unfavorable karyotype, and transfusion need: 1 point each

IPSS: International Prognostic Scoring System. Low risk: 0 points; intermediate-1 risk: 1 point; intermediate-2 risk: 2 points; high risk: 3-5 points. DIPPS: Dynamic IPSS. Low risk: 0 points; intermediate-1 risk: 1-2 points; intermediate-2 risk: 3-4 points; high risk: 5-6 points. DIPPS-plus. This is based on the DIPSS, to which the other three posible risk factors are added. Low-risk: 0 points; intermediate-1 risk: 1 point; intermediate-2 risk: 2-3 points; high risk: 4-6 points.

conservative approach seems a reasonable option, especially taking into account that, with current dynamic prognostic models, this decision can be reconsidered at anytime during the disease evolution if the clinical situation and prognosis change.

In the majority of MF patients, symptom type and burden are the main determinants of therapy choice. The predominant symptoms are those derived from anemia and splenomegaly and constitutional symptoms (weight loss, excessive sweats and low-grade fever).[9,13] Beside these, extramedullary hematopoiesis in sites other than the spleen and liver, aquagenic pruritus, bone pain, and thrombosis can represent a problem in some patients. Since, for the time being, no treatment modality other than allo-SCT covers all clinical manifestations of MF, therapy choice is basically guided by the predominant symptom in each patient. This means that we usually administer drugs for the anemia or therapies for the hyperproliferative manifestations of MF, such as splenomegaly and the constitutional symptoms. Again, the situation is often more complex, as many patients share several symptoms, whereas a therapy instituted for one symptom can worsen the other, as is the case of the anemia, frequently triggered or accentuated by the agents given for the splenomegaly.[20] This fact frequently leads to use of a combination of agents, the most typical being a cytoreductive agent plus a drug to alleviate anemia. Finally, an important consideration is that the possible survival benefit derived from the institution of a specific therapy must be balanced with its effect on the patient's quality of life.[21]

Based on the above premises, a tentative algorithm for the treatment of MF is shown in Figure 1, in which patients are allocated to different therapeutic strategies, ranging from the more conservative 'wait-and-see' approach to allo-SCT.

Treatment of anemia

Once treatable causes of anemia are excluded, such as iron, folate or vitamin B12 deficiency or immune hemolysis, there are several options to treat the anemia of MF.

Androgens

Nandrolone, fluoxymesterolone, methandrostenolone and oxymetholone were reported to improve the anemia in 30-60% of patients.[22,23] Similar results, with less toxicity, can be obtained with danazol, a semisynthetic attenuated androgen that can also correct thrombocytopenia.[24] The overall response approaches 40%, and half of these are durable over time.[24] A sufficient dose (600 mg a day) must be administered and should be maintained for a minimum of six months, unless toxicity develops, since most responses are seen between three and six months. After this, the dose must be progressively reduced to the minimum necessary to maintain the response, usually 200 mg/day. Liver function must be monitored and periodic ultrasound imaging surveillance performed to detect the possible appearance of liver tumors. Systematic screening for prostate cancer must also be carried out.

Erythropoiesis stimulating agents (ESA)

Recombinant human erythropoietin (rHuEPO) or darbepoetin-alfa achieve an improvement in anemia for approximately 40% of patients.[25,26] Median duration of responses is 12 months, and half of these are maintained in the long term. Responses are limited to patients with inappropriate serum erythropoietin levels (<125 mU/mL). As response is usually seen within a few weeks of treatment initiation, if no response is observed after three months, therapy should be definitively stopped. Increase in spleen size has

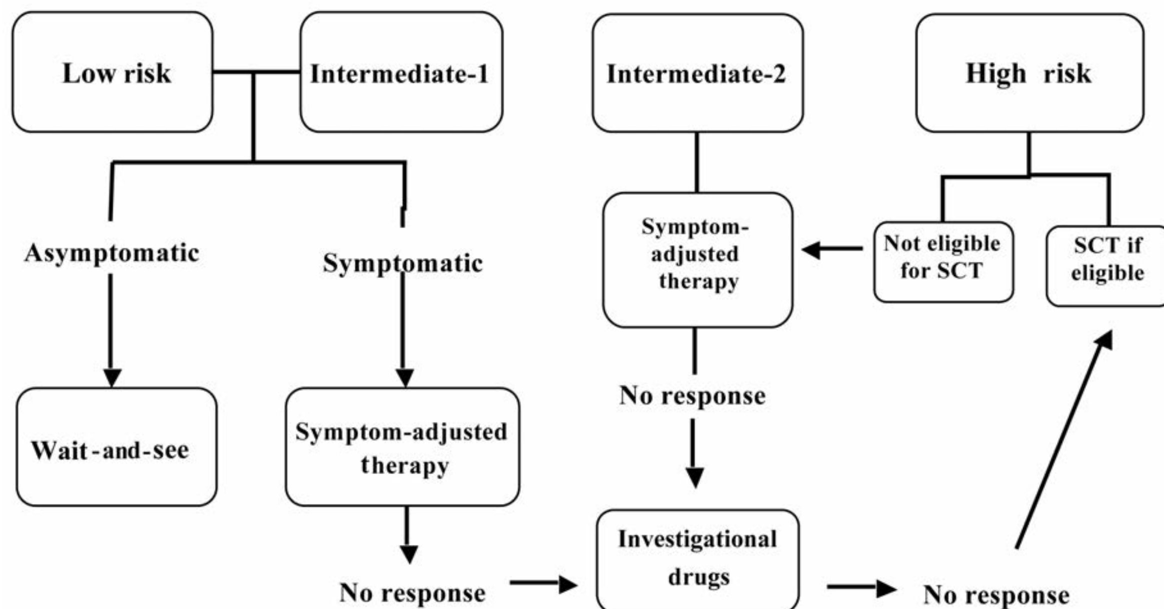

Figure 1. Proposed algorithm for the treatment of myelofibrosis.

occasionally been reported during ESA treatment, but this side effect is not a problem in the majority of patients.

Immunomodulating drugs

Thalidomide, at a starting daily dose of 100-200 mg, was associated with high treatment withdrawal due to the frequent toxicity, mainly constipation, fatigue, paresthesia, sedation, hematologic toxicity, and myeloproliferative acceleration.[27] Among assessable patients, anemia improved in 29% of cases. To minimize toxicity, lower doses of thalidomide (50 mg daily) are currently being given in combination with oral prednisone,[28] resulting in less treatment withdrawal and slightly higher response rates. However, the efficacy of thalidomide in MF has been put into question,[29] suggesting that the efficacy of low-dose thalidomide plus prednisone might be ascribed rather to the prednisone.

Lenalidomide, a thalidomide derivative, produces 22% response in the anemia of MF, with normalization of Hb in some patients, as well as some response in splenomegaly. Again, treatment discontinuation is high (30-50%) due to side effects, mainly hematologic toxicity.[30]

Treatment of splenomegaly

Cytoreductive drugs

Therapy for the 'hyperproliferative' forms of MF has been traditionally based on the use of cytoreductive agents, with hydroxyurea as the drug of choice. Hydroxyurea can reduce spleen and liver size, improve constitutional symptoms, pruritus and bone pain, and control leukocytosis and thrombocytosis, with an overall response rate of 40% and a median duration of response of 13.2 months.[20] The dose must be adjusted to the hematologic tolerability, and addition of agents to treat anemia is often required due to worsening of the anemia in half of the patients. Other oral cytoreductive drugs, such as busulfan[31] or melphalan,[32] have also been used. However, due to their associated risks, mainly long-lasting cytopenias, and their possible leukemogenic potential, they are rarely employed. 2-chlorodeoxyadenosine, a purine nucleoside analog for intravenous administration, has been reported to have a role for the treatment of progressive hepatomegaly and symptomatic thrombocytosis that develop after splenectomy.[33]

Splenectomy

Splenectomy can be considered in patients with massive and painful splenomegaly or refractory cytopenias. However, the procedure involves substantial risk. Thus, in a single institution series[34] operative morbidity was 31% and mortality 9%. Main complications are bleeding (especially hemoperitoneum), infections and thrombosis. In addition, massive hepatomegaly due to compensatory myeloid metaplasia of the liver develops in 16-24% of patients, some of whom die from liver failure.[35] Post-splenectomy thrombocytosis increases the risk of thrombosis, especially in the splenoportal vein tract.[36] Splenectomy can be considered for symptomatic splenomegaly refractory to treatment, severe constitutional symptoms, uncontrollable hemolysis, transfusion-dependent anemia unresponsive to therapy, and portal hypertension secondary to increased portal flow.[34] In the Mayo Clinic series, durable responses in constitutional symptoms, transfusion-dependent anemia, and portal hypertension were obtained in 67%, 23% and 50%, respectively.[34] In every patient, the risks of splenectomy should be balanced against the possible advantages. The availability of the JAK2 inhibitors will probably lead to even more limited use of splenectomy in MF.

Radiation therapy

Splenic radiation reduces spleen size and procures rapid symptom relief. Doses are variable, ranging from 0.15 Gy to 65 Gy per course, administered on a fractioned basis.[37] Splenic radiation can be considered for patients not responding to JAK2 inhibitors who are poor candidates for surgery and for palliation of severe pain from spleen infarction. However, its effect is transient, whereas the risk of provoking severe and long-lasting cytopenias is high, due to an effect on circulating progenitors.[38] This latter complication is observed in up to one-third of patients and can be life-threatening due to severe infection or bleeding.[39] Therefore, routine use of splenic irradiation in MF patients is not recommended. Also, it should be noted that an increased risk of postoperative bleeding has been observed in patients submitted to splenic radiation to reduce spleen size before splenectomy.[39]

Treatment of extramedullary hematopoiesis

Low-dose radiation therapy is the therapy of choice for symptomatic extramedullary hematopoiesis in places other than the spleen, such as the spinal cord, the peritoneum or the pleura, granulocytic sarcomas of the bone causing local pain, and pulmonary hypertension secondary to myeloid metaplasia of the lung.[40,41] Further experience with patients receiving JAK inhibitors is needed to determine whether these drugs are also effective in this complication of MF.

Treatment of constitutional symptoms

Until the introduction of the JAK2 inhibitors, treatment of the constitutional symptoms associated with MF was largely unsatisfactory, although a proportion of patients may obtain transient benefit from cytoreductive therapy[20] or corticosteroids.

Allogeneic stem cell transplantation

Allo-SCT remains the only curative therapy for MF. However, most published series of conventional conditioning allo-SCT include patients transplanted one or two decades ago. Therefore, these early reports are no longer appropriate to use as a basis for the decision to transplant. In 1999, Guardiola *et al.*[42] analyzed the results of allo-SCT in 55 MF patients. Graft failure was 9%, transplant-related mortality 27%, and 5-year probability of survival and disease-free survival 47% and 39%, respectively. In an update of the series, only 14% of patients transplanted over the age of 45 years survived in the long term *versus* 62% of the younger patients.[43] A more recent publication of the international bone marrow transplant registry

(IBMTR) analyzed 289 MF patients transplanted worldwide from 1989 to 2002.[44] Survival at five years was 37%, 30% and 40%, depending on the donors being identical siblings, unrelated individuals or non-identical relatives, whereas disease-free survival was 33%, 27% and 22%, respectively. In a more recent study from Seattle[45] in 170 patients transplanted between 1990 and 2009, engraftment failure was 7% and transplant-related mortality 34%, whereas overall survival and disease-free survival were both 57% at five years, with DIPSS group and comorbidity index being the main factors predictive for transplant-related mortality and overall survival. Concerning the conditioning regimen, busulfan, at doses adjusted to achieve adequate plasma levels, is associated with lower transplant-related mortality and higher survival than total body irradiation (TBI)-based regimens.[46]

Based on the demonstration of a graft-*versus*-myelofibrosis effect,[47] reduced intensity conditioning (RIC) allo-SCT was started in MF. After a pioneering experience,[48] many reports have followed. The larger series of RIC allo-SCT[49] included 103 patients conditioned with fludarabine, busulfan and anti-thymocyte globulin and transplanted from family (n=33) or unrelated (n=70) donors. Graft failure was 2% and transplant-related mortality 20%, being higher in patients over 50 years of age. Resolution of splenomegaly and marrow fibrosis was slow, taking even more than one year in some patients. A recent survey of the literature on allo-SCT in MF[50] reported a procedure-related mortality for conventional allo-SCT of 20-42% and a 5-year survival of 31-61%; the corresponding figures for RIC allo-SCT being 0-37% and 50-67%, respectively. In summary, RIC allo-SCT has lower treatment-related mortality than conventional allo-SCT while maintaining the capacity to eradicate MF. Concerning the use of conventional or RIC conditioning, although the RIC modality is also being performed in young patients, it is generally applied to patients over the age of 40-45 years. The upper age limit for transplantation is usually established at 65 years, but a slightly higher limit can be considered in individual patients.

In addition to transplant-related mortality and relapse, morbidity is also an issue. Therefore, the risks of allo-SCT *versus* the patient's expected survival must be carefully balanced. Whereas everybody agrees with the indication of allo-SCT in eligible patients with high-risk MF, for the remainder, drug treatment first seems a reasonable option, delaying transplantation until the appearance of poor-risk features or resistance to therapy. However, the transplantation results are better in patients in the favorable prognostic categories of MF.[45,51] In this sense, the long expected survival and the low risk of evolution to acute leukemia of patients with low- and intermediate-1 risk MF would argue against submitting these patients to the risk of transplantation. Patients with intermediate-2 risk MF, whose median survival is around four years, would be a more difficult group. Although there is no solid evidence to support a recommendation in these patients, a reasonable approach could be to proceed to allo-SCT in patients with unfavorable cytogenetic abnormalities and to consider it also in good candidates who fail one line of current best available therapy.

Controversy remains on the need for splenectomy before transplantation. The faster hematologic recovery and the debulking effect in patients with massive splenomegaly would favor spleen removal. In turn, the morbidity and mortality associated with the procedure, and the observation that even marked splenomegaly can resolve following transplantation,[49] would argue against splenectomy. Because of this, it seems reasonable to restrict the procedure to patients with osteosclerosis or massive splenomegaly, *i.e.* those at higher risk of graft failure.[42] Trials are ongoing to evaluate the role of the JAK2 inhibitors to reduce spleen size and improve the patient's general condition before transplantation.

New drugs

JAK2 inhibitors

The discovery of the *JAK*2 mutation triggered the development of molecular targeted therapies for the MPNs, especially for MF. However, the expectations that the JAK2 inhibitors could reproduce the enormous success of the tyrosine kinase inhibitors in chronic myeloid leukemia have not been substantiated. These agents mainly inhibit dysregulated *JAK-STAT* signaling, present in all MF patients irrespective of their *JAK*2 mutational status. Beside, all agents have overlapping activity against other members of the *JAK* family (that includes JAK1, JAK2, JAK3 and Tyk2) and sometimes against other tyrosine kinases, while they are not specific for the mutated *JAK*2.[52] Consequently, the differences in toxicity and efficacy may be ascribed to their variability in target selectivity, potency and pharmacokinetics. To date, information on the use of JAK2 inhibitors in MF is available for five drugs: ruxolitinib (formerly known as INCB018424), SAR302503 (formerly known as TG101348), CYT387, SB1518, and CEP-701, whereas other agents are at an earlier stage of clinical development (Table 2). In clinical trials, the JAK2 inhibitors have been administered to patients with intermediate-2 or high-risk MF.

Ruxolitinib, an oral JAK1/JAK2 inhibitor, was well tolerated in a phase I/II trial, with thrombocytopenia as the dose-limiting toxicity.[53] At the dose of 15 mg twice daily, half of the patients had a response in splenomegaly and constitutional symptoms. The response was usually dramatic but also drug- and dose-dependent, since treatment discontinuation or dose reductions were rapidly followed

Table 2. JAK2 inhibitors in development in myelofibrosis.

Agent	Other targets	Phase
Ruxolitinib	JAK1	III completed
SAR302503	FLT3, Ret	III
SB1518 (pacritinib)	FLT3	Entering III
CYT387	JAK1, JNK1, TYK2, CDK2	II
CEP-701	FLT3, TrkA	II
AZD1480	JAK1, JAK3	II
LY2784544	NA	I
NS-018	Src	I
BMS-911543	-	I

NA: not available.

by increase in spleen size and reappearance of the constitutional symptoms. A small proportion of patients became transfusion independent and the same proportion had accentuation of pre-existing anemia. The response was independent of the patient's *JAK2* mutational status, whereas no difference was seen between PMF and post-PV/ET MF. The effect on *JAK2*V617F allele burden was limited and there was no significant reduction in marrow fibrosis. Normalization of several pro-inflammatory cytokines was observed and was correlated with symptomatic improvement, a fact that may be ascribed to the anti-JAK1 activity of the drug. Two different phase III multicenter studies (COMFORT-I and COMFORT-II) were subsequently carried out and their early results have been published. COMFORT-I[54] compared ruxolitinib with placebo in 309 patients, whereas COMFORT-II[55] compared the drug with best-available therapy (mostly hydroxyurea) in 219 patients. Both trials attained the primary end point of 35% or over reduction in spleen size, as measured by imaging techniques, at 24 or 48 weeks of treatment start, respectively. Based on these results, ruxolitinib has been approved for the treatment of patients with high- or intermediate-risk MF with symptomatic splenomegaly. Historical comparison of the patients of the phase I-II study of ruxolitinib with a matched MF population has also shown a survival advantage for patients treated with ruxolitinib,[56] whereas extended follow up of the two phase III studies indicates a survival advantage for patients assigned to the ruxolitinib arm.[57,58] From a practical point of view, it is important to remember that sudden withdrawal of the drug can result in a shock-like syndrome, due to the re-emergence of the suppressed cytokines[59] and, because of this, the drug must be tapered. Given the palliative nature of ruxolitinib, cost considerations will be important in deciding whether it should be given to every MF patient with significant splenomegaly or if it could be used as second-line therapy in those patients with moderate symptoms who do not respond or who lose the response to hydroxyurea.

SAR302503 has preferential activity in JAK2. In a phase I/II study with 59 patients,[60] the dose-limiting toxicity was an increase in serum amylases, without clinical pancreatitis. Gastrointestinal adverse events were frequent. Worsening of anemia, thrombocytopenia and neutropenia occurred in 35%, 24% and 10% of patients, respectively. At six months, almost 60% achieved a 50% or over decrease in splenomegaly. The responses were independent of the *JAK2*V617F mutational status, but a 50% or over decrease in the allele burden was reported in 40% of mutated patients. Symptomatic response was achieved in 50-75% and, as opposed to ruxolitinib, improvement in constitutional symptoms did not correlate with changes in pro-inflammatory cytokines. A phase III study comparing SAR302503 with placebo is currently ongoing.

CYT387, a JAK1/JAK2 inhibitor, produced 45% spleen responses in a phase I/II study including 108 MF patients; resolution of constitutional symptoms was observed in the majority of patients.[61] Interestingly, of 42 patients evaluable for anemia response, 50% responded, including 58% of those who were transfusion dependent. Grade 3-4 thrombocytopenia was observed in 25% of patients, while hyperlipasemia and headache were the most characteristic non-hematologic side effects. The above important anemia response, as compared with other JAK2 inhibitors, could be of clinical interest and warrants confirmation in a larger number of patients.

SB1518 (pacritinib), a selective JAK2 inhibitor, was effective in reducing splenomegaly in 57% of 33 patients included in a phase I/II study, with scarce myelosuppresion and some gastrointestinal side effects.[62]

CEP-701 achieved clinical improvement in 6 of 22 MF patients[63] but was associated with substantial gastrointestinal toxicity. No effect on *JAK2*V617F allele burden or pro-inflammatory cytokines was documented.

The approval of ruxolitinib has led to its incorporation into current treatment algorithms of MF, in which it has a major role in the therapy for splenomegaly and constitutional symptoms. Quite likely, other JAK2 inhibitors will follow. Longer follow up is required to establish the definitive role of these drugs in MF treatment. The trade offs between clinical activity and toxicity will help choose the right drug. Information on their possible extra-hematologic effects in the long term is also needed.

Other new drugs and new strategies

Table 3 summarizes new drugs other than the JAK2 inhibitors that have been or are being tested in MF. Pomalidomide is a new thalidomide derivative with higher response rates and less toxicity than thalidomide and lenalidomide;[64] a phase III study comparing pomalidomide with placebo is ongoing. Everolimus is an m-TOR inhibitor with efficacy in the splenomegaly and the constitutional symptoms of MF.[65] In addition, given the increasing awareness of the contribution of epigenetic alterations to the pathogenesis of MF,[66] hypomethylating agents and histone deacetylase inhibitors, such as givinostat[67] and panobinostat,[68] are currently being tested. Hedgehog pathway inhibitors have also been investigated.

Ongoing clinical trials are combining the JAK inhibitors with other drugs, some of which are anemia agents, including danazol, ESA, lenalidomide or pomalidomide. Other combinations with newer drugs, such as panobinostat or hedgehog inhibitors, are aimed at achieving a disease modifying effect.

Table 3. Novel drugs other than the JAK inhibitors for myelofibrosis.

Agent	Drug class	Phase
Pomalidomide	Immunomodulator	III
Azacitidine	Hypomethylating agent	II
Decitabine	Hypomethylating agent	I-II
Givinostat	Histone deacetylase inhibitor	II
Panobinostat	Histone deacetylase inhibitor	II
Everolimus	m-TOR inhibitor	II
Obatoclax mesylate	Bcl-2 inhibitor	II
IPI926	Hedgehog inhibitor	I
LDE225	Hedgehog inhibitor	I
ABT-737	BCL-XL inhibitor	Pre-clinical
PU-H71	HSP90 inhibitor	Pre-clinical

Finally, attention has recently been paid to the possible ability of interferon, given at the early stages of MF, to alter the natural course of the disease.[69] However, although the pegylated formulation of interferon is better tolerated, tolerability remains an issue, especially considering that many patients in the early stages of MF are asymptomatic or have a low symptom burden, whereas they have a long life expectancy.

Conclusion

Myelofibrosis is a heterogeneous disease in which treatment decisions must be individualized as much as possible. These should be based mainly on the patient's prognostic assessment and the type and severity of the symptoms, and should always take into consideration that these two elements are not stable but dynamic. For the time being, conventional modalities still have a role in MF treatment. However, better understanding of the pathogenesis of MF has provided the basis for promising therapeutic advances in this disease. Hopefully, a better knowledge of the mechanisms underlying MF will lead to the availability of newer therapies with higher potential to modify the natural course and eventually cure MF.

References

1. Tefferi A, Thiele J, Orazi A, Kvasnicka HM, Barbui T, Hanson CA, et al. Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an ad hoc international expert panel. Blood. 2007;110:1092-7.
2. Mesa RA, Verstovsek S, Cervantes F, Barosi G, Reilly JT, Dupriez B, et al. Primary myelofibrosis (PMF), post polycythemia vera myelofibrosis (post-PV MF), post essential thrombocythemia myelofibrosis (post-ET MF), blast phase PMF (PMF-BP): Consensus on terminology by the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT). Leuk Res. 2007;31:737-40.
3. Jacobson RJ, Salo A, Fialkow PJ. Agnogenic myeloid metaplasia: a clonal proliferation of hematopoietic stem cells with secondary myelofibrosis. Blood. 1978;51:189-94.
4. Buschle M, Janssen JW, Drexler H, Lyons J, Anger B, Bartram CR. Evidence of pluripotent stem cell origin of idiopathic myelofibrosis: clonal analysis of a case characterized by a N-ras gene mutation. Leukemia. 1988;2:658-60.
5. Barosi G. Myelofibrosis with myeloid metaplasia: diagnostic definition and prognostic classification for clinical studies and treatment guidelines. J Clin Oncol.1999;17:2954-70.
6. Kralowics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweig JR, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352:1779-90.
7. Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PloS Med. 2006;3:1140-51.
8. Milosevic JD, Kralowics R. Genetic and epigenetic alterations of myeloproliferative disorders. Int J Hematol. 2012 [Epub ahead of print].
9. Tefferi A, Lasho T, Jimma T, Finke CM, Gangat N, Vaidya R, et al. One thousand patients with primary myelofibrosis: the Mayo Clinic experience. Mayo Clin Proc. 2012;87:25-33.
10. Tam CS, Nussenzveig RM, Popat U, Bueso-Ramos CE, Thomas DA, Cortes JA, et al. The natural history and treatment outcome of blast-phase BCR-ABL-negative myeloproliferative neoplasms. Blood. 2008;112:1628-37.
11. Cervantes F, Dupriez B, Pereira A, Passamonti F, Reilly JT, Morra E, et al. A new prognostic scoring system for primary myelofibrosis based on a study of the International Working Group for Myelofibrosis Research and Treatment. Blood. 2009;113:2895-901.
12. Ballen KK, Shrestha S, Sobocinski KA, Zhang MJ, Bashey A, Bolwell BJ, et al. Outcome of transplantation for myelofibrosis. Biol Bone Marrow Transplant. 2010;16:358-67.
13. Cervantes F, Dupriez B, Passamonti F, Vannucchi AM, Morra E, Reilly JT, et al. Improving survival trends in primary myelofibrosis: an international study. J Clin Oncol. 2012;30:2981-7.
14. Wilkins BS, Erber WN, Bareford D, Buck G, Wheatley K, East CL, et al. Bone marrow pathology in essential thrombocythemia: inter-observer reliability and utility for identifying disease subtypes. Blood. 2008;111:60-70.
15. Buhr T, Hebeda K, Kaloutsi V, Porwit A, Van der Walt J, Kreipe H. European Bone Marrow Working Group trial on reproducibility of World Health Organization criteria to discriminate essential thrombocythemia from prefibrotic primary myelofibrosis. Haematologica. 2012;97:360-5.
16. Barbui T, Thiele J, Passamonti F, Rumi E, Boveri E, Ruggeri M, et al. Survival and risk of leukemic transformation in essential thrombocythemia are significantly influenced by accurate morphologic diagnosis: an international study of 1,104 patients. J Clin Oncol. 2011;29:3179-84.
17. Barosi G, Mesa RA, Thiele J, Cervantes F, Campbell PJ, Verstovsek S, et al. Proposed criteria for the diagnosis of post-polycythemia vera and post-essential thrombocythemia myelofibrosis: a consensus statement from the International Working Group for Myelofibrosis Research and Treatment. Leukemia. 2008;22:437-8.
18. Passamonti F, Cervantes F, Vannucchi A, Morra E, Rumi E, Pereira A, et al. A dynamic prognostic model to predict survival in primary myelofibrosis: a study by the International Working Group for Myeloproliferative Neoplasms Research and Treatment. Blood. 2010;115:1703-8.
19. Gangat N, Pardanani A, Hanson CA, Van Dyke DL, Begna KH, Vaidya R, et al. DIPSS-Plus: A refined dynamic international prognostic scoring system (DIPSS) for primary myelofibrosis that incorporates prognostic information from karyotype, platelet count and transfusion status. J Clin Oncol. 2011;29:392-7.
20. Martínez-Trillos A, Gaya A, Maffioli M, Arellano-Rodrigo E, Calvo X, Díaz-Beyá M, et al. Efficacy and tolerability of hydroxyurea in the treatment of the hyperproliferative manifestations of myelofibrosis: results in 40 patients. Ann Hematol. 2010;89:1233-7.
21. Barbui T, Barosi G, Birgegard G, Cervantes F, Finazzi G, Griesshammer M, et al. Philadelphia–negative classical myeloproliferative neoplasms: Critical concepts and management recommendations from European LeukemiaNet. J Clin Oncol. 2011;29:761-70.
22. Hast R, Engstedt L, Jameson S, Killander A, Lundh B, Reizenstein P, et al. Oxymetholone treatment in myelofibrosis. Blut. 1978;37:19-26.
23. Brubaker LH, Briere J, Laszlo J, Kraut E, Landaw SA, Peterson P, et al. Treatment of anemia in myeloproliferative disorders: A randomized study of fluoxymesterone v transfusions only. Arch Intern Med. 1982;142:1533-7.
24. Cervantes F, Alvarez-Larrán A, Domingo A, Arellano-Rodrigo E, Montserrat E. Efficacy and tolerability of danazol as a treatment for the anemia of myelofibrosis with myeloid metaplasia: long-term results in 30 patients. Br J Haematol. 2005;129:771-5.
25. Cervantes F, Alvarez-Larrán A, Hernández-Boluda JC, Sureda A, Torrebadell M, Montserrat E. Erythropoietin treatment of the anaemia of myelofibrosis with myeloid metaplasia: results in 20 patients and review of the literature. Br J Haematol. 2004;127:399-403.
26. Cervantes F, Alvarez-Larrán A, Hernández-Boluda JC, Sureda A, Granell M, Vallansot R, et al. Efficacy and safety of darbepoetin-alpha in the anaemia of myelofibrosis with myeloid metaplasia. Br J Haematol. 2006;134:184-6.
27. Barosi G, Elliott M, Canepa M, Ballerini F, Piccaluga PP, Visani G, et al. Thalidomide in myelofibrosis with myeloid metaplasia: a pooled-analysis of individual patient data from 5 studies. Leuk Lymph. 2002;43:2301-7.
28. Mesa RA, Steensma DP, Pardanani A, Li CY, Elliott M, Kaufmann SH, et al. A phase 2 trial of combination low-dose thalidomide and prednisone for the treatment of myelofibrosis with myeloid metaplasia. Blood. 2003;101:2534-41.
29. Abgrall JF, Guibaud I, Bastie JN, Flesch M, Rossi JF, Lacotte-Thierry L, et al. Thalidomide versus placebo in myeloid metaplasia with myelofibrosis: a prospective, randomized, double-blind, multicenter study. Haematologica. 2006;91:1027-32.
30. Tefferi A, Cortes J, Verstovsek S, Mesa RA, Thomas D, Lasho TL, et al. Lenalidomide therapy in myelofibrosis with myeloid metaplasia. Blood. 2006;108:1158-64.
31. Oishi N, Swisher SN, Troup SB. Busulfan therapy in myeloid

metaplasia. Blood. 1960;15:863-72.
32. Petti MC, Latagliata R, Spadea T, Spadea A, Montefusco E, Aloe Spiriti MA, et al. Melphalan treatment in patients with myelofibrosis with myeloid metaplasia. Br J Haematol. 2002;116:576-81.
33. Tefferi A, Silverstein MN, Li C-Y. 2-Chlorodeoxyadenosine treatment after splenectomy in patients who have myelofibrosis with myeloid metaplasia. Br J Haematol. 1997;99:352-7.
34. Tefferi A, Mesa RA, Nagomey DN, Schroeder G, Silverstein MN. Splenectomy in myelofibrosis with myeloid metaplasia: a single-institution experience with 223 patients. Blood. 2000;95:2226-33.
35. López-Guillermo A, Cervantes F, Bruguera M, Pereira A, Feliu E, Rozman C. Liver dysfunction following splenectomy in idiopathic myelofibrosis: a study of 10 patients. Acta Haematol. 1991;85:184-8.
36. Chaffanjon PC, Brichon PY, Ranchoup Y. Portal vein thrombosis following splenectomy for hematologic disease: Retrospective study with Doppler color flow imaging. World J Surg. 1998;22:1082-6.
37. Bouabdallah R, Coso D, Gonzague-Casabianca L, Alzieu C, Resbeut M, Gastaut JA. Safety and efficacy of splenic irradiation in the treatment of patients with idiopathic myelofibrosis: a report on 15 patients. Leuk Res. 2000;24:491-5.
38. Koeffler HP, Cline MJ, Golde DW. Splenic irradiation in myelofibrosis: effect on circulating myeloid progenitor cells. Br J Haematol. 1979;43:69-77.
39. Elliott MA, Chen MG, Silverstein MN, Tefferi A. Splenic irradiation for symptomatic splenomegaly associated with myelofibrosis with myeloid metaplasia. Br J Haematol. 1998;103:505-11.
40. Barlett RP, Greipp PR, Tefferi A, Cupps RE, Mullan BP, Trastek VF. Extramedullary hematopoiesis manifesting as a symptomatic pleural effusion. Mayo Clin Proc. 1995;70:1161-4.
41. Steensma DP, Hook CC, Stafford SL, Tefferi A. Low-dose, single-fraction, whole-lung radiotherapy for pulmonary hypertension associated with myelofibrosis with myeloid metaplasia. Br J Haematol. 2002;118:813-6.
42. Guardiola P, Anderson JE, Bandini G, Cervantes F, Runde V, Arcese W, et al. Allogeneic stem cell transplantation for agnogenic myeloid metaplasia: a European Group for Blood and Marrow Transplantation, Societé Française de Greffe de Moelle, Gruppo Italiano per il Trapianto del Midollo Osseo, and Fred Hutchinson Cancer Research Center Collaborative Study. Blood. 1999;93:2831-8.
43. Guardiola P, Anderson JE, Gluckman E. Myelofibrosis with myeloid metaplasia. N Engl J Med. 2000;343:59.
44. Ballen KK, Shrestha S, Sobocinski KA, Zhang MJ, Bashey A, Bolwell BJ, et al. Outcome of transplantation for myelofibrosis. Biol Bone Marrow Transplant. 2010;16:358-67.
45. Scott BL, Gooley TA, Sorror ML, Rezvani AR, Linenberger ML, Grim J, et al. The Dynamic International Prognostic Scoring System for myelofibrosis predicts outcomes after hematopoietic cell transplantation. Blood. 2012;119:2657-64.
46. Deeg HJ, Gooley TA, Flowers ME, Sale GE, Slattery JT, Anasetti C, et al. Allogeneic hematopoietic stem cell transplantation for myelofibrosis. Blood. 2003;102:3912-8.
47. Cervantes F, Rovira M, Urbano-Ispizua A, Rozman M, Carreras E, Montserrat E. Remission of idiopathic myelofibrosis following donor lymphocyte infusion after failure of allogeneic stem cell transplantation. Bone Marrow Transplant. 2000;26:697-9.
48. Devine SM, Hoffman R, Verma A, Shah R, Bradlow BA, Stock W, et al. Allogeneic blood cell transplantation following reduced-intensity conditioning is effective therapy for older patients with myelofibrosis with myeloid metaplasia. Blood. 2002;99:2255-8.
49. Kröger N, Holler E, Kobbe G, Bornhäuser M, Schwerdtfeger R, Baurmann H, et al. Allogeneic stem cell transplantation after reduced-intensity conditioning in patients with myelofibrosis: a prospective, multicenter study of the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Blood. 2009;114:5264-70.
50. Alchalby H, Kröger N. Reduced-intensity conditioning followed by allogeneic hematopoietic stem cell transplantation in myelofibrosis. Curr Hematol Malig Rep. 2010;5:53-61.
51. Ditschowski M, Elmaagacli AH, Trenschel R, Gromke T, Steckel NK, Koldehoof M, et al. Dynamic International Prognostic Scoring sytem scores, pre-transplant therapy and chronic graft-versus-host disease determine outcome after allogeneic hematopoietic stem cell transplantation for myelofibrosis. Haematologica. 2012;97:1574-84.
52. Santos FPS, Verstovsek S. JAK2 inhibitors: what's the true potential? Blood Rev. 2011;25:53-63.
53. Verstovsek S, Kantarjian HM, Mesa R, Levy RS, Gupta V, Di Persio JF, et al. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. N Engl J Med. 2010;363:1117-27.
54. Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V, DiPersio JF, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. N Engl J Med. 2012;366:7998-8007.
55. Harrison C, Kiladjian JJ, Al-Ali HK, Gisslinger H, Waltzman R, Stalbovskaya V, et al. JAK inhibition with ruxolitinib vs best available therapy in myelofibrosis. N Engl J Med. 2012;366:787-98.
56. Verstovsek S, Kantarjian HM, Estrov Z, Cortes JE, Thomas DA, Kadia T, et al. Long-term outcomes of 107 patients with myelofibrosis receiving JAK1/JAK2 inhibitor ruxolitinib: survival advantage in comparison to matched historical controls. Blood. 2012;120:1202-9.
57. Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V, DiPersio JF, et al. Long-term outcome of ruxolitinib treatment in patients with myelofibrosis: durable reductions in spleen volume, improvements in quality of life, and overall survival advantage in COMFORT-I. Blood. 2012;120:800.
58. Cervantes F, Kiladjian JJ, Niederwieser D, Sirulnik A, Stalbovskaya V, McQuity M, et al. Long-term safety, efficacy, and survival findings from Comfort-II, a phase 3 study comparing ruxolitinib with best available therapy (BAT) for the treatment of myelofibrosis (MF). Blood. 2012;120:801.
59. Tefferi A, Pardanani A. Serious adverse events during ruxolitinib treatment discontinuation in patients with myelofibrosis. Mayo Clin Proc. 2011;86:1188-91.
60. Pardanani A, Gotlib JR, Jamieson C, Cortes JE, Talpaz M, Stone RM, et al. Safety and efficacy of TG101348, a selective JAK2 inhibitor, in myelofibrosis. J Clin Oncol. 2011;29:789-96.
61. Pardanani AD, Caramazza D, George G, Lasho T, Hogan WJ, Litzow MR, et al. Safety and efficacy of CYT387, a JAK 1 / 2 inhibitor, for the treatment of myelofibrosis. J Clin Oncol. 2011;29(Suppl):Abstract 6614.
62. Verstovsek S, Deeg HJ, Odenike O, et al. Phase 1-2 study of SB1518, a novel JAK2/FLT3 inhibitor, in the treatment of primary myelofibrosis. Blood. 2010;116:3082.
63. Santos FP, Kantarjian HM, Jain N, Manshouri T, Thomas DA, Garcia-Manero G, et al. Phase II study of CEP-701, an orally available JAK2 inhibitor, in patients with primary or post polycythemia vera/essential thrombocythemia myelofibrosis. Blood. 2010;115:1131-6.
64. Tefferi A, Verstovsek S, Barosi G, Passamonti F, Roboz GJ, Gisslinger H, et al. Pomalidomide is active in the treatment of anemia associated with myelofibrosis. J Clin Oncol. 2009;27:4563-9.
65. Guglielmelli P, Barosi G, Rambaldi A, Marchioli R, Masciulli A, Tozzi L, et al. Safety and efficacy of everolimus, a mTOR inhibitor, as single agent in a phase 1/2 study in patients with myelofibrosis. Blood. 2011;118:2069-76.
66. Vannucchi AM, Biamonte F. Epigenetics and mutations in chronic myeloproliferative neoplasms. Haematologica. 2011;96:1398-402.
67. Rambaldi A, Dellacasa CM, Finazzi G, Carobbio A, Ferrari ML, Guglielmelli P, et al. A pilot study of the Histone-Deacetylase inhibitor Givinostat in patients with JAK2V617F positive chronic myeloproliferative neoplasms. Br J Haematol. 2010;150:446-55.
68. De Angelo DJ, Tefferi A, Fiskus W, et al. A phase II trial of panobinostat, an orally available deacetylase inhibitor (DACi), in patients with primary myelofibrosis (PMF), post-essential thrombocythemia (ET), and post-polycythemia vera (PV) myelofibrosis. Blood. 2010;116:630.
69. Silver RT, Vandris K, Goldman JJ. Recombinant interferon-α may retard progression of early primary myelofibrosis: a preliminary report. Blood. 2011;117:6669-72.

Pharmacogenetics of childhood acute lymphoblastic leukemia: an actor looking for a role

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A B S T R A C T

The 5-year overall survival of childhood acute lymphoblastic leukemia (ALL) is now 85-90% in the best contemporary protocols, but this reflects an overall intensification of therapy, and as many as one-third of all deaths in childhood ALL are caused by toxicities or secondary neoplasms. Many survivors are burdened by life-long sequelae that emphasize the need to develop more individualized treatment approaches. The treatment of ALL may include more than ten different anticancer agents that are used in different doses, combinations, and routes of administration for a total period of 2-3 years. In general, the pathways (and genes) affecting the pharmacokinetics and pharmacodynamics of these drugs are well known. As with all other drugs, the absorption, distribution, elimination and effect of the drugs varies widely between patients. Much of this diversity is genetically determined, reflecting the millions of genome variants between patients. Due to the complexity of the treatment, single pharmacogenetic variants will have little influence on cure rates or risk of toxicities. Instead, extensive panels of genetic variants need to be addressed. This review summarizes the advantages and challenges for implementing pharmacogenetic testing in the treatment strategies for childhood ALL.

Learning goals

At the conclusion of this activity, participants should be able to:
- understand and address challenges for implementation of pharmacogenetics in the treatment of childhood AL;
- describe characteristics of genomic variants that can be expected to have a significant impact on cure rates of childhood AL;
- describe strategies for identification of genomic variants that could potentially influence cure rates;
- describe the potential role of thiopurine methyltransferase variants for risk of relapse and risk of secondary cancer.

Introduction

Antileukemic therapy

The complex treatment of childhood acute lymphoblastic leukemia (ALL) includes an induction phase with 3-4 drugs to obtain clinical remission, followed by multi-agent consolidation and delayed intensification, central nervous system (CNS)-directed therapy and, finally, a maintenance phase lasting 2-3 years from the time of diagnosis. The induction and delayed intensification phases generally consist of a glucocorticosteroid, vincristine, an anthracycline and/or asparaginase. During the consolidation phase, other anticancer agents are introduced, such as the alkylating agent cyclophosphamide, and the antimetabolites methotrexate (MTX, a folate antagonist), thiopurines (purine analogs), and cytarabine (a pyrimidine analog). The backbone of maintenance therapy nearly always consists of MTX and a thiopurine with or without intermittent addition of other anticancer drugs.[1]

Through improved diagnostics, risk grouping and chemotherapy, the 5-year overall survival of childhood ALL has reached an impressive 85-90% in the best contemporary protocols.[2-18] However, this reflects an overall intensification of therapy, and as many as one-third of all deaths in childhood ALL are caused by toxicities (mostly infections) or secondary neoplasms (SMN).[19-23] This emphasizes the need to develop more individualized treatment approaches. Through drug dosing by body weight or body surface area, oncologists attempt to obtain the same treatment intensity for their patients. This is in vain since all anticancer agents vary several fold in critical pharmacokinetic parameters such as bioavailability, volume of distribution, peak concentration, clearance, and area under the plasma concentration-time curve.[24] This variation is fully compatible with clinicians' experience: some patients are cured, while others with the same leukemia subtype relapse; some patients tolerate chemotherapy well, while others are burdened by or even die from a variety of serious adverse events (SAE). Clinicians seem to be confronted with an impossible triangle: the disease, the treatment, and the host (Figure 1).

The leukemic motor (karyotype, gene expression profile, methylation pattern),[25-28] the degree of disease dissemination (white blood cell count (WBC), mediastinal mass, central nervous system or testicular leukemia),[29] and leukemic

chemosensitivity (*in vitro* drug resistance, post-induction minimal residual disease (MRD))[30-32] have revealed significant correlations between such leukemia characteristics and cure rates. But the leukemia is not a self-sufficient entity. It grows within, depends on, thrives on, and eventually may kill its host; all this is strongly dependent on, precisely, the host. As no two patients have leukemias with the exact same genetic aberrations, neither are any two patients identical with respect to the gene sequences that affect drug absorption, metabolism, excretion, cellular transport, targets and target pathways, i.e. drug-response phenotypes, including occurrence of toxicities.[33,34]

Genome variants

Each week hundreds of healthy and sick individuals have their genome sequenced and cancer patients have their aberrant tumor genome sequenced. But although we are flooded by genomic data, we are still far from understanding the impact of genomic variations on the phenotype of cancer patients. Ninety percent of the genomic variations consist of single nucleotide polymorphisms (SNP), *i.e.* single base differences in the DNA sequence occurring in at least 1% of the population or on average at every 100-300 base sites. Public databases such as the Single Nucleotide Polymorphism Database (dbSNP) (http://www.ncbi.nlm.nih.gov/snp?db=snp&otool=umnbmlib) offer information on these SNPs, including their unique reference identifier (rsID) (*e.g.* rs1045642 for *MDR1* 3435C>T), their genomic location, whether they are haplotype-tagged, and whether they are synonymous (confer no amino acid change) or non-synonymous (changes the amino acid). In addition to these estimated 15 million SNPs, other genetic variations include insertions, deletions, a variable number of tandem repeats (VNTR) of 2-60 bases, and copy number variation (CNV) in sizes ranging from 1 kilobase to several megabases. In addition, individual phenotypes may be affected by DNA methylation and histone modifications, of which at least the former is heritable and can remain stable through cell divisions. Since the completion of the Human Genome Project in 2001, several public databases have offered information on these genomic variations and their functional impact, *e.g.* hosted by the National Center for Biotechnology Information (NCBI), the International HapMap Project, the 1000 Genomes Project, and the Pharmacogenetics and Pharmacogenomics Knowledge Base (PharmGKB).

Childhood ALL: a model disease

There are multiple reasons for childhood ALL being a model cancer for understanding both the clinical potential and the practical challenges of pharmacogenetics[35] (Tables 1 and 2). First, ALL is the most common cancer in child-

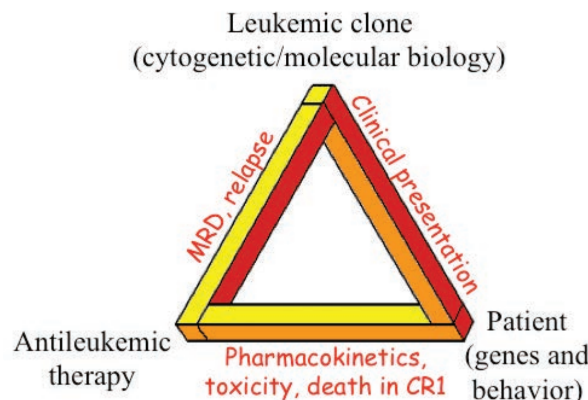

Figure 1. The impossible triangle.

Table 1. Advantages of using childhood ALL as a model for exploring the potential of pharmacogenetics.

Issue	Comment
1. Incidence	Most common cancer in childhood with an annual incidence in Europe and the US of approximately 3.5 cases per 100,000 children 0-14.9 years of age.
2. Subtype classification	The cytogenetic diversity and, *e.g.* gene expression profiles of childhood ALL is well described.
3. Collaborative trials	Patients are treated within large collaborative groups.
4. High chemosensitivity	Childhood ALL generally have high *in vivo* chemosensitivity, that is easily testable.
5. Precise MRD monitoring	Early treatment response can be determined precisely through quantification of minimal residual disease.
6. Toxicities	Serious adverse events are common, well described and potentially avoidable.
7. Huge variation in drug disposition	Huge variation in the pharmacokinetics of anticancer drugs
8. Narrow therapeutic index	The difference between the dose that causes effect and the dose that causes toxicity is small.
9. Individualized therapy by TDM ís complex or impossible	Individualized therapy by drug level measurements has had little influence on cure rates.
10. Pre-clinical testing	Leukemic clones can be cultured for testing of *in vitro* chemosensitivity and impact of pharmacogenetic polymorphisms, and data then correlated to clinical phenotypes.

TDM: therapeutic drug monitoring.

hood with an annual incidence in Europe and the US of approximately 3.5 cases per 100,000 children aged 0-14.9 years.[36] Second, the cytogenetic diversity and the gene expression profiles of childhood ALL is well described with respect to epidemiology, clinical characteristics, and biological profiles, which allows the clinical impact of pharmacogenetic polymorphisms to be explored within well-defined biological subsets.[25,37-39] Third, children with ALL are in general treated within collaborative national or multinational groups[2-18] with risk adapted and body size-based therapy, which allows large-scale exploration of whether interindividual variations in pharmacokinetics, treatment response, or toxicity can be explained by genetic polymorphisms.[33,40-42] Fourth, childhood ALL generally have a high *in vivo* chemosensitivity which increases the likelihood that variations in drug disposition significantly influence cure rates. Fifth, the early response to antileukemic therapy can be determined precisely through quantification of bone marrow minimal residual disease (MRD) using flowcytometry, chimeric gene transcripts, or clonal immune gene rearrangements.[32,43,44] This also allows us to explore the impact of pharmacogenetic variants during early phases of treatment, when patients receive a limited number of anticancer agents.[45,46] Sixth, serious adverse events (SAEs) are common. Not only will virtually all patients experience significant bone marrow and immunosuppression accompanied by a high risk of potentially life-threatening infections, but a number of other severe toxicities (*e.g.* osteonecrosis, pancreatitis, thrombosis, veno-occlusive disease) will occur in a small percentage of patients. These may be as burdensome as the leukemia itself, and their etiology can be questioned in genotype-phenotype association studies. Seventh, although drug dosing by body surface area facilitates comparison of the efficacy and toxicity of different protocols, it does not secure equal systemic drug exposure.[47,48] Eighth, the therapeutic index is very narrow for anticancer agents. Genetically determined variations in pharmacokinetics can thus affect the chances of cure or the risk of SAEs. Ninth, individualized therapy by drug level measurements has not consistently improved the cure rates,[49,50] and pharmacogenetic data can be added to drug concentration measurements to predict outcome. Finally, the malignant cells are readily available for *in vitro* chemosensitivity studies of the impact of the pharmacogenetic polymorphisms,[30,51,52] and clinical pharmacogenetic results can thus be explored through interference studies in leukemia cell lines. With all these clinical, technical and logistical advantages, and with the huge amount of cancer genome and host genome data pouring out worldwide from the laboratories of research institutions and collaborative childhood ALL groups, why then is pharmacogenetic information not routinely implemented in the treatment strategy of childhood ALL? It is not just because pediatric oncologists are conservative or skeptical, although psychological and cultural factors certainly may play a role. More important-

Table 2. Challenges for pharmacogenetically based individualization of ALL treatment.

Issue	Comment
1. Legitimized dose adjustment	*E.g.* dose reductions: risk of unacceptable toxicity (*e.g.* toxic death) outweighs the risk of relapse in higher risk patients or gives life-long sequelae in lower risk patients (*e.g.* osteonecrosis).
2. Convincing and defendable tailored therapy	PG-based dose adjustments must be defendable *statistically* (*i.e.* PG-outcome associations are supported by independent data sets), *biologically* (*i.e.* the PG-outcome associations are 'understood'), and *therapeutically* (*i.e.* effective alternatives to traditional treatment have been identified).
3. Prediction	Treatment adjustments by genetic polymorphism have predictable effects on efficacy/toxicity in individual patients.
4. Better strategy	Dose adjustments by pharmacogenetic data better/easier/cheaper than by toxicity and/or by drug concentration measurements.
5. No reverse effect	Reducing toxicity or increasing efficacy by PG-based drug dosing must not be upset by increased risk of 'reverse' events (*i.e.* less efficacy or more toxicity).
6. The target population for dose adjustments is well defined	Patient populations are not homogeneous, and PG-based drug dosing should be limited to the relevant leukemia subsets: a) the overall risk of a specific toxicity in the total population (e.g. AVN) should be significantly reduced (optimally >50%); b) interaction with leukemia subtype has been mapped.
7. Multiple genetic variants and long follow up	Multiple anticancer agents are given and their individual impact on cure rates is uncertain. Pharmacokinetics and pharmacodynamics of anticancer agents are complex and multiple genes are involved, and the effect of their genetic variants may be redundant or counteractive.
8. 'Conventional' therapy is not conventional	Clinicians already face the adverse effect of known or yet unidentified genetic variants and may on clinical grounds adjust therapy according to *e.g.* toxicity that can affect cure rates.
9. Clinical testing	PG-based treatment amendments must be tested in randomized trials of the specific and overall toxicities and relapse pattern.

AVN: avascular necrosis; PG: pharmacogenetic.

ly, there is an array of challenges that complicates the clinical implementation of pharmacogenetic data (Table 2).

First, upward or downward dose adjustments and, to an even greater extent, even elimination of specific drugs due to expected severe toxicities, need to be justified by an unacceptable balance between treatment efficacy and toxicity. Second, to implement pharmacogenetic data into first-line treatment strategies, such treatment adaptations must be defendable *statistically* (i.e. validated by independent data sets), *biologically* (clinicians as well as patients are likely to require that they 'understand' the genotype-phenotype associations), and *therapeutically* (effective treatment alternatives have been identified). Third, treatment adjustments guided by genetic profiles must have predictable effects on efficacy/toxicity in the individual patient. Thus, it is not sufficient to know that patients with a specific SNP profile are at increased risk of a specific toxicity (*e.g.* severe immunosuppression), clinicians will need to know the relevant degree of dose adjustments to compensate for the adverse genetic profile. Fourth, already today patients may receive modified therapy according to plasma concentrations of the maternal drug (*e.g.* MTX concentrations or asparaginase activity),[53] the intracellular levels of cytotoxic metabolites (*e.g.* 6-meracptopurine, 6MP, metabolites = thioguanine nucleotides),[50] or the target effect (*e.g.* post-induction MRD, or absolute neutrophil counts (ANC) during maintenance therapy).[31,42] In such cases, we need data that demonstrate that host genotyping will offer more effective / less toxic treatment guidelines compared to such traditional phenotyping. Fifth, the current childhood ALL drug combinations and doses have evolved through decades of empirical testing, including numerous randomized trials. Even though retrospective host genomic data may indicate that certain patients are at increased risk of relapse with standard dosing, there are virtually no published data that demonstrate that pharmacokinetic-based treatment intensification will not lead to more toxic deaths. Sixth, the target population needs to be precisely defined since the benefit of treatment adjustments may differ among the childhood ALL subsets, and very few of the published studies have been performed within well-defined ALL subsets.[38,45] In addition, most publications on genotype-phenotype associations report odds ratios of 2.0-3.0 at most. Figure 2A and B illustrates that even if all events could be avoided in a subpopulation with an odds ratio of 3.0, this may have little impact on the overall risk of the toxicity in question and, furthermore, will not be beneficial for most patients with the specific phenotype. Seventh, unless the end point is early MRD monitoring,[34,45,54,55] quantification of efficacy requires years of follow up, and a polymorphism that influences the efficacy of a drug given during the early treatment phases is likely to be modified by subsequent treatment with other agents. This may, in part, help explain the diverse results obtained in childhood ALL pharmacogenetic studies.[33,56,57] In addition, thousands of genetic polymorphisms may affect the most commonly used antileukemic agents, which hampers the interpretation of their individual significance, and increases the risk of type I errors. And it certainly does not help that SNPs are sometimes reported differently with respect to their genomic position.[33] Also, due to the low frequency of many of the SNPs, the statistical power of most published studies has been very low. Thus, even with a 1:1 distribution of two alleles, more than 500 patients will be needed to show a 10% reduction in cure for one of the SNPs, if the overall cure rate is 75%. Finally, many polymorphisms are linked in haplotypes, which complicates interpretation of their individual clinical impact and also the understanding on how a specific gene variant affects phenotype. A polymorphism that reduces clearance of one drug, *e.g.* glucocorticosteroids, may induce increased transcription of CYP3A enzymes, which subsequently may increase the clearance of other anticancer agents.[58] Eighth, the relationship between a given polymorphism and relapse rate may easily be misinterpreted, since some polymorphisms increase the risk of, for example, myelo- or hepatotoxicity, which subsequently may lead clinicians to decrease the dose intensity for such patients and thus potentially increase their risk of relapse.[42,59,60] Ninth, since multiple rare toxicities and relapse sites are to be questioned in trials of individualized therapy, addressing only one of these events may have little impact on the overall risk of SAEs or on cure rates. Alternatively, randomized trials can compare conventional ALL treatment with complex genetic profiling and multiple treatment amendments to explore to what extent this tailored therapy approach influences the *overall* burden of therapy and *overall* cure rates, i.e. a proof of principle strategy.

How then can we identify the genomic variants that have the strongest impact on the efficacy and/or toxicity of specific anticancer agents? Such variants should either: i) markedly influence the disposition or target of drugs that are critical for cure and widely used (*e.g.* glucocorticosteroids, asparaginase, antimetabolites); or ii) alter the activity of metabolizing enzymes or transporters that address several important antileukemic agents; or iii) have a combined effect on a complex pathway affected by widely used antileukemic agents (thiopurine therapy or DNA repair), where the cumulative effect of many SNPs are significantly

A N= 1000 pts	N	Risk	OR	N w/ AVN	Fraction AVN
PG-group A	900	5%	1.0	45	75%
PG-group B	100	15%	3.0	15	25%

B N= 1000 pts	N	Risk	OR	N w/ AVN	Fraction AVN
PG-group A	900	5%	1.0	45	45%
PG-group B	100	55%	11.0	55	55%

Figure 2. (A) Risk of AVN in PG-defined subsets. Even if a change in therapy completely eliminates the risk of AVN for group B who have a 3-fold increased risk of AVN, the overall *occurrence* of AVN among all patients (group A+B) would be reduced by only 25%, and 6 of 7 group B patients will not benefit from the intervention. (B) Risk of AVN in PG-defined subsets. If a change in therapy eliminated the risk for group B, the overall occurrence of AVN would be reduced by 55%; 55% of the patients may benefit from the intervention. AVN: avascular bone necrosis; PG: pharmacogenetic; OR: odds ratio.

associated with outcome even though the effect of each individual SNP is small. The lengthy and complex multidrug approach to childhood ALL with an interplay of thousands of genetic variants makes it likely that only very few pharmacogenetic variants will have a significant independent influence. Instead, thousands of variants should be questioned in parallel by genome-wide association studies,[34,61,62] targeted sequencing,[63] or whole genome sequencing,[64] although the latter is still too costly to be widely applied. Subsequently, extensive bioinformatic data mining and complex pathway analysis is required.

Drug disposition

Drug metabolizing enzymes are divided into phase I enzymes which metabolize the functional part of the drug leading to activation or inactivation, and phase II enzymes which conjugate drugs with endogenous substances making them more water-soluble and suitable for excretion. These detoxifying pathways and drug efflux systems are very polymorphic and often share the same anticancer agent as a substrate, and polymorphisms in such genes are thus likely to influence treatment response.[58,65,66]

Cytochrome P450 enzymes

The cytochrome P450 (CYP) phase I enzymes, and particularly the CYP3A subfamily, are involved in the activation (*e.g.* cyclophosphamide and epipodophyllotoxins) or inactivation (*e.g.* glucocorticosteroids and vinca alkaloids) of many anticancer agents. Furthermore, the glucocorticosteroids induce CYP3A enzymes, which may influence the clearance of the glucocorticosteroids themselves, but also of other anticancer agents such as vincristine.[58,67,68] Most of the *CYP* genes are highly polymorphic, and although the clinical consequences remain uncertain, several studies have indicated that these variants may influence the risk of relapse in childhood ALL.[33,69-71]

Glutathione S-transferases

The phase II enzymes glutathione *S*-transferases (GSTs) include *GSTP1* 313A>G which alters substrate affinity, *GSTP1* A114V which changes the catalytic activities, and the *GSTM1* and *GSTT1* null alleles both of which, in the case of homozygozity, lead to absence of activity. Since the GST enzymes metabolize a number of anticancer agents, including glucocorticosteroids, vincristine, anthracyclines, methotrexate, cyclophosphamide, and epipodophyllotoxins, polymorphisms in these genes are likely to influence the prognosis in childhood ALL, and at least a few, but not all, studies have shown that poor metabolizers have a decreased risk of relapse.[72-76]

Drug transporters

Many cancer cells have a multi-drug resistance (MDR) phenotype.[77] The classic form of MDR is caused by increased activity of transmembrane protein-mediated efflux of anticancer drugs. Most of the multidrug efflux proteins belong to the superfamily of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP). The lung resistance protein (LRP) is not an ABC transporter, but is also part of the MDR scenario.[77] The genes encoding these transporters are highly polymorphic, and their substrates include many anticancer agents, including vincristine, anthracyclines, methotrexate, thiopurines, and epipodophyllotoxins.[33] Yet, these polymorphisms do not seem to have a significant influence on relapse rate or toxicity in childhood ALL (reviewed by Borst *et al.*[33]).

Pharmacogenetics of childhood ALL illustrated by 6-mercaptopurine and methotrexate

The thiopurines 6-mercaptopurine (6MP) and 6-thioguanine (6TG) are among the most important drugs in the treatment of ALL.[78,79] The bioavailability of oral 6MP is highly variable.[79] A major fraction is broken down in first pass metabolism to the inactive thiouric acid. The remainder is methylated by thiopurine methyltransferase (*TPMT*) or enzymatically converted into 6TG and then to 6-thioguanine nucleotides (6TGN). 6TGN are the most important cytotoxic metabolites of thiopurines as they are incorporated into DNA (DNA-6TGN) causing DNA-damage and cell death.[80] Red blood cell levels of free 6TGN (Ery-6TGN) have been related to the risk of relapse,[50,81,82] but dosing 6MP according to Ery-6TGN does not improve cure rates[50] because Ery-6TGN levels are inadequate surrogates for events in the nucleated target cells, where the end point metabolites are DNA-6TGN.[83,84] Methylated 6MP metabolites enhance DNA-6TGN incorporation, due to inhibition of purine *de novo* synthesis[83,85-88] and is, furthermore, associated with hepatotoxicity.[89] This may explain why patients with low methylated 6MP metabolite levels in red blood cells (Ery-MeMP), *e.g.* in *TPMT* deficient patients, may tolerate Ery-6TGN levels ten times higher than *TPMT* wild-type patients.[90] Similarly, lack of methylated 6MP metabolites may explain why replacing 6MP with 6TG, as tested by the US CCG, the German COALL and the British UKALL groups, failed to improve ALL cure rates, even though children receiving 6TG had 6-fold higher Ery-6TGN levels.[85,91]

MTX inhibits folate-dependent processes, such as nucleotide *de novo* synthesis, and this affects cell proliferation and survival.[92,93] MTX is transported by Reduced Folate Carrier (*RFC1*) into target cells[94] and is then conjugated with up to seven glutamates (MTXpg).[95,96] MTX molecules with longer polyglutamate tails are retained longer intracellularly and have higher affinities for the target enzymes.[97,98]

Since patients differ widely in 6MP and MTX disposition, all international study groups recommend dose adjustments by the degree of myelotoxicity.[99] However,

since the WBC varies between healthy individuals,[100,101] the on-treatment WBC is a weak surrogate for the treatment intensity.

Pharmacogenetic variants may significantly influence the response to 6MP therapy[102] with low activity TPMT variants being studied most extensively.[103] The intermediate low-activity *TPMT* heterozygous patients have high intracellular 6TGN levels, reduced tolerance to 6MP,[103-105] a higher cure rate,[42] but also a higher risk of second cancers,[20,106] although not all groups have confirmed this, possibly due to different 6MP dosing strategies.[46] In the most extreme situation, TPMT deficient patients (homozygous for low-activity alleles) may develop life-threatening myelosuppression at standard 6MP doses.[90,107] Unfortunately, dose increments of 6MP in *TPMT* wild-type patients to obtain higher intracellular 6TGN levels and improved chance of cure, will not mirror the situation in TPMT low-activity patients, since the extra 6MP is shunted to methylated metabolites causing more liver toxicity.[89,108] and in some patients even increased risk of relapse.[50] Interestingly, the superior cure rates for patients with TPMT low-activity does not seem to be dependent on the degree of myelosuppression during maintenance therapy.[42] Still, there are no studies to demonstrate that 6MP dose reductions for patients heterozygous for *TPMT* low activity alleles to reduce their risk of secondary cancer will not lead to an increased risk of relapse.

A large number of studies have shown that the clinical variation in response to MTX reflects polymorphisms in genes involved in MTX and folate disposition (reviewed in[33,56,109]). However, many of the studies have been small, most only address one or a few of the genetic polymorphisms involved in the disposition of MTX, and subsequent larger studies or meta-analyses have not confirmed previous results.[57]

The reduced folate carrier *RFC1* 80G>A is the most investigated polymorphism in the *RCF1* gene (=*SCL19A1*). The *RFC1* gene is located on chromosome 21, which probably explains the high MTX sensitivity in children with high hyperdiploidy (which nearly always includes trisomy 21[27]) and in Down syndrome.[110,111] The A allele results in higher MTX plasma concentrations in AA homozygous patients[38,112] and has, furthermore, been more associated with gene dose-related higher cure rates in patient cohorts treated on protocols with high cumulative doses of MTX.[38] Other alleles related to higher plasma MTX concentrations include variants of the *ABC C-family* (=*MRP2*).[62,113]

Several functional polymorphisms have been found in the gene encoding folyl-polyglutamyl synthetase (*FPGS*),[114] but pharmacogenetic studies in ALL are lacking, and in rheumatoid arthritis, SNPs in the *FPGS* gene seem not to affect MTX efficacy or toxicity.[115] In contrast, low activity SNPs in γ-glutamyl hydrolase, such as *GGH* 452C>T, may increase intracellular MTX_{PG} and MTX cytotoxicity on leukaemic cells.[116] In addition, the *GGH* –401C>T genotype has been associated with decreased MTX_{PG} levels in patients with rheumatoid arthritis, indicating increased GGH activity,[117] but the clinical significance of these SNPs has not been mapped in childhood ALL. Numerous studies have been performed on several other genes related to folate metabolism, including *thymidylate synthetase* and its triple repeat (3R) polymorphism in the enhancer region of the gene,[73,118-122] *methylene-tetrahydrofolate reductase* (an important enzyme in the folate-homocysteine cycle)[123] where two SNPs in the gene encoding MTHFR have been extensively studied (*i.e.* *MTHFR* 677C>T and *MTHFR* 1298A>C) but with limited association with MTX effects,[57] and m*ethionine synthetase* and *methionine synthase reductase* that both play a role in the homocysteine-methionine pathway.[124,125] Finally, *methylene-tetrahydrofolate dehydrogenase* plays a role in purine *de novo* synthesis, and has been associated with risk of relapse, but with no association to toxicity.[126]

Conclusion and perspectives

The low-activity alleles of *TPMT* so far represent the only example of implementation of pharmacogenetically-based drug dosing in ALL protocols, and then only in a few treatment centers.[99,127] This shows that it has been difficult, other than *TPMT* polymorphisms, to establish clear associations between polymorphisms and treatment response. Ideally, clinical pharmacogenetic studies should be performed in a protocol- and ALL cytogenetic subtype-specific manner and should address both cure rates and pattern of toxicities. The genetic screening of patients needs to explore hundreds of SNPs to give a combined gene-dosage effect (*e.g.* individual SNP risk profile) rather than just question one or a few variants.[63] Until whole genome sequencing can be offered at sufficiently low costs to allow its application to all patients on a protocol, targeted *SNP profiling* will require extensive preparatory work in order to identify the genes and variants most relevant to include in an extensive targeted genotyping approach. Subsequently, a customized genotyping platform for childhood ALL needs to be designed to fully explore pharmacogenetics relating to efficacy and toxicity to allow individually tailored therapy.

References

1. Schmiegelow K, Gustafsson G. Acute Lymphoblastic Leukemia. In: Voute PA BASM, Caron M (eds.) Cancer in Children. 5th ed. Oxford: Oxford University Press; 2005:138-70.
2. Schmiegelow K, Forestier E, Hellebostad M, et al. Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2): 345-54.
3. Kamps WA, van der Pal-de Bruin KM, Veerman AJ, Fiocco M, Bierings M, Pieters R. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. Leukemia. 2010;24(2):309-19.
4. Pui CH, Pei D, Sandlund JT, et al. Long-term results of St Jude Total Therapy Studies 11, 12, 13A, 13B, and 14 for childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2):371-82.
5. Tsuchida M, Ohara A, Manabe A, et al. Long-term results of Tokyo Children's Cancer Study Group trials for childhood acute lymphoblastic leukemia, 1984-1999. Leukemia. 2010;24(2):383-96.
6. Tsurusawa M, Shimomura Y, Asami K, et al. Long-term results of the Japanese Childhood Cancer and Leukemia Study Group studies 811, 841, 874 and 911 on childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2):335-44.
7. Liang DC, Yang CP, Lin DT, et al. Long-term results of Taiwan Pediatric Oncology Group studies 1997 and 2002 for childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2):397-405.
8. Silverman LB, Stevenson KE, O'Brien JE, et al. Long-term results of Dana-Farber Cancer Institute ALL Consortium protocols for children with newly diagnosed acute lymphoblastic

leukemia (1985-2000). Leukemia. 2010;24(2):320-34.
9. Conter V, Arico M, Basso G, et al. Long-term results of the Italian Association of Pediatric Hematology and Oncology (AIEOP) Studies 82, 87, 88, 91 and 95 for childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2):255-64.
10. Stary J, Jabali Y, Trka J, et al. Long-term results of treatment of childhood acute lymphoblastic leukemia in the Czech Republic. Leukemia. 2010;24(2):425-8.
11. Stark B, Nirel R, Avrahami G, et al. Long-term results of the Israeli National Studies in childhood acute lymphoblastic leukemia: INS 84, 89 and 98. Leukemia. 2010;24(2):419-24.
12. Gaynon PS, Angiolillo AL, Carroll WL, et al. Long-term results of the children's cancer group studies for childhood acute lymphoblastic leukemia 1983-2002: a Children's Oncology Group Report. Leukemia. 2010;24(2):285-97.
13. Kamps WA, van der Pal-de Bruin KM, Veerman AJ, Fiocco M, Bierings M, Pieters R. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. Leukemia. 2010;24(2):309-19.
14. Salzer WL, Devidas M, Carroll WL, et al. Long-term results of the pediatric oncology group studies for childhood acute lymphoblastic leukemia 1984-2001: a report from the children's oncology group. Leukemia. 2010;24(2):355-70.
15. Moricke A, Zimmermann M, Reiter A, et al. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia. 2010;24(2):265-84.
16. Escherich G, Horstmann MA, Zimmermann M, Janka-Schaub GE. Cooperative study group for childhood acute lymphoblastic leukaemia (COALL): long-term results of trials 82,85,89,92 and 97. Leukemia. 2010;24(2):298-308.
17. Mitchell C, Richards S, Harrison CJ, Eden T. Long-term follow-up of the United Kingdom medical research council protocols for childhood acute lymphoblastic leukaemia, 1980-2001. Leukemia. 2010;24(2):406-18.
18. Schrappe M, Nachman J, Hunger S, et al. 'Educational symposium on long-term results of large prospective clinical trials for childhood acute lymphoblastic leukemia (1985-2000)'. Leukemia. 2010;24(2):253-4.
19. Hijiya N, Hudson MM, Lensing S, et al. Cumulative incidence of secondary neoplasms as a first event after childhood acute lymphoblastic leukemia. JAMA. 2007;297(11):1207-15.
20. Schmiegelow K, Al-Modhwahi I, Andersen MK, et al. Methotrexate/6-mercaptopurine maintenance therapy influences the risk of a second malignant neoplasm after childhood acute lymphoblastic leukemia - results from the NOPHO ALL-92 study. Blood. 2009;113:6077-84.
21. Prucker C, Attarbaschi A, Peters C, et al. Induction death and treatment-related mortality in first remission of children with acute lymphoblastic leukemia: a population-based analysis of the Austrian Berlin-Frankfurt-Munster study group. Leukemia. 2009;23(7):1264-9.
22. Lund B, Asberg A, Heyman M, et al. Risk factors for treatment related mortality in childhood acute lymphoblastic leukaemia. Pediatr Blood Cancer. 2011;56(4):551-9.
23. Schmiegelow K, Levinsen M, Attarbaschi A, et al. Second neoplasms after treatment of childhood acute lymphoblastic leukemia. J Clin Oncol. 2013. [Epub ahead of print.]
24. Rodman JH, Relling MV, Stewart CF, et al. Clinical pharmacokinetics and pharmacodynamics of anticancer drugs in children. Semin Oncol. 1993;20(1):18-29.
25. Yeoh EJ, Ross ME, Shurtleff SA, et al. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell. 2002;1(2):133-43.
26. Forestier E, Heim S, Blennow E, et al. Cytogenetic abnormalities in childhood acute myeloid leukaemia: a Nordic series comprising all children enrolled in the NOPHO-93-AML trial between 1993 and 2001. Br J Haematol. 2003;121(4):566-77.
27. Forestier E, Schmiegelow K. The incidence peaks of the childhood acute leukemias reflect specific cytogenetic aberrations. J Pediatr Hematol Oncol. 2006;28(8):486-95.
28. Milani L, Lundmark A, Kiialainen A, et al. DNA methylation for subtype classification and prediction of treatment outcome in patients with childhood acute lymphoblastic leukemia. Blood. 2010;115(6):1214-25.
29. Vaitkeviciene G, Forestier E, Hellebostad M, et al. High white blood cell count at diagnosis of childhood acute lymphoblastic leukaemia: biological background and prognostic impact. Results from the NOPHO ALL-92 and ALL-2000 studies. Eur J Haematol. 2011;86(1):38-46.
30. Schmiegelow K, Nyvold C, Seyfarth J, et al. Post-induction residual leukemia in childhood acute lymphoblastic leukemia quantified by PCR correlates with in-vitro prednisolone resistance. Leukemia. 2001;15:1066-71.
31. Campana D. Minimal residual disease in acute lymphoblastic leukemia. Semin Hematol. 2009;46(1):100-6.
32. Bruggemann M, Schrauder A, Raff T, et al. Standardized MRD quantification in European ALL trials: proceedings of the Second International Symposium on MRD assessment in Kiel, Germany, 18-20 September 2008. Leukemia. 2010;24(3):521-35.
33. Davidsen ML, Dalhoff K, Schmiegelow K. Pharmacogenetics influence treatment efficacy in childhood acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 2008;30(11):831-49.
34. Yang JJ, Cheng C, Devidas M, et al. Genome-wide association study identifies germline polymorphisms associated with relapse of childhood acute lymphoblastic leukemia. Blood. 2012;120(20):4197-204.
35. Cheok MH, Pottier N, Kager L, Evans WE. Pharmacogenetics in acute lymphoblastic leukemia. Semin Hematol. 2009;46(1):39-51.
36. Hjalgrim LL, Rostgaard K, Schmiegelow K, et al. Age- and sex-specific incidence of childhood leukemia by immunophenotype in the Nordic countries. J Natl Cancer Inst. 2003;95(20):1539-44.
37. Forestier E, Johansson B, Gustafsson G, et al. Prognostic impact of karyotypic findings in childhood acute lymphoblastic leukaemia: a Nordic series comparing two treatment periods. For the Nordic Society of Paediatric Haematology and Oncology (NOPHO) Leukaemia Cytogenetic Study Group. Br J Haematol. 2000;110(1):147-53.
38. Gregers J, Christensen IJ, Dalhoff K, et al. The association of reduced folate carrier 80G>A polymorphism to outcome in childhood acute lymphoblastic leukemia interacts with chromosome 21 copy number. Blood. 2010;115(23):4671-7.
39. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007;446(7137):758-64.
40. Evans WE, McLeod HL. Pharmacogenomics—drug disposition, drug targets, and side effects. N Engl J Med. 2003;348(6):538-49.
41. Aplenc R, Lange B. Pharmacogenetic determinants of outcome in acute lymphoblastic leukaemia. Br J Haematol. 2004;125(4):421-34.
42. Schmiegelow K, Forestier E, Kristinsson J, et al. Thiopurine methyltransferase activity is related to the risk of relapse of childhood acute lymphoblastic leukemia: results from the NOPHO ALL-92 study. Leukemia. 2009;3:557-64.
43. Nyvold C, Madsen HO, Ryder LP, et al. Precise quantification of minimal residual disease at day 29 allows identification of children with acute lymphoblastic leukemia and an excellent outcome. Blood. 2002;99(4):1253-8.
44. Obro NF, Ryder LP, Madsen HO, et al. Identification of residual leukemic cells by flow cytometry in childhood B-cell precursor acute lymphoblastic leukemia: verification of leukemic state by flow-sorting and molecular/cytogenetic methods. Haematologica. 2012;97(1):137-41.
45. Davies SM, Borowitz MJ, Rosner GL, et al. Pharmacogenetics of minimal residual disease response in children with B-precursor acute lymphoblastic leukemia: a report from the Children's Oncology Group. Blood. 2008;111(6):2984-90.
46. Stanulla M, Schaeffeler E, Moricke A, et al. Thiopurine methyltransferase genetics is not a major risk factor for secondary malignant neoplasms after treatment of childhood acute lymphoblastic leukemia on Berlin-Frankfurt-Munster protocols. Blood. 2009;114(7):1314-8.
47. Newell DR, Pearson AD, Balmanno K, et al. Carboplatin pharmacokinetics in children: the development of a pediatric dosing formula. The United Kingdom Children's Cancer Study Group. J Clin Oncol. 1993;11(12):2314-23.
48. Canal P, Chatelut E, Guichard S. Practical treatment guide for dose individualisation in cancer chemotherapy. Drugs. 1998;56(6):1019-38.
49. Evans WE, Relling MV, Rodman JH, Crom WR, Boyett JM, Pui CH. Conventional compared with individualized chemotherapy for childhood acute lymphoblastic leukemia. N Engl J Med. 1998;338(8):499-505.
50. Schmiegelow K, Bjork O, Glomstein A, et al. Intensification of mercaptopurine/methotrexate maintenance chemotherapy may increase the risk of relapse for some children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21(7):1332-9.
51. Pieters R, Klumper E, Kaspers GJ, Veerman AJ. Everything you always wanted to know about cellular drug resistance in childhood acute lymphoblastic leukemia. Crit Rev Oncol Hematol. 1997;25(1):11-26.
52. Kaspers GJ, Zwaan CM, Veerman AJ, et al. Cellular drug

resistance in acute myeloid leukemia: literature review and preliminary analysis of an ongoing collaborative study. Klin Padiatr. 1999;211(4):239-44.
53. Skarby TV, Anderson H, Heldrup J, Kanerva JA, Seidel H, Schmiegelow K. High leucovorin doses during high-dose methotrexate treatment may reduce the cure rate in childhood acute lymphoblastic leukemia. Leukemia. 2006;20(11):1955-62.
54. Anderer G, Schrappe M, Brechlin AM, et al. Polymorphisms within glutathione S-transferase genes and initial response to glucocorticoids in childhood acute lymphoblastic leukaemia. Pharmacogenetics. 2000;10(8):715-26.
55. Stanulla M, Schaeffeler E, Flohr T, et al. Thiopurine methyltransferase (TPMT) genotype and early treatment response to mercaptopurine in childhood acute lymphoblastic leukemia. JAMA. 2005;293(12):1485-9.
56. Schmiegelow K. Advances in individual prediction of methotrexate toxicity: a review. Br J Haematol. 2009;146(5): 489-503.
57. Lopez-Lopez E, Martin-Guerrero I, Ballesteros J, Garcia-Orad A. A systematic review and meta-analysis of MTHFR polymorphisms in methotrexate toxicity prediction in pediatric acute lymphoblastic leukemia. Pharmacogenomics J. 2012.[Epub ahead of print.]
58. Pichard L, Fabre I, Daujat M, Domergue J, Joyeux H, Maurel P. Effect of corticosteroids on the expression of cytochromes P450 and on cyclosporin A oxidase activity in primary cultures of human hepatocytes. Mol Pharmacol. 1992;41(6): 1047-55.
59. Schmiegelow K. Prognostic significance of methotrexate and 6-mercaptopurine dosage during maintenance chemotherapy for childhood acute lymphoblastic leukemia. Pediatr Hematol Oncol. 1991;8(4):301-12.
60. Relling MV, Hancock ML, Boyett JM, Pui CH, Evans WE. Prognostic importance of 6-mercaptopurine dose intensity in acute lymphoblastic leukemia. Blood. 1999;93(9):2817-23.
61. Yang JJ, Cheng C, Yang W, et al. Genome-wide interrogation of germline genetic variation associated with treatment response in childhood acute lymphoblastic leukemia. JAMA. 2009;301(4):393-403.
62. Trevino LR, Shimasaki N, Yang W, et al. Germline genetic variation in an organic anion transporter polypeptide associated with methotrexate pharmacokinetics and clinical effects. J Clin Oncol. 2009;27(35):5972-8.
63. Wesolowska A, Dalgaard MD, Borst L, et al. Cost-effective multiplexing before capture allows screening of 25 000 clinically relevant SNPs in childhood acute lymphoblastic leukemia. Leukemia. 2011;25(6):1001-6.
64. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-10.
65. Relling MV, Pui CH, Sandlund JT, et al. Adverse effect of anticonvulsants on efficacy of chemotherapy for acute lymphoblastic leukaemia. Lancet. 2000;356(9226):285-90.
66. Kishi S, Yang W, Boureau B, et al. Effects of prednisone and genetic polymorphisms on etoposide disposition in children with acute lymphoblastic leukemia. Blood. 2004;103(1):67-72.
67. Kishi S, Yang W, Boureau B, et al. Effects of prednisone and genetic polymorphisms on etoposide disposition in children with acute lymphoblastic leukemia. Blood. 2004;103(1):67-72.
68. Villikka K, Kivisto KT, Maenpaa H, Joensuu H, Neuvonen PJ. Cytochrome P450-inducing antiepileptics increase the clearance of vincristine in patients with brain tumors. Clin Pharmacol Ther. 1999;66(6):589-93.
69. Kuehl P, Zhang J, Lin Y, et al. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. Nat Genet JID - 9216904 2001;27(4):383-91.
70. Agundez JA. Cytochrome P450 gene polymorphism and cancer. Curr Drug Metab. 2004;5(3):211-24.
71. Borst L, Wallerek S, Dalhoff K, et al. The impact of CYP3A5*3 on risk and prognosis in childhood acute lymphoblastic leukemia. Eur J Haematol. 2011;86(6):477-83.
72. Stanulla M, Schrappe M, Brechlin AM, Zimmermann M, Welte K. Polymorphisms within glutathione S-transferase genes (GSTM1, GSTT1, GSTP1) and risk of relapse in childhood B-cell precursor acute lymphoblastic leukemia: a case-control study. Blood. 2000;95(4):1222-8.
73. Rocha JC, Cheng C, Liu W, et al. Pharmacogenetics of outcome in children with acute lymphoblastic leukemia. Blood. 2005;105(12):4752-8.
74. Kishi S, Cheng C, French D, et al. Ancestry and pharmacogenetics of antileukemic drug toxicity. Blood. 2007;109(10):4151-7.
75. Meissner B, Stanulla M, Ludwig WD, et al. The GSTT1 deletion polymorphism is associated with initial response to glucocorticoids in childhood acute lymphoblastic leukemia. Leukemia. 2004;18(11):1920-3.
76. Borst L, Buchard A, Rosthoj S, et al. Gene dose effects of GSTM1, GSTT1 and GSTP1 polymorphisms on outcome in childhood acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 2012;34(1):38-42.
77. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer. 2002;2(1):48-58.
78. Van Scoik KG, Johnson CA, Porter WR. The pharmacology and metabolism of the thiopurine drugs 6-mercaptopurine and azathioprine. Drug Metab Rev. 1985;16(1-2):157-74.
79. Lennard L. The clinical pharmacology of 6-mercaptopurine. Eur J Clin Pharmacol. 1992;43(4):329-39.
80. Uribe-Luna S, Quintana-Hau JD, Maldonado-Rodriguez R, et al. Mutagenic consequences of the incorporation of 6-thioguanine into DNA. Biochem Pharmacol. 1997;54(3):419-24.
81. Lennard L, Lilleyman JS, Van Loon J, Weinshilboum RM. Genetic variation in response to 6-mercaptopurine for childhood acute lymphoblastic leukaemia. Lancet. 1990;336 (8709):225-9.
82. Schmiegelow K, Schroder H, Gustafsson G, et al. Risk of relapse in childhood acute lymphoblastic leukemia is related to RBC methotrexate and mercaptopurine metabolites during maintenance chemotherapy. Nordic Society for Pediatric Hematology and Oncology. J Clin Oncol. 1995;13(2):345-51.
83. Hedeland RL, Hvidt K, Nersting J, et al. DNA incorporation of 6-thioguanine nucleotides during maintenance therapy of childhood acute lymphoblastic leukaemia and non-Hodgkin lymphoma. Cancer Chemother Pharmacol. 2010;66(3):485-91.
84. Jacobsen JH, Schmiegelow K, Nersting J. Liquid chromatography-tandem mass spectrometry quantification of 6-thioguanine in DNA using endogenous guanine as internal standard. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;881-882:115-8.
85. Harms DO, Gobel U, Spaar HJ, et al. Thioguanine offers no advantage over mercaptopurine in maintenance treatment of childhood ALL: results of the randomized trial COALL-92. Blood. 2003;102(8):2736-40.
86. Vogt MH, Stet EH, De Abreu RA, Bokkerink JP, Lambooy LH, Trijbels FJ. The importance of methylthio-IMP for methylmercaptopurine ribonucleoside (Me-MPR) cytotoxicity in Molt F4 human malignant T-lymphoblasts. Biochim Biophys Acta. 1993;1181(2):189-94.
87. Bokkerink JP, Bakker MA, Hulscher TW, De Abreu RA, Schretlen ED. Purine de novo synthesis as the basis of synergism of methotrexate and 6-mercaptopurine in human malignant lymphoblasts of different lineages. Biochem Pharmacol. JID - 0101032 1988;37(12):2321-7.
88. Bokkerink JP, Bakker MA, Hulscher TW, et al. Sequence-, time- and dose-dependent synergism of methotrexate and 6-mercaptopurine in malignant human T-lymphoblasts [published erratum appears in Biochem Pharmacol 1987 Mar 1;36(1):781]. Biochem Pharmacol. 1986;35(20):3549-55.
89. Nygaard U, Toft N, Schmiegelow K. Methylated metabolites of 6-mercaptopurine are associated with hepatotoxicity. Clin Pharmacol Ther. 2004;75(4):274-81.
90. Andersen JB, Szumlanski C, Weinshilboum RM, Schmiegelow K. Pharmacokinetics, dose adjustments, and 6-mercaptopurine/methotrexate drug interactions in two patients with thiopurine methyltransferase deficiency. Acta Paediatr. 1998;87(1):108-11.
91. Vora A, Mitchell CD, Lennard L, et al. Toxicity and efficacy of 6-thioguanine versus 6-mercaptopurine in childhood lymphoblastic leukaemia: a randomised trial. Lancet. 2006; 368(9544):1339-48.
92. Schmiegelow K. Advances in individual prediction of methotrexate toxicity. Br J Haematol 2009;146:489-503.
93. Nersting J, Schmiegelow K. Pharmacogenomics of methotrexate: moving towards individualized therapy. Pharmacogenomics. 2009;10(12):1887-9.
94. Baslund B, Gregers J, Nielsen CH. Reduced folate carrier polymorphism determines methotrexate uptake by B cells and CD4+ T cells. Rheumatology (Oxford). 2008;47(4):451-3.
95. Kager L, Evans WE. Pharmacogenomics of acute lymphoblastic leukemia. Curr Opin Hematol. 2006;13(4):260-5.
96. Kager L, Cheok M, Yang W, et al. Folate pathway gene expression differs in subtypes of acute lymphoblastic leukemia and influences methotrexate pharmacodynamics. J Clin Invest. 2005;115(1):110-7.

97. Chabner BA, Donehower RC, Schilsky RL. Clinical pharmacology of methotrexate. Cancer Treat Rep. 1981;65(Suppl 1):51-4.
98. Chabner BA, Allegra CJ, Curt GA, et al. Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest. 1985;76(3):907-12.
99. Arico M, Baruchel A, Bertrand Y, et al. The seventh international childhood acute lymphoblastic leukemia workshop report: Palermo, Italy, 2005. Leukemia. 2005;19(7):1145-52.
100. Schmiegelow K, Pulczynska MK. White-cell counts in childhood acute lymphoblastic leukemia. Eur J Haematol. 1990; 44(1):72-4.
101. Schmiegelow K, Ifversen M. Myelotoxicity, pharmacokinetics, and relapse rate with methotrexate/6-mercaptopurine maintenance therapy of childhood acute lymphoblastic leukemia. Pediatr Hematol Oncol. 1996;13(5):433-41.
102. de Beaumais TA, Jacqz-Aigrain E. Intracellular disposition of methotrexate in acute lymphoblastic leukemia in children. Curr Drug Metab. 2012;13(6):822-34.
103. Wang L, Weinshilboum R. Thiopurine S-methyltransferase pharmacogenetics: insights, challenges and future directions. Oncogene. 2006;25(11):1629-38.
104. Lennard L, Lilleyman JS. Variable mercaptopurine metabolism and treatment outcome in childhood lymphoblastic leukemia. J Clin Oncol. JID - 8309333 1989;7(12):1816-23.
105. Lennard L. The clinical pharmacology of 6-mercaptopurine. Eur J Clin Pharmacol. 1992;43(4):329-39.
106. Relling MV, Rubnitz JE, Rivera GK, et al. High incidence of secondary brain tumours after radiotherapy and antimetabolites. Lancet. 1999;354(9172):34-9.
107. McBride KL, Gilchrist GS, Smithson WA, Weinshilboum RM, Szumlanski CL. Severe 6-thioguanine-induced marrow aplasia in a child with acute lymphoblastic leukemia and inhibited thiopurine methyltransferase deficiency [In Process Citation]. J Pediatr Hematol Oncol. 2000;22(5):441-5.
108. Janka GE, Harms DO, Graubner U, Spaar HJ, Jorch N, Koerholz D. Randomized comparison of 6-mercaptopurine vs 6-thioguanine in childhood ALL; differing cytotoxicity and interim results. Med Ped Oncol. 1998;31:267.
109. Krajinovic M, Moghrabi A. Pharmacogenetics of methotrexate. Pharmacogenomics. 2004;5(7):819-34.
110. Taub JW, Ge Y. Down syndrome, drug metabolism and chromosome 21. Pediatr Blood Cancer. 2005;44(1):33-9.
111. Belkov VM, Krynetski EY, Schuetz JD, et al. Reduced folate carrier expression in acute lymphoblastic leukemia: a mechanism for ploidy but not lineage differences in methotrexate accumulation. Blood. 1999;93(5):1643-50.
112. Laverdiere C, Chiasson S, Costea I, Moghrabi A, Krajinovic M. Polymorphism G80A in the reduced folate carrier gene and its relationship to methotrexate plasma levels and outcome of childhood acute lymphoblastic leukemia. Blood. 2002;100(10):3832-4.
113. Rau T, Erney B, Gores R, Eschenhagen T, Beck J, Langer T. High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations. Clin Pharmacol Ther. 2006;80(5):468-76.
114. Leil TA, Endo C, Adjei AA, et al. Identification and characterization of genetic variation in the folylpolyglutamate synthase gene. Cancer Res. 2007;67(18):8772-82.
115. van der Straaten RJ, Wessels JA, de Vries-Bouwstra JK, et al. Exploratory analysis of four polymorphisms in human GGH and FPGS genes and their effect in methotrexate-treated rheumatoid arthritis patients. Pharmacogenomics. 2007;8(2):141-50.
116. Cheng Q, Wu B, Kager L, et al. A substrate specific functional polymorphism of human gamma-glutamyl hydrolase alters catalytic activity and methotrexate polyglutamate accumulation in acute lymphoblastic leukaemia cells. Pharmacogenetics. 2004;14(8):557-67.
117. Dervieux T, Kremer J, Lein DO, et al. Contribution of common polymorphisms in reduced folate carrier and gamma-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. Pharmacogenetics. 2004;14(11):733-9.
118. Horie N, Aiba H, Oguro K, Hojo H, Takeishi K. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. Cell Struct Funct. 1995;20 (3):191-7.
119. Krajinovic M, Labuda D, Mathonnet G, et al. Polymorphisms in genes encoding drugs and xenobiotic metabolizing enzymes, DNA repair enzymes, and response to treatment of childhood acute lymphoblastic leukemia. Clin Cancer Res. 2002;8(3):802-10.
120. Krajinovic M, Robaey P, Chiasson S, et al. Polymorphisms of genes controlling homocysteine levels and IQ score following the treatment for childhood ALL. Pharmacogenomics. 2005;6(3):293-302.
121. Pakakasama S, Kanchanakamhaeng K, Kajanachumpol S, et al. Genetic polymorphisms of folate metabolic enzymes and toxicities of high dose methotrexate in children with acute lymphoblastic leukemia. Ann Hematol. 2007;86(8):609-11.
122. Lauten M, Asgedom G, Welte K, Schrappe M, Stanulla M. Thymidylate synthase gene polymorphism and its association with relapse in childhood B-cell precursor acute lymphoblastic leukemia. Haematologica. 2003;88(3):353-4.
123. Fotoohi AK, Albertioni F. Mechanisms of antifolate resistance and methotrexate efficacy in leukemia cells. Leuk Lymphoma. 2008;49(3):410-26.
124. Huang L, Tissing WJ, de JR, van Zelst BD, Pieters R. Polymorphisms in folate-related genes: association with side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia. Leukemia. 2008;22(9):1798-800.
125. Wilson A, Platt R, Wu Q, et al. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab. 1999;67(4):317-23.
126. Krajinovic M, Lamothe S, Labuda D, et al. Role of MTHFR genetic polymorphisms in the susceptibility to childhood acute lymphoblastic leukemia. Blood. 2004;103(1):252-7.
127. Relling MV, Gardner EE, Sandborn WJ, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. Clin Pharmacol Ther. 2011;89(3):387-91.

Immunotherapy in pediatric hemato-oncology

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A B S T R A C T

Immunotherapy has progressively acquired an important role in the treatment of children with refractory/resistant hemato-oncological diseases. Its ultimate goal is that of increasing the immunological driven anti-cancer effect without causing further immunological complications. Recent experience has made clear some basic principles in the use of immunotherapy: it has a better success rate when applied in the pre-emptive setting and it should be preceded by lymphodepletion. It is, therefore, reasonable to think that the stem cell transplantation setting could be a perfect match for this kind of therapy. The present review summarizes some of the many different strategies that are currently under pre-clinical and clinical evaluation, mostly in the pediatric field, and hints at their possible application in the allogeneic stem cell transplantation setting.

Learning goals

At the conclusion of this activity, participants should know that:
- immunotherapy refers to a complex group of treatments, which all aim, through different strategies, to increase innate or adaptive immune activity against tumor cells;
- immunotherapy can be applied in the allogeneic transplantation setting with the aim of further boosting the graft-versus-leukemia effect, a role already played by the donor cells in the recipient body;
- immunotherapeutic strategies are nowadays highly experimental. Therefore, it is of the utmost importance that patients receiving some kind of immunotherapy be included in clinical studies to allow clinical and immunological readouts to be correctly evaluated.

Introduction

In the last 30 years, the success rate of treatment for most hemato-oncological disease in the pediatric field has substantially increased. The development of risk-tailored chemotherapeutic protocols for children has resulted in greater success of disease treatment, and the improvement in supportive care has translated into less treatment-related mortality. Nowadays, it is estimated that 76-86% of acute lymphoblastic leukemia (ALL) and 49-63% of acute myeloid leukemia (AML) affected patients can be cured with front-line conventional treatments.[1,2] Second-line treatment for relapsing or resistant patients often includes stem cell transplantation (SCT). Progress in immunology, supportive care and pre-emptive treatments also allowed a progressive increase in overall survival for transplant recipients, disease relapse remaining the most important limiting factor for higher success rate.[3]

The use of SCT for patients with aggressive diseases is based on the statement that immunological surveillance and killing of tumor cells from the transplanted immune-system can exert a powerful and long-lasting antitumor action as compared to conventional chemo- and radiotherapy. Nonetheless, so far, a desirable graft-*versus*-leukemia (GvL) effect cannot be separated from an unwanted graft-*versus*-host disease (GvHD).[4] This explains why stem cell transplantation can be considered a platform for further immunotherapy in transplant recipients.[5]

Donor selection, conditioning regimens, GvHD prophylaxis and stem cell processing before infusion already play an important role in determining the immune-reconstitution of the procedure, and therefore also the possible GvL effect. Nevertheless, over the last 20 years, an ever increasing number of possibilities in the field of immunotherapy have been explored.

The proof of principle for the use of immunotherapy after stem cell transplantation was set by Kolb in 1990.[6] By infusing unprocessed donor-derived lymphocytes (DLI) he was able to achieve a clinical response in up to 73% of patients with a chronic myeloid leukemia (CML) relapsed after transplantation. Unfortunately, less promising results were obtained with patients affected with acute leukemias, the response rate of whom, in case of relapse, varied between 3% and 30%.[7] A first attempt to improve survival for relapsed AML/ALL patients using DLI was to use them in a pre-emptive setting in order to anticipate morphological relapse. Minimal residual disease (MRD) and chimerism monitoring set the standard for

continuous and frequent posttransplantation monitoring in high-risk patients.[8,9] According to this strategy, a relatively simple immunotherapeutic tool allowed good prognostic results to be achieved in the pre-emptive setting for ALL and AML patients after transplantation.[10-13]

The drawbacks of DLI administration, however, remain the reduced efficacy in advanced stage of disease, as well as the risk of GvHD and severe immunological complications which can sometimes be life-threatening.[14,15]

Immunologists, therefore, tried to elaborate other strategies to try and split the GvL from the GvHD effect of the infused cells. Furthermore, they of course aimed at producing newer tools that could be effective for a wider cohort of patients, and eventually provide a possible therapeutic option also for more advanced stage of disease.

This is an updated review of the different immunotherapeutic tools that are currently being evaluated in ongoing clinical studies (Table 1).

Peptide- and cell-based cancer vaccine

Autologous T-cell response against leukemia and other solid tumors has been extensively documented and this has paved the way to the possibility of using a vaccine strategy as anti-cancer therapy.[16] The availability of an increasing number of recognized tumor associated antigens (TAAs) has become the starting point for the development of different peptide-based anti-cancer vaccines, as the isolation of cancer-specific proteins gave hope of a possible patient immunization. In this context, hundreds of TAAs have been evaluated as possible efficacious peptides. The first studies were concentrated on BCR-ABL antigenic epitopes. In CML, it had already been demonstrated through the use of DLI that the immunological action could play a substantial role. Therefore, multiple phase I and II studies evaluated a combination of different peptides, associated to adjuvants and eventually to interferon-gamma. Different studies were almost always able to document an increased specific CD4 T-cell immunity, but clinical results could only be seen at molecular level and not for all patients treated.[17-19]

Extensive research into TAAs has involved AML, and among the most targeted peptide for this disease, Wilms Tumor Suppressing Gene-1 (WT1), has emerged to be one of the most promising. Tumor regression could be demonstrated in 12 of 20 patients exhibiting MDS by using a WT1 peptide associated to an adjuvant.[20] Positive findings were also reported in a phase II study by Keilholz and colleagues.[21]

PR1 was also considered a good peptide target for vaccine delivery since it is a peptide derived from neutral serin protease that is over-expressed in leukemic progenitor cells as well as in CML and AML blasts. A randomized phase II study with 66 leukemia patients demonstrated a tendency towards a better overall survival and event-free survival for those patients receiving PR1-based vaccine plus adjuvant and chemotherapy as compared to chemotherapy alone.[22]

The subsequent testing of a combined WT1 and PR3 vaccine made it evident for the first time that the weak point of peptide vaccine strategy relies on the tolerance which is achieved after subsequent inoculations. The same problem was seen in a phase I study of 10 patients treated with a CD168-based vaccine who progressively developed immunological tolerance.[23]

To overcome the problem of a weak and fading immunological signal, cellular vaccines and combined adoptive T-cell transfer and vaccination have been developed. The first cellular vaccines were mostly based on dendritic cells, which can be expanded and loaded with a specific peptide. Dendritic cells are specialized antigen presenting cells (APC) that play a critical role in the adaptive immune response. Clinical responses to APC-based vaccines have been reported in pediatric trials for solid tumors.[24] The combination of adoptive T-cell transfer and

Table 1. Overview of the currently available immunotherapy strategies.

Immunotherapy strategy	Targets	Advantages	Disadvantages	Further development
Vaccine	CML AML Solid tumors	Possible multi-targeted therapy	Short-lasting efficacy because of development of tolerance	Combined adoptive T-cell transfer with vaccination or immunotransplantation
Natural killer (NK) cell infusion	AML Solid tumors	GvT without GvHD	NK isolation and culture is technically challenging. Restricted pool of alloreactive donors available	NK cell line pre-production
Monoclonal antibody	AML NHL CLL ALL	Specific target	Potential toxicity to be evaluated	Combined bispecific targeted therapy with the BiTe technology
Cytokine induced killer cells (CIK)	AML Solid tumors	GvT without GvHD	Repeated administrations needed, possible tolerance induction mechanism	Gene elaboration of CIK cells through the chimeric antigen receptor technique
Chimeric antigen receptors (CARs)	ALL CLL Solid tumors AML?	No HLA restriction, long-lasting expansion in the host	Production is technically challenging, safety profile needs further testing	Wide variety of possible cells to be engineered (NK, CIK) and antibodies to be targeted

CML: chronic myeloid leukemia; AML: acute myeloid leukemia; NHL: non-Hodgkin's lymphoma; CLL: chronic lymphocytic leukemia; GvT: graft-versus-tumor; GvHD: graft-versus-host disease.

vaccination on the other hand, relies on vaccinating the patient, collecting lymphocytes before treatment, and re-infusing them with further vaccination after lymphoreductive chemotherapy. Studies in this direction demonstrated at least a clear immunological response to treatment for myeloma patients with no benefit on overall survival.[25]

The vaccination strategy has so far been implemented mostly in the autologous setting. There are convincing immunological reasons to think that the early post-allogeneic transplant setting could be an ideal milieu in which to develop vaccine-based strategies.[26] As a matter of fact, not only the tumor burden is limited after SCT, but also the lymphopenic environment would allow a strong expansion of the transferred T cells. The possibility of pursuing this strategy was demonstrated in 1995, when tumor-specific T cells where induced in a stem cell donor and later transferred to the recipient.[27] Nowadays, such an 'immunotransplantation' model is being implemented in a pre-clinical model of lymphoma at Stanford University.[28] Moreover, a novel allogeneic vaccine trial that utilizes WT1 peptide-loaded dendritic cells generated from healthy SCT donors is being conducted at the National Cancer Institute for children and adults with WT1-expressing hematologic malignancies.[29]

Natural killer cells

Natural killer (NK) cells were first identified in 1975.[30] As part of the innate immunity system, they are able to rapidly react towards infected or transformed cells without MHC restriction. Their cytotoxicity develops through perforin and granzyme B as well as through triggering apoptosis pathways. Through complex activating and inhibiting signals, NK cells are endowed with a spontaneous anti-tumor activity.[31] A possible graft-*versus*-tumor (GvT) effect through alloreactive NK cells was first illustrated in the studies by Ruggeri and colleagues.[32] In HLA haplotype mismatched hematopoietic transplantation, donor *versus* recipient NK cell alloreactions are associated with enhanced control of AML and ALL relapse and no risk of graft-*versus*-host disease, through a complex interaction of activation and inhibition signals, the mechanism of which is beyond the intent of this review.[33] To extend this effect, several attempts have been made to boost the NK-cell response in the allogeneic setting, for example through the administration of purified or interleukin-stimulated donor NK cell products. Rubniz and colleagues proposed the isolated infusion of haplo-identical donor-derived NK cells following a fludarabine and cyclophosphamyde immunosuppressive cycle, as consolidation therapy for children affected with AML. The feasibility and safety of this approach was successfully tested in 10 patients, who also demonstrated an *in vivo* expansion of the infused cells.[34] In the transplantation setting, however, the simple isolation and reinfusion of NK cells from the donor did not result in a superior outcome in a cohort of haplo-identical transplanted patients. Therefore, different NK-cell expansion protocols have been developed, and these were able to increase the NK cell activity through cytokine stimulation. Though more active, these NK cells are very difficult to expand, are unstable, and need to be strictly depleted from other T cells to avoid risk of GvHD.[35,36] This results in a very expensive and long expansion procedure. To overcome these difficulties, permanent NK-cell lines have been developed under good manufacturing practice (GMP) conditions and are currently being tested in different protocols.[37] NK-cell lines also represent an optimal target for genetic modification to enhance cytotoxic potential.[38]

Antibodies

Since the discovery of hybridoma technology by Kohler and Milstein in 1975, the availability of monoclonal antibodies (mAbs) has continued to increase. mAbs targeting cell clusters of differentiation (CD) today represent a potential targeted therapy for several malignancies. MoAbs can kill cancer cells by means of direct and indirect pathways. Specific antibody-receptor binding can directly cause apoptosis through intracellular signaling. Indirect killing can occur by complement-activation, antibody-dependent cellular cytotoxicity (ADCC), complement-dependant cytotoxicity (CDC) or cell-mediated cytokine release.[39] The first mAbs to receive approval from the Food and Dug Administration (FDA) for clinical purpose was anti-CD20 rituximab in 1997. Its mechanisms of action include inhibition of B-cell proliferation, ADCC, CDC and possible induction of apoptosis. This drug now represents a consolidated treatment, in combination with chemotherapy, for $CD20^+$ non-Hodgkin's lymphomas (NHL). Around 30% of adults and 48% of children with B-lymphoblastic ALL also express CD20[40], the upregulation of which has been demonstrated in resistant blasts.[41] Based on these findings, rituximab has been studied in patients with *de novo* Philadelphia chromosome negative, precursor B-lineage, $CD20^+$ ALL in combination with hyper-CVAD regimen (fractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone). Among patients under 60 years of age, those who received rituximab had a statistically significant improvement in 3-year overall survival (75% *vs.* 47%; *P*=0.03).[42]

Alemtuzumab targets anti-CD52 positive cells. Its use has been widely explored in the transplantation setting, as part of the preparatory regimen and anti-GvHD prophylaxis. It mediates ADCC, and induces apoptosis of tumor cells. Alemtuzumab is officially approved for the treatment of adults with B-cell chronic lymphocytic leukemia (CLL). This drug has also been studied in combination with chemotherapy in adult and pediatric patients with relapsed ALL. A phase II study was conducted within the Children Oncology Group for the treatment of relapsed pediatric ALL. Only one of 13 patients showed a complete response to single-agent treatment.[43] The most important drawback in its use of alemtuzumab is the high risk of toxicity and infectious complications that it causes.[44]

Epratuzumab targets the extracellular domain of CD22, an antigen expressed in over 95% of pediatric B-lymphoblastic ALL.[45] Its proposed mechanisms of action include ADCC, CD22 phosphorylation and the inhibition of cell proliferation. Epratuzumab was first studied in adults with indolent and aggressive B-NHL, displaying good safety and efficacy. Epratuzumab alone and in association with chemotherapy for $CD22^+$ ALL with first or subsequent relapse was tested in a pediatric cohort of patients. While the overall remission rate did not differ from historical controls, the MRD negative rate was better

for the patients receiving epratuzumab (42% *vs.* 25% MRD-negative $P<0.01\%$).[46] The safety profile of the drug was confirmed.

Blinatumomab is a bi-specific antibody that binds CD19 and CD3. Its function is to attract CD3+ cytotoxic T cells to CD19-expressing leukemic blasts with a so-called BiTE (bi-specific T-cell engagers). Blinatumomab was first tested in adults with relapsed/refractory B-NHL and B-lymphoblastic ALL. A recent update of an ongoing German multicenter trial documented a high rate of clinical and molecular response (67%).[47] Though concerns remain about the toxicity of this drug, its promising efficacy has led to the opening of a pediatric study for relapsed/resistant ALL in Europe as well as in the USA.

In the field of AML malignancies, the availability of antibodies has not so far been so extensive, especially after the official withdrawal of gemtuzumab ozogamicin from the market due to increased fatal infectious events. However, some promising tools are being tested in the pre-clinical and early clinical phase. A promising 3rd generation antibody with improved capability to recruit Fc receptor-bearing effector cells has been created against CD135, an antigen often expressed in AML blasts.[48] Moreover, the same BiTE technology used with blinatumomab is now being applied to an anti-CD33/CD3 double antibody, which could potentially have a high impact on the treatment of relapsed/refractory AML.[49]

It is worth adding that antibodies can often improve their efficacy by being combined either with radioisothops or with chemotherapy/toxins. Among the combinations that are now undergoing clinical trials, gemtuzumab ozogamicin has been confirmed as an innovative anti-AML treatment. Its combination of anti-CD33 and calicheamicin demonstrated its activity in relapsed adult and pediatric AML even as single agent therapy (30% remission rate).[50,51] However, the high toxicity profile (myelosuppression, systemic infections, transaminitis, veno-occlusive disease) led to its withdrawal from the market in 2010. A recent detailed review about the use of gemtuzumab ozogamicin in the treatment of adult AML has, however, advocated the re-introduction of this drug in combination with cytarabine and anthracycline for the treatment of patients with a favorable cytogenetic profile on the bases of five reported randomized studies that suggested an overall survival benefit for this subgroup of patients.[52] Conjugated anti-CD22 antibodies have also been developed. Inotuzumab ozogamicin (anti-CD22 plus calicheamicin), BL22 and moxetumomab pasudotox (both anti-CD22 combined with Pseudomonas exotoxin A) have begun tests in clinical trials. The results seem promising, but the number of pediatric patients treated is so far too small to allow conclusions to be drawn.[53-55] At the present time, there are very studies involving antibodies combined to radioactive isotopes in the pediatric population, and these are mostly feasibility phase studies. In the adult setting, radioisotope-antibody conjugates directed against surface markers of leukemia cells (CD33, CD45) are available for routine clinical use. These agents concentrate in the bone marrow, generating a severe myelosuppression. Given as an adjunct to TBI, no increased side effects were observed.

Cytokine-induced killer cells

Cytokine-induced killer (CIK) cells are *ex vivo* expanded T lymphocytes (CD3+) that share a natural killer (NK) phenotype (CD56+). CIK cells display a high anti-leukemic activity, independently of MHC restriction while having negligible alloreactive potential. They can kill a broad array of tumor targets, including hematologic and solid malignancies. In this way, cell-cell interaction is mediated via TNF-related apoptosis-inducing ligand (TRAIL) on CIK cells and death receptors on tumor targets (Figure 1A and B) that results in an activation of the caspase cascade enrolling the intrinsic apoptotic pathway. But the molecule that probably plays the most important role in CIK cell-mediated killing, as shown by blocking experiments (Figure 1C), is the NKG2D receptor, which is an activating NK-cell receptor. The ligands of this receptor known so far are relatively restricted to tumor cells. However, the NKG2D only mediates the interaction between CIK cells and tumor targets while the final execution of apoptosis is mediated via a perforin and granzyme release (Figure 1A).

CIK cells can be expanded from peripheral blood, from cord blood, and also from washout of leftover mononuclear cells from cord blood unit bags.[56] One of their hallmarks is that they can be easily produced under GMP conditions through different cytokine protocols, some of which only require ten days of expansion before harvesting.[57] In the allogeneic setting, these cells have been tested in 3 different clinical trials. All of them included adult patients who had relapsed from hematologic malignancies after stem cell transplantation. All of them showed a good safety profile, with only a few GvHD cases and no severe toxicity. In all studies, a clinical transient response of the disease could be observed in 30-50% of the patients treated.[58-60] These trials therefore, suggest a true activity of CIK cells in hematologic malignancies, but also underline the absence of long-lasting efficacy, thus questioning possible resistance mechanisms developed by the target cells. Interestingly, the rate of immunological complications (GvHD) for patients receiving those cells is low, and this holds true even when those cells are applied to haplo-identical settings (P Bader, personal communication, 2012).

Moreover, Introna and colleagues demonstrated that these cells retain a dual function, being active both as CD8-specific effector T and NK cells. In the posttransplantation setting, this would allow their use not only as cancer specific treatment, but also in the treatment of life-threatening viral reactivation.[61]

Anti-tumor cytotoxic T cells

After the first attempt at infusing donor-derived lymphocytes or specific T lymphocytes in relapsing recipients, the first real documentation of specific T-cell production was linked to anti-viral treatment. Riddel was the first to produce clinical grade cytotoxic T cells,[62] followed by Rooney and Heslop, who developed EBV-specific T cells and even proved their activity against posttransplant lympho-proliferative disease (PTLD).[63] In the same era, Rosenberg was attempting to expand T cells from tumor mass and re-infusing them to patients affected with metastatic melanoma, obtaining only an occasional

response.[64] As the experience with T-cell production grew, a number of factors presented themselves and had to be considered. First of all, it was made clear that anti-viral immunity could only be delivered if both CD4 and CD8 cells were expanded, a lympho-depleted setting was necessary to achieve cell expansion, central memory T cells represented a better cell population as compared to effector T cells as they could expand better. Moreover, it became clear that, when elaborating possible anti-tumor specific T cells, a variety of means had to be used to take into account the possibility of tumor escape. Last but not least, the production system evolved so as to allow GMP manipulation of cells. In general, it has also become clear that targeting tumor cells with T cells was more challenging than targeting virus, first of all because of their immunological escape capability, and because T-cell therapy could only address residual tumor mass, being insufficient to treat overt relapses.[65] The first technique which was used to overcome tumor escaping was to elaborate multitumor-specific or gene-modified T cells, the construction of which could be one of the next clinical achievements.[66] Moreover, combining multitumor specific T cells with demethylating agents or T-cell activators or with proliferative stimuli could also be considered a further evolution of this technique.[67-69]

Chimeric antigen receptors

Chimeric antigen receptors (CARs) have been developed through advanced gene-transfer technology in order to overcome HLA restriction limitation of conventional T-cell therapy. Through genetic reprogramming, immune effector cells can be redirected to target antigens expressed by leukemic cells. In most clinical applications to date, a patient's own T cells may be reprogrammed to express these tumor-specific receptors, largely minimizing the potential immunological risks. CARs are composed of an Ag-specific binding domain (most commonly a single-chain variable fragment derived from the fused variable heavy- and light-chain domains of a tumor-targeted mAb) fused to a transmembrane domain followed by one or more cytoplasmic signaling domains. The evolution of CARs from 1st to 3rd generation has progressively combined activating to co-stimulatory signaling domain, so as to achieve not only T-cell activation, but also T-cell expansion on long-lasting antigen exposure and, therefore, T-cell persistence.

Nowadays, Cars are designed to recognize several surface antigens, and more than 30 phase I trials are ongoing in the field of hemato-oncology and solid tumors.

An elegant review by Davila and colleagues has recently analyzed the results of the first 28 patients treated at 5 different clinical centers with CARs-based protocols.[70] These studies presented several differences in terms of treated disease, stage of disease, type of CARs used, gene technology used, and number of infused T cells, making a direct comparison of the obtained results inappropriate. However, the overall consideration that can be derived from these studies is that anti CD19 CARs have shown some degree of clinical activity in patients affected with CLL and lymphomas.

Tumor burden was directly related to the degree of response shown by these patients even if a high tumor burden did not prevent some degree of clinical response. The use of lymphodepletion before CARs infusion proved to be fundamental, whereas the number of infused T cells was not shown to have a great impact on outcome. In all the clinical studies, a number of acute reactions were associated to CARs infusion but no lethal complication was

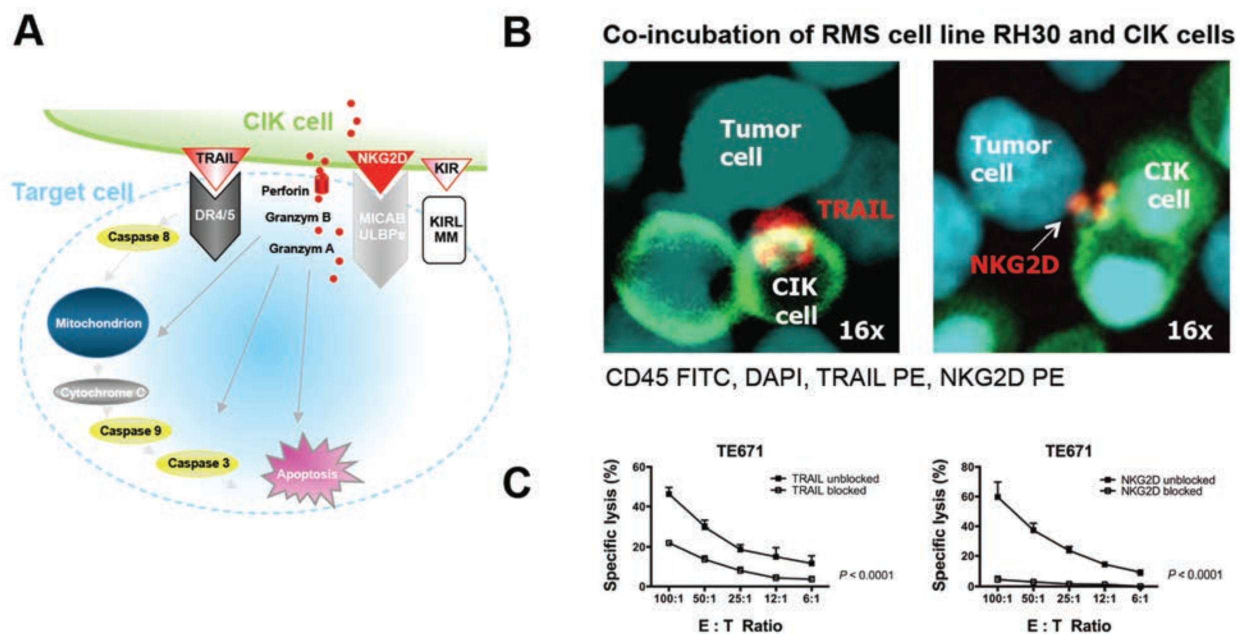

Figura 1. Mechanism of action of citokin induced killer cells.

observed, producing reassuring results on the safety of these products. Most recently, very promising clinical results were reported at the ASH meeting in Atlanta 2012 by Carl June and colleagues. A 7-year girl affected with relapsed refractory ALL received autologous CART19 after chemotherapy. The cells where transduced with a lentivirus encoding CD-19 scFv linked to 4-1BB and CD3-z signaling domains. CART19 were documented in the girl's bone marrow as well as in her central nervous system on Day +23 after infusion; the maximal expansion of CART cells occurred on Day +11 after infusion. The treated child achieved a complete morphological and molecular remission of the disease and this was maintained at a 4-month follow up, with stable levels of CAR^+ CD3 cells in peripheral blood as well as in bone marrow. Notably, the girl displayed a severe cytokine release syndrome (CRS) which required admission to the intensive care unit and respiratory support, and which was successfully treated with IL-6 inhibitor and steroid.

Another 9 adult patients affected with relapsed refractory CLL were treated with the same CAR cells (3 of them have already been reported in the review by Davila and colleagues[70]) and 4 of them achieved a complete remission at a median follow up of 5.6 months. All responding patients developed a mild to moderate CRS, which temporally always correlated with the peak of T-cell expansion in peripheral blood.[71] While the pioneering centers for the development of CARs try to set up common standard criteria and evaluation tools to be able to perform comparable clinical evaluations, other pre-clinical studies are reaching out to new possible CARs targets. In AML, for example, Marin *et al.* developed a 3rd generation CAR complexing a CD33-specific CAR with CD28 and OX-40 co-stimulatory signaling. The study was able to show that cytokine-induced killer cells inherited increased proliferative, migratory, and lytic functions at a variety of leukemic cell lines.[72] A further application of this technique involved the targeting of CD123 and this is being developed by the same group.[73]

Conclusion

The range of immunotherapeutic strategies under development to address relapsed or resistant leukemia is steadily increasing, and the ultimate goal of achieving clinical success for high-risk patients may be closer. As far as published reports show, these techniques have an adequate safety profile as referred to the high-risk patients in which they need to be used. It is possible that different techniques will emerge and will prove to be more advantageous for different diseases at different time points, and it is not to be excluded that some of the described techniques will be fused and combined to achieve better results. In such an ample repertoire of possible new therapies, it is of the utmost importance that all the patients treated in predefined protocols are included in results analysis according to pre-set end points.

References

1. Pui CH, Mullighan CG, Evans WE, Relling MV. Pediatric acte lymphoblastic leukemia: where are we going and how do we get there? Blood. 2012;120:1165-74.
2. Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol. 2011;29:551-65.
3. MacMillan ML, Davies SM, Nelson GO, Chitphakdithai P, Confer DL, King RJ, et al. Twenty years of unrelated donor bone marrow transplantation for pediatric acute leukemia facilitated by the National Marrow Donor Program. Biol Blood Marrow Transplant. 2008;14:16-22.
4. Vincent K, Roy DC, Perreault C. Next-generation leukemia immunotherapy. Blood. 2011;118:2951-9.
5. Li HW, Sykes M. Emerging concepts in haematopoietic cell transplantation. Nat Rev Immunol. 2012;12:403-16.
6. Kolb HJ, Mittermüller J, Clemm C, Holler E, Ledderose G, Brehm G, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. Blood. 1990;76(12):2462-5.
7. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood. 1995:86:2041-50.
8. Kröger N, Bacher U, Bader P, Böttcher S, Borowitz MJ, Dreger P, et al. NCI First International Workshop on the Biology, Prevention, and Treatment of Relapse after Allogeneic Hematopoietic Stem Cell Transplantation: report from the Committee on Disease-Specific Methods and Strategies for Monitoring Relapse following Allogeneic Stem Cell Transplantation. Part I: Methods, acute leukemias, and myelodysplastic syndromes. Biol Blood Marrow Transplant. 2010;16:1187-211.
9. Bader P, Willasch A, Klingebiel T. Monitoring of post-transplant remission of childhood malignancies: is there a standard? Bone Marrow Transplant. 2008;42(Suppl 2):S31-4.
10. Rettinger E, Willasch AM, Kreyenberg H, Borkhardt A, Holter W, Kremens B, et al. Preemptive immunotherapy in childhood acute myeloid leukemia for patients showing evidence of mixed chimerism after allogeneic stem cell transplantation. Blood. 2011;118:5681-8.
11. Formánková R, Sedlácek P, Krsková L, Ríhová H, Srámková L, Star J; Czech Pediatric Hematology Working Group. Chimerism-directed adoptive immunotherapy in prevention and treatment of post-transplant relapse of leukemia in childhood. Haematologica. 2003;88:117-8.
12. Gorczyñska E, Turkiewicz D, Toporski J, Kalwak K, Rybka B, Ryczan R, et al. Prompt initiation of immunotherapy in children with an increasing numberof autologous cells after allogeneic HCT can induce complete donor-type chimerism: a report of 14 children. Bone Marrow Transplant. 2004;33:211-7.
13. Bader P, Kreyenberg H, Hoelle W, Dueckers G, Handgretinger R, Lang P, et al. Increasing mixed chimerism is an important prognostic factor for unfavorable outcome in children with acute lymphoblastic leukemia after allogeneic stem-cell transplantation: possible role for pre-emptive immunotherapy? J Clin Oncol. 2004;22:1696-705.
14. Krishnamurthy P, Potter VT, Barber LD, Kulasekararaj AG, Lim Z, Pearce RM, et al. Outcomes Following Donor Lymphocyte Infusions Post-T-cell Depleted Allogeneic Haematopoietic Stem Cell Transplants for Acute Myeloid Leukaemia and Myelodysplastic Syndromes. Biol Blood Marrow Transplant. 2012 Dec 20. [Epub ahead of print].
15. Slavin S, Morecki S, Weiss L, Or R. Donor lymphocyte infusion: the use of alloreactive and tumor-reactive lymphocytes for immunotherapy of malignant and nonmalignant diseases in conjunction with allogeneic stem cell transplantation. J Hematother Stem Cell Res. 2002;11:265-76.
16. Rice J, Ottensmeier CH, Stevenson FK. DNA vaccines: precision tools for activating effective immunity against cancer. Nat Rev Cancer. 2008;8:108-20.
17. Cathcart K, Pinilla-Ibarz J, Korontsvit T, Schwartz J, Zakhaleva V, Papadopoulos EB, et al. A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. Blood. 2004;103:1037-42.
18. Jain N, Reuben JM, Kantarjian H, Li C, Gao H, Lee BN, et al. Synthetic tumor-specific breakpoint peptide vaccine in patients with chronic myeloid leukemia and minimal residual disease: a phase 2 trial. Cancer. 2009;115:3924-34.
19. Bocchia M, Defina M, Aprile L, Ippoliti M, Crupi R, Rondoni M, et al. Complete molecular response in CML after p210 BCR-ABL1-derived peptide vaccination. Nat Rev Clin Oncol. 2010;7:600-3.
20. Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. Proc Natl Acad Sci USA. 2004;101:13885-90.

21. Keilholz U, Letsch A, Busse A, Asemissen AM, Bauer S, Blau IW, et al. A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. Blood. 2009;113:6541-8.
22. Qazilbash MH, Wieder ED, Thall PF, Wang X, Rios RL, Lu S, et al. PR1 vaccine elicited immunological response after hematopoietic stem cell transplantation is associated with better clinical response and event-free survival. Blood. ASH Annual Meeting Abstracts. 2007;110:577.
23. Rezvani K, Yong AS, Mielke S, Jafarpour B, Savani BN, Le RQ, et al. Repeated PR1 and WT1 peptide vaccination in Montanide-adjuvant fails to induce sustained high-avidity, epitope-specific CD8+ T cells in myeloid malignancies. Haematologica. 2011;96:432-40.
24. Mackall CL, Rhee EH, Read EJ, Khuu HM, Leitman SF, Bernstein D, et al. A pilot study of consolidative immunotherapy in patients with high-risk pediatric sarcomas. Clin Cancer Res. 2008;14:4850-8.
25. Rapoport AP, Aqui NA, Stadtmauer EA, Vogl DT, Fang HB, Cai L, et al. Combination immunotherapy using adoptive T-cell transfer and tumor antigen vaccination on the basis of hTERT and survivin after ASCT for myeloma. Blood. 2011;117:788-97.
26. Rezvani K, Brody JD, Kohrt HE, Logan AC, Advani R, Czerwinski DK, et al. Cancer vaccines and T cell therapy. Biol Blood Marrow Transplant. 2013;19(Suppl 1):S97-S101.
27. Kwak LW, Taub DD, Duffey PL, Bensinger WI, Bryant EM, Reynolds CW, et al. Transfer of myeloma idiotype-specific immunity from an actively immunised marrow donor. Lancet. 1995;345:1016-20.
28. Brody JD, Goldstein MJ, Czerwinski DK, Levy R. Immunotransplantation preferentially expands T-effector cells over T-regulatory cells and cures large lymphoma tumors. Blood. 2009;113:85-94.
29. Wayne AS, Capitini CM, Mackall CL. Immunotherapy of childhood cancer: from biologic understanding to clinical application. Curr Opin Pediatr. 2010;22:2-11.
30. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. Distribution of reactivity and specificity. Int J Cancer. 1975; 16:216-29.
31. Sutlu T, Alici E. Natural killer cell-based immunotherapy in cancer: current insights and future prospects. J Intern Med. 2009;266:154-81.
32. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295:2097-100.
33. Velardi A, Ruggeri L, Mancusi A, Aversa F, Christiansen FT. Natural killer cell allorecognition of missing self in allogeneic hematopoietic transplantation: a tool for immunotherapy of leukemia. Curr Opin Immunol. 2009;21:525-30.
34. Rubniz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. J Clin Oncol. 2010; 28:955-9.
35. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105:3051-7.
36. Stern M, Passweg JR, Meyer-Monard S, Esser R, Tonn T, Soerensen J, et al. Pre-emptive immunotherapy with purified natural killer cells after haploidentical SCT: a prospective phase II study in two centers. Bone Marrow Transplant. 2012 Sep 3. [Epub ahead of print].
37. Cheng M, Zhang J, Jiang W, Chen Y, Tian Z. Natural killer cell lines in tumor immunotherapy. Front Med. 2012;6:56-6.
38. Shook DR, Campana D. Natural killer cell engineering for cellular therapy of cancer. Tissue Antigens. 2011 Dec;78:409-15.
39. Barth M, Raez E, Cairo MS. The future role of monoclonal antibody therapy in childhood acute leukaemias. Br J Haematol. 2012;159:3-17.
40. Jeha S, Behm F, Pei D, Sandlund JT, Ribeiro RC, Razzouk BI, al. Prognostic significance of CD20 expression in childhood B-cell precursor acute lymphoblastic leukemia. Blood. 2006;108: 3302-4.
41. Dworzak MN, Schumich A, Printz D, Pötschger U, Husak Z, Attarbaschi A, et al. CD20 up-regulation in pediatric B-cell precursor acute lymphoblastic leukemia during induction treatment: setting the stage for anti-CD20 directed immunotherapy. Blood. 2008;112:3982-8.
42. Thomas DA, O'Brien S, Faderl S, Garcia-Manero G, Ferrajoli A, Wierda W, et al. Chemoimmunotherapy with a modified hyper-CVAD and rituximab regimen improves outcome in de novo Philadelphia chromosome-negative precursor B-lineage acute lymphoblastic leukemia. J Clin Oncol. 2010;28:3880-9.
43. Angiolillo AL, Yu AL, Reaman G, Ingle AM, Secola R, Adamson PC. A phase II study of Campath-1H in children with relapsed or refractory acute lymphoblastic leukemia: a Children's Oncology Group report. Pediatr Blood Cancer. 2009;53:978-8.
44. Elter T, Hallek M, Montillo M. Alemtuzumab: what is the secret to safe therapy? Clin Adv Hematol Oncol. 2011;9:364-73.
45. Gudowius S, Recker K, Laws HJ, Dirksen U, Tröger A, Wieczorek U, et al. Identification of candidate target antigens for antibody-based immunotherapy in childhood B-cell precursor ALL. Klin Padiatr. 2006;218:327-33.
46. Raetz EA, Cairo MS, Borowitz MJ, Xiaomin Lu P, Devidas M, Reid JM, et al. Reinduction chemoimmunotherapy with epratuzumab in relapsed acute lymphoblastic leukemia (ALL) in children, adolescents and young adults: results from children´s oncology group (COG) study ADVL04P2. Blood. ASH Annual Meeting Abstracts. 2011;118:573.
47. Topp MS, Goekbuget N, Zugmaier G, Viardot A, Stelljes M, Neumann S, et al. Anti-CD19 BiTE blinatumomab induces high complete remission rate in adult patients with relapsed B-precursor ALL: updated results of an ongoing phase II trial. Blood. ASH Annual Meeting Abstracts. 2011;118:252
48. Hofmann M, Große-Hovest L, Nübling T, Pyż E, Bamberg ML, Aulwurm S, et al. Generation, selection and preclinical characterization of an Fc-optimized FLT3 antibody for the treatment of myeloid leukemia. Leukemia. 2012;26:1228-37.
49. Arndt C, Bonin MV, Cartellieri M, Feldmann A, Koristka S, Michalk I, et al. Redirection of T cells with a first fully humanized bispecific CD33-CD3 antibody efficiently eliminates AML blasts without harming hematopoietic stem cells. Leukemia. 2013 Jan 17. [Epub ahead of print].
50. Sievers EL, Larson RA, Stadtmauer EA, Estey E, Lowenberg B, Dombret H. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. J Clin Oncol. 2001;19: 3244-54.
51. Arceci RJ, Sande J, Lange B, Shannon K, Franklin J, Hutchinson R, et al. Safety and efficacy of gemtuzumab ozogamicin in pediatric patients with advanced CD33+ acute myeloid leukemia. Blood. 200515;106:1183-8.
52. Ravandi F, Estev EH, Appelbaum FR, Lo-Coco F, Schiffer CA, Larson RA, et al. Gemtuzumab ozogamicin: time to resurrect? J Clin Oncol 2012;30:3921-3.
53. Mussai F, Campana D, Bhojwani D, Stetler-Stevenson M, Steinberg SM, Wayne AS, et al. Cytotoxicity of the anti-CD22 immunotoxin HA22 (CAT-8015) against paediatric acute lymphoblastic leukaemia. Br J Haematol. 2010;150:352-8.
54. Kantarjian H, Thomas D, Jorgensen J, Jabbour E, Kebriaei P, Rytting M, et al. Inotuzumab ozogamicin, an anti-CD22-calecheamicin conjugate, for refractory and relapsed acute lymphocytic leukaemia: a phase 2 study. Lancet Oncol. 2012;13403-11.
55. Wayne AS, Bhojwani D, Silverman LB, Richards K, Stetler-Stevenson M, Shah NN, et al. A novel anti-CD22 immunotoxin, moxetumomab pasudotox: phase I study in pediatric acute lymphoblastic leukemia (ALL). Blood. ASH Annual Meeting Abstracts. 2011;118:248.
56. Introna M, Franceschetti M, Ciocca A, Borleri G, Conti E, Golay J, et al. Rapid and massive expansion of cord blood-derived cytokine-induced killer cells: an innovative proposal for the treatment of leukemia relapse after cord blood transplantation. Bone Marrow Transplant. 2006;38:621-7.
57. Rettinger E, Kuçi S, Naumann I, Becker P, Kreyenberg H, Anzaghe M, et al.The cytotoxic potential of interleukin-15-stimulated cytokine-induced killer cells against leukemia cells. Cytotherapy. 2012;14:91-103.
58. Introna M, Borleri G, Conti E, Franceschetti M, Barbui AM, Broady R, et al.Repeated infusions of donor-derived cytokine-induced killer cells in patients relapsing after allogeneic stem cell transplantation: a phase I study. Haematologica. 2007;92:952-9.
59. Laport GG, Sheehan K, Baker J, Armstrong R, Wong RM, Lowsky R, et al. Adoptive immunotherapy with cytokine-induced killer cells for patients with relapsed hematologic malignancies after allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2011;17:1679-87.
60. Linn YC, Niam M, Chu S, Choong A, Yong HX, Heng KK, et al. The anti-tumour activity of allogeneic cytokine-induced killer cells in patients who relapse after allogeneic transplant for haematological malignancies. Bone Marrow Transplant. 2012l;47:957-66.

61. Pievani A, Borleri G, Pende D, Moretta L, Rambaldi A, Golay J, et al. Dual-functional capability of CD3+CD56+ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. Blood. 2011;118:3301-10.
62. Riddell SR, Greenberg PD. Principles for adoptive T cell therapy of human viral diseases. Annu Rev Immunol. 1995:13;545-6.
63. Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood. 2010;115:925-35.
64. Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, et al. Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N Engl J Med. 1990;323:570-8.
65. Barrett J, Bollard CM. T-cell therapy for cancer. Immunotherapy. 2012;4:347-50.
66. Bollard CM, Rössig C, Calonge MJ, Huls MH, Wagner HJ, Massague J, et al. Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity. Blood. 2002;99:3179-87.
67. Craddock C. Epigenetic manipulation of the immune response: a novel treatment strategy in hematologic malignancies. Cytotherapy. 2011;13:516-7.
68. Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. Leukemia. 2010;24:1160-70.
69. Huang RR, Jalil J, Economou JS, Chmielowski B, Koya RC, Mok S, et al. CTLA4 blockade induces frequent tumor infiltration by activated lymphocytes regardless of clinical responses in humans. Clin Cancer Res. 2011;17:4101-9.
70. Davila M, Brentjens R, Wang X, Riviere I, Sadelain M. How do CARs work? Oncoimmunol. 2012;9:1577-83.
71. Porter DL, Grupp SA, Kalos M, Loren AW, Lledo L, Gilmore J, et al. Chimeric antigen receptor T cells directed against CD19 induce durable response and transient cytokine release syndrome in relapsed, refractory CLL and ALL. Oral communication, 54th ASH Annual Meeting.
72. Marin V, Pizzitola I, Agostoni V, Attianese GM, Finney H, Lawson A, et al. Cytokine-induced killer cells for cell therapy of acute myeloid leukemia: improvement of their immune activity by expression of CD33-specific chimeric receptors. Haematologica. 2010;95:2144-52.
73. Tettamanti S, Marin V, Pizzitola I, Magnani CF, Giordano Attaniese GMP, Cribioli E, et al. Targeting of acute myeloid leukaemia by cytokine.induced killer cells redirected with a novel CD123-specific chimeric antigen receptor. Br J Haematol.2013;doi: 10.1111/bjh.12282. [E-pub ahead of print].

Novel drugs for childhood hematologic malignancies and the challenges of clinical trials

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A B S T R A C T

Despite the increasing success of front-line therapies for children with newly diagnosed acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), the majority of children with these diseases who relapse will ultimately die of their disease. New therapies for relapsed childhood ALL and AML are desperately needed. A number of new agents currently in development for hematologic malignancies, including cytotoxic agents, immunotoxins, other antibody-based immunological agents, and signal transduction agents, are relevant to childhood ALL and AML and are reviewed here. However, investigators face a number of challenges in developing novel agents for childhood ALL, including: 1) declining numbers of subjects for relapse trials, due to the increasing success of front-line therapies for childhood ALL; 2) a plethora of novel agents; 3) an increasing number of agents which target a molecular lesion present only in a subset of patients; 4) variations in clinical trial design; and 5) the reluctance of some pharmaceutical companies to evaluate novel agents in the relapsed childhood ALL population. Potential approaches and solutions to these challenges are discussed.

Learning goals

At the conclusion of this activity, participants should:
- be familiar with novel drugs being developed for the treatment of childhood ALL and AML;
- be familiar with challenges facing the conduct of clinical trials of novel agents in childhood ALL and AML.

Introduction

Children with acute lymphoblastic leukemia (ALL) treated with contemporary protocols enjoy 5-year event-free survival rates of between 80% and 90%.[1-4] Despite these remarkable successes, relapsed childhood ALL and AML remain a significant burden for society and a clinical challenge for the treating physician; more children suffer from relapsed childhood ALL than from childhood osteosarcoma or rhabdomyosarcoma, and the majority of these children will die of their disease despite advances in the application of conventional chemotherapeutic agents and hematopoietic stem cell transplantation (HSCT).[5] Thus, effective new therapies for relapsed childhood ALL and AML are needed. In recent years, a number of novel agents have been developed which show promising activity in pre-clinical studies of pediatric malignancies as well as in early-phase trials in adult lymphoid malignancies, and which therefore hold great promise to improve outcome in children with relapsed or newly diagnosed high-risk ALL.

Novel agents relevant to childhood ALL

A comprehensive review of all novel agents relevant to childhood ALL and AML is beyond the scope of this review, which will therefore be limited to those novel agents which are presently under investigation or in development for childhood ALL and AML.

Cytotoxic agents

Clofarabine

Clofarabine is a nucleoside analog that combines the most favorable properties of fludarabine and cladribine. It demonstrated significant activity in multiply relapsed pediatric ALL patients in a phase I trial;[6] a subsequent phase II trial of single-agent clofarabine in relapsed and refractory pediatric ALL demonstrated a response rate of 30%.[7] Subsequent development of clofarabine has focused on combination therapy; while clofarabine in combination with cytarabine (AAML0523) did not show significant activity in relapsed childhood ALL,[8] clofarabine in combination with cyclophosphamide and etoposide demonstrated activity in this setting in both ALL and AML,[9-11] with an overall response rate of 44% in a phase II trial of relapsed/refractory childhood ALL. Further evaluation of this regimen is underway in a very high-risk subset of newly diagnosed childhood ALL (AALL1131) by the Children's Oncology Group (COG) and is planned in high-risk first relapse childhood ALL in the International Study for Treatment

of Childhood Relapsed ALL 2010 conducted by the Resistant Disease Committee of the International BFM Study Group.

Nelarabine

Nelarabine is a nucleoside analog with a high level of activity in T-ALL due to its conversion to 9-beta-D-arabinosylguanine (araG) that is resistant to cleavage by purine nucleoside phosphorylase (PNP). A phase I trial of nelarabine in patients with recurrent T-ALL demonstrated a remarkable response rate of 54% in T-ALL;[12] a pediatric phase II trial demonstrated response rates of 55% in first marrow relapse and 27% in second or subsequent relapse, together with grade 3 or greater adverse neurological events in 18% of patients.[13] Nelarabine is presently being evaluated in combination with etoposide and cyclophosphamide in a phase I trial of relapsed pediatric T-ALL and T-lymphoblastic lymphoma (T2008-002) conducted by the Therapeutic Advances in Childhood Leukemia & Lymphoma (TACL) consortium in collaboration with the Innovative Therapies for Children with Cancer (ITCC) consortium, and in newly diagnosed T-ALL in a phase III COG trial (AALL0434).

Bendamustine

Bendamustine is an alkylating agent with activity in adults with rituximab-refractory non-Hodgkin's lymphoma (NHL) and chronic lymphoblastic leukemia. A pediatric phase I trial of bendamustine in pediatric patients with relapsed or refractory acute leukemia demonstrated safety but suggested that bendamustine has minimal activity in heavily pre-treated children with relapsed and refractory ALL, but not in AML.[14]

Novel formulations of conventional agents

Liposomal vincristine (Marqibo)

Vincristine sulfate liposomes injection (VSLI, Marqibo) is a nanoparticle formulation of vincristine (VCR) that facilitates dose-intensive treatment by minimizing the neurotoxicity associated with the administration of the traditional formulation of vincristine. A phase II trial of VSLI in patients with Philadelphia (Ph)-negative ALL in second relapse or who progressed after two prior lines of treatment showed a response rate of 36%,[15] leading to an accelerated approval of VSLI in this setting by the FDA. An ongoing pediatric phase I trial of VSLI at the Pediatric Oncology Branch of the National Cancer Institute (NCI) has demonstrated a complete response in relapsed/refractory pediatric ALL.[16]

Tyrosine kinase inhibitors

Dasatinib, nilotinib and ponatinib

The addition of imatinib, a 1st generation tyrosine kinase inhibitor (TKI) directed against the BCR-ABL fusion protein, to conventional cytotoxic chemotherapy has transformed the treatment of Ph-positive ALL in children, more than doubling 3-year event-free survival (EFS) rates from approximately 35% in the pre-TKI era to 80% with the addition of continuous exposure to imatinib.[17] Newer 2nd generation TKIs such as nilotinib and dasatinib produce more rapid and complete cytogenetic and molecular responses than imatinib in CML,[18,19] but these favorable properties have yet to be demonstrated to translate into improved outcomes in Ph-positive ALL. The COG and EsPhALL groups are presently conducting a phase II trial of dasatinib combined with standard chemotherapy in children with newly diagnosed Ph-positive ALL (CA180372, COG AALL1122). Ponatinib, a 3rd generation TKI with activity *in vitro* against all tested resistant BCR-ABL mutants including the previously resistant T315I mutation, has significant clinical activity in patients with CML and Ph-positive ALL who fail other TKIs.[20]

Monoclonal antibodies

Epratuzumab

Epratuzumab is a humanized monoclonal antibody targeting CD22 that appears to modulate B-cell activation and signaling, and has demonstrated activity against adult non-Hodgkin's lymphoma. Epratuzumab was evaluated by COG in a phase I/II trial (ADVL04P2) in combination with multi-agent chemotherapy for the reinduction of relapsed childhood ALL.[21] Although the trial did not meet its primary end point of a statistically significant increase in CR2 rate at the end of the first block of chemotherapy plus epratuzumab as compared to historical control of chemotherapy alone, among those who attained a CR, subjects receiving epratuzumab were significantly more likely to become minimal residual disease (MRD) negative as compared to those who did not.[22] Epratuzumab is being further evaluated in standard-risk first relapse childhood ALL in the International Study for Treatment of Childhood Relapsed ALL 2010 conducted by the Resistant Disease Committee of the International BFM Study Group.

Immunoconjugates

Gemtuzumab ozogamicin

Gemtuzumab ozogamicin (GO) is a humanized monoclonal antibody directed against the CD33 surface antigen (present on approximately 90% of cases of childhood AML) which is conjugated to calicheamicin, a potent antitumor antibiotic that cleaves DNA. In a phase II trial of relapsed or refractory pediatric $CD33^+$ AML, GO produced a 28% overall remission rate.[23] GO has shown activity and tolerability in combination with standard chemotherapy in a COG trial (AAML00P2) for relapsed pediatric AML,[24] and has been randomly evaluated in newly diagnosed pediatric AML in combination with standard chemotherapy by COG[25] and NOPHO.[26] The equivocal results of several randomized trials of GO in adult AML led to its withdrawal from the market by the FDA in 2010 (reviewed in[27]). While the results of the COG AAML0531 trial are not yet available, the NOPHO-2004 trial showed no survival benefit for GO given to children after consolidation.

Moxetumomab

Moxetumomab (HA22, CAT-8015) is a 2nd generation recombinant anti-CD22 immunotoxin composed of the variable domain of an anti-CD22 monoclonal antibody fused to a 38 Kda truncated form of *Pseudomonas* exotox-

in A. Studies of moxetumomab in adults have demonstrated significant activity against hairy cell leukemia.[28] In an ongoing pediatric phase I trial of moxetumomab in North America, among 19 heavily pre-treated childhood ALL patients, 4 achieved CRs and one had a partial response, for an objective response rate of 26%; the most significant recurring toxicity has been capillary leak syndrome, leading to dexamethasone premedication.[29]

Blinatumomab

Blinatumomab is the first of a new class of immunoconjugates characterized as bispecific T-cell engager (BiTEs) which engage and activate $CD3^+$ T cells in close proximity to the target cell.[30] Blinatumomab is a single-chain bispecific antibody construct with specificity for CD19 and CD3 which has demonstrated significant activity in both adult and pediatric ALL,[31,32] with an 80% MRD response rate in a phase II trial of adult B-lineage ALL patients with persistent or relapsed MRD, and a relapse-free survival of 61% after a median follow up of 33 months.[33] A pediatric phase I trial of blinatumomab in patients with frank relapse of ALL (MT103-205) is underway through the Resistant Disease Committee of the International BFM Study Group and COG, in collaboration with Amgen.

Inotuzumab ozogamicin

Inotuzumab ozogamicin is a CD22 monoclonal antibody conjugated to calicheamicin. In a phase II study of inotuzumab in adults with refractory/relapsed ALL, the overall response rate was 57%; the most frequent adverse event was severe but reversible liver function abnormalities in 31% of patients.[34] A pediatric phase I trial of inotuzumab in relapsed/refractory childhood ALL is in development by the Therapeutic Advances in Childhood Leukemia & Lymphoma (TACL) and Innovative Therapies for Children with Cancer (ITCC) consortia, in collaboration with Pfizer. (Permission obtained by the author from Pfizer to disclose this study in development).

SAR3419

SAR3419 is a humanized CD19 monoclonal antibody conjugated to maytansin, a potent tubulin inhibitor.[35] In adults with recurrent/refractory NHL, SAR3419 demonstrated a response rate of 55% when administered at the maximum tolerated dose.[36] An adult phase I trial of SAR3419 in relapsed/refractory adult ALL is ongoing at the MD Anderson Cancer Center. In pre-clinical testing of SAR3419 by the NCI's Pediatric Preclinical Testing Program, significant activity was seen in multiple $CD19^+$ ALL xenografts, including MLL^+ ALLs.[37] A pediatric phase I trial of SAR3419 in relapsed/refractory childhood ALL is under development by the Therapeutic Advances in Childhood Leukemia & Lymphoma (TACL) and Innovative Therapies for Children with Cancer (ITCC) consortia, in collaboration with Sanofi. (Permission obtained by the author from Sanofi to disclose this study in development).

Proteosome inhibitors

Bortezomib

Bortezomib is a 1st generation proteosome inhibitor with little single-agent activity against acute leukemias, but with additive or synergistic *in vitro* effects against acute leukemias when combined with commonly used chemotherapeutic agents.[38] This observation led to a phase I/II trial of bortezomib phase I/II trial combined with multi-agent chemotherapy for the reinduction of relapsed childhood ALL (T2005-003) by the TACL consortium; the overall response rate was 73% and 80% in B-precursor ALL.[39,40] A phase II trial of bortezomib in combination with multi-agent chemotherapy for the reinduction of early first relapse of childhood ALL is being conducted by COG, and a pilot trial of bortezomib in combination with multi-agent chemotherapy for the reinduction of relapsed childhood ALL is being conducted by ITCC.

COG conducted a phase II pilot study of bortezomib in combination with two different multi-agent chemotherapy regimens (one containing an anthracycline and one without) for recurrent or refractory AML (AAML07P1); both regimens were tolerated and active in this setting.[41] The addition of bortezomib to standard chemotherapy is currently being evaluated in a randomized trial of newly diagnosed pediatric AML patients without high allelic ratio $FLT3/ITD^+$ (AAML1031) by COG.

mTOR inhibitors

Sirolimus, temsirolimus and everolimus

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that functions as a key regulator of cell growth and cell-cycle progression via multiple signaling pathways, including PI3K/AKT, ras and BCR/ABL. Pre-clinical studies have demonstrated that mTOR inhibitors are active, both as single agents and in combination therapy, in a variety of hematologic malignancies.[42] Although a phase I/II trial of sirolimus (rapamycin) added to standard graft-*versus*-host disease prophylaxis in children undergoing HSCT for ALL demonstrated safety and promising activity,[43] a subsequent randomized phase III trial demonstrated no decrease in relapse.[44] Phase I studies to evaluate the safety of adding temsirolimus or everolimus to multi-agent chemotherapy for the reinduction of relapsed childhood ALL are underway by COG (ADVL1114) and Dana-Farber Cancer Institute, respectively.

FLT3 inhibitors

The presence of FLT3 mutations as well as the allelic ratio of FLT3/ITD (ITD-AR, mutant-wild type ratio) have been shown to have prognostic significance in pediatric AML.[45] Infant leukemias with MLL gene rearrangements have high-level expression of constitutively activated FLT3.[46] Inhibition of FLT3 is an attractive strategy to improve outcome of both poor-prognosis FLT3/ITD-positive pediatric AML and MLL-rearranged infant ALL.

Midostaurin (PKC412)

The staurosporine derivative midostaurin (N-benzoyl staurosporine; PKC412), is an inhibitor of multiple protein kinases including FLT3. Midostaurin is cytotoxic to acute lymphoblastic leukemia cell lines with MLL translocations or activated FLT3 receptors.[47] The safety and efficacy of midostaurin is currently being evaluated in an inter-

national phase I/II trial of pediatric patients with recurrent MLL+ or FLT3+ acute leukemias (NCT00866281) conducted by Novartis.

Lestaurtinib

In vitro inhibition of FLT3 in AML[48] and in MLL-rearranged infant leukemias[46] by lestaurtinib (CEP-701), a highly selective FLT3 inhibitor, suppresses FLT3-driven cell survival, leading to leukemic cell death. The safety and efficacy of adding lestaurtinib to standard multi-agent chemotherapy is currently being evaluated in a randomized trial of newly diagnosed infant ALL patients with MLL rearrangements (AALL0631) conducted by COG.

Sorafenib

Sorafenib is a multi-target tyrosine kinase inhibitor that targets *FLT3, c-KIT, PDGF, VEGF*, and other signaling pathways. Sorefenib combined with multi-agent cytotoxic chemotherapy in young adults with AML resulted in inhibition of FLT3 signaling and a significant increase in the proportion of FLT3/ITD+ AML patients attaining remission.[49] The addition of sorefenib to standard multi-agent chemotherapy is currently being evaluated in a randomized trial of newly diagnosed pediatric AML patients with high allelic ratio FLT3/ITD+ (AAML1031) conducted by COG.

Quizartinib (AC220)

AC220 is a 2nd generation FLT3 inhibitor with increased potency and selectivity compared to other currently available FLT3 inhibitors; using the PIA assay, AC220 is the first compound to completely suppress FLT3 phosphorylation *ex vivo* at doses that are easily achievable and sustainable in the clinic.[50] The safety and efficacy of AC220 is currently being evaluated in a phase I trial of pediatric patients with recurrent MLL+ or FLT3+ acute leukemias (T2009-004) conducted by the TACL consortium.

JAK inhibitors

Ruxolitinib

Recurring activating mutations in genes encoding the Janus kinase (JAK) family of signal transduction enzymes have been identified in myeloproliferative disorders[51,52] and in a subset of high-risk pediatric ALLs.[53] The efficacy of JAK inhibitors in myeloproliferative disorders with JAK mutations[54] led to the development of an ongoing pediatric phase I trial of the JAK inhibitor ruxolitinib (ADVL1011) in COG.

Epigenetic modification

Decitabine and vorinostat

Epigenetic alterations are common in both AML and ALL.[55,56] The use of epigenetic modifiers such as demethylating agents and/or histone deacetylase (HDAC) inhibitors may increase response to conventional chemotherapeutic agents. The combination of the histone deacetylase inhibitor vorinostat with idarubicin and cytarabine was safe and active in a phase II study of newly diagnosed adult AML.[57] TACL is conducting a phase I trial of the demethylating agent decitabine and vorinistat in combination with multi-agent chemotherapy for the reinduction of relapsed childhood ALL (T2009-003).

Panobinostat

Panobinostat, a novel HDAC inhibitor with increased potency compared to vorinostat and other HDAC inhibitors,[58] is currently being evaluated in a pediatric phase I trial for relapsed ALL, AML, Hodkgin's lymphoma and non-Hodgkin's lymphoma conducted by TACL.

Stem cell mobilization

Plerixafor

The resistance of leukemic stem cells (LSCs) to conventional chemotherapeutic agents may play an important role in treatment failure. Resistance of LSCs to treatment may be mediated in part by the stromal environment, including interactions between stem cell-derived growth factor (CXCL-12/SDF-1α) and its receptor, CXCR4.[59] Plerixifor (AMD3100) a bicyclam inhibitor of CXCL12-CXCXR4 binding and signaling, is approved by the FDA for mobilization of normal hematopoetic stem cells for autologous transplantation in hematologic malignancies. The administration of plerixafor prior to conventional chemotherapy in order to mobilize LSCs from the protective stromal environment and thus restore chemosensitivity demonstrated safety and efficacy in a murine model of AML.[60] Plerixafor in combination with cytotoxic chemotherapy is presently being investigated in a pediatric phase I trial for relapsed acute leukemias (POE 10-03) conducted by the Pediatric Oncology Experimental Therapeutics Investigators' Consortium (POETIC).

What is the proper efficacy end point in the evaluation of novel agents for childhood ALL?

The rate of attainment of a complete remission (CR) as measured by morphological bone marrow response is the traditional benchmark for evaluation of efficacy, in the context of a phase II trial, in the evaluation of novel anti-leukemic agents. Although most investigators would agree that EFS is the ideal end point for efficacy in the evaluation of a new therapy for hematologic malignancies, the heterogeneity of post-remission therapies such as allogeneic HSCT makes unbiased evaluation of EFS difficult to attain in phase II trials evaluating novel agents during reinduction. However, concern about the validity of CR rate and its correlation with outcome was raised with the publication of the UK's ALLR3 trial for children with first relapse of ALL. The R3 trial randomized idarubicin and mitoxantrone during induction therapy; mitoxantrone conferred a significant benefit in both progression-free and overall survival, despite conveying no advantage in end-induction morphological CR rates or levels of MRD response.[61] This puzzling discrepancy remains unexplained, and raises the concern that novel agents which do not improve early response may nonetheless convey therapeutic advantage in relapsed childhood ALL. Thus, studies that utilize CR rate as the primary end point may fail to identify effective agents. However, the limited number of

Table 1. A summary of agents and clinical trials/development status in pediatrics.

Agent	Class	Mechanism of action	Target	Clinical trials/ development status in pediatrics
Clofarabine	Cytotoxic	Nucleoside analog	Non-specific	Ind: phase II reinduction trial (with cyclophosphamide and etoposide) in relapsed ALL (*completed*) COG: phase I/II reinduction trial (with cytarabine) in relapsed AML and ALL (*completed*) ITCC: phase I/II reinduction trial (with cytarabine and liposomal daunorubicin) in relapsed AML (*completed*) COG: phase III post-induction combination trial in very high-risk newly diagnosed ALL (*active*) IntReALL: phase II randomized reinduction trial (with cyclophosphamide and etoposide) in HR 1st relapse ALL (*planned*)
Nelarabine	Cytotoxic	Nucleoside analog	T cells	COG: phase II single-agent reinduction in relapsed T-ALL/LL (*completed*) COG: phase III post-induction randomized trial in higher risk newly diagnosed T-ALL (*active*) TACL: phase I reinduction trial (with cyclophosphamide and etoposide) in 1*st* relapse T-ALL/T-LL (*active*)
Bendamustine	Cytotoxic	Alkylating agent	Non-specific	Ind: phase I single agent reinduction trial in relapsed pediatric ALL and AML (*completed*)
Liposomal vincristine (Marqibo)	Cytotoxic	Mitotic inhibitor	Microtubules	POB: phase I single agent reinduction trial in relapsed pediatric malignancies (*active*) TACL: phase I multi-agent reinduction trial in relapsed ALL (*planned*)
Dasatinib	Signal transduction inhibitor	Tyrosine kinase inhibitor	bcr-abl	COG: phase I single agent reinduction trial in relapsed pediatric malignancies (*completed*) Ind: phase II single agent reinduction trial in relapsed pediatric Ph+ malignancies (*active*) Ind/ EsPhALL/COG: phase II multi-agent trial in newly diagnosed Ph+ ALL (*active*)
Nilotinib	Signal transduction inhibitor	Tyrosine kinase inhibitor	bcr-abl	Ind/ITCC/COG: Pharmacokinetic single agent reinduction trial in relapsed pediatric Ph+ malignancies (*active*)
Ponatinib	Signal transduction inhibitor	Tyrosine kinase inhibitor	bcr-abl	Ind: phase I single agent reinduction trial in relapsed pediatric Ph+ malignancies (*planned*)
Epratuzumab	Immunotherapy	Monoclonal antibody	CD22	COG: pilot/phase II multi-agent reinduction trial in relapsed ALL (*completed*) IntReALL: phase III multi-agent post-reinduction trial in SR 1st relapse ALL (*planned*)
Gemtuzumab ozogamicin	Immunotherapy	Immunotoxin (calicheamicin)	CD34	COG: phase I combination reinduction trial in relapsed AML (*completed*) NOPHO: randomized post-induction multi-agent trial in higher risk newly diagnosed T-ALL (*active*) COG: phase III post-induction multi-agent trial in newly diagnosed AML (*completed*)
Moxetumomab (HA22, CAT8015)	Immunotherapy	Immunotoxin (Pseudomonas exotoxin A)	CD22	Ind: phase I single agent trial in relapsed pediatric CD22+ malignancies (*active*) Ind: phase I combination trial in relapsed pediatric CD22+ malignancies (*planned*)
Blinatumomab (MT103	Immunotherapy	Bispecific T-cell engager (BiTE)	CD19	Ind/IBFM-SG/COG: phase I/II single agent reinduction trial in relapsed pediatric ALL (*active*) COG: phase III multi-agent post-induction trial in 1st relapse ALL (*planned*)
Inotuzumab ozogamicin	Immunotherapy	Immunotoxin (calicheamicin)	CD22	TACL/ITCC: phase I trial in pediatric ALL/NHL (*planned*)
SAR3419	Immunotherapy	Immunotoxin (calicheamicin)	CD19	TACL/ITCC: phase I single agent reinduction trial in relapsed pediatric ALL/NHL (*planned*)
Bortezomib (PS-341)		Proteosome inhibition	26S proteasome	TACL: phase I/II combination reinduction trial in relapsed pediatric ALL (*completed*) COG: phase II combination reinduction trial in relapsed pediatric AML (*completed*) COG: phase II combination reinduction trial in relapsed pediatric ALL/NHL (*active*) ITCC: pilot combination reinduction trial in relapsed pediatric ALL (*active*) COG: phase III multi-agent reinduction trial in newly diagnosed pediatric AML (*active*) COG: phase III multi-agent trial in newly diagnosed pediatric T-ALL (*planned*)
Sirolimus (rapamycin)		mTOR inhibition	FKBP12	COG: phase III post-transplantation trial in pediatric ALL (*completed*)
Temsirolimus (CCI-779)		mTOR inhibition	FKBP-12	COG: phase I multi-agent reinduction trial in relapsed pediatric ALL/NHL (*active*) TACL: phase I multi-agent reinduction trial in relapsed pediatric ALL/NHL (*planned*)
Everolimus (RAD001)		mTOR inhibition	FKBP12	DFCI: phase I combination reinduction trial in relapsed pediatric ALL/NHL (*active*)
Midostaurin (PKC412)	Signal transduction inhibitor	Receptor tyrosine kinase inhibition	FLT3	ITCC: phase I single-agent trial in relapsed pediatric ALL and AML (*active*)
Lestaurtinib (CEP-701)	Signal transduction inhibitor	Receptor tyrosine kinase inhibition	FLT3	COG: pilot combination reinduction trial in relapsed pediatric AML (*completed*) COG: phase III multi-agent trial in newly diagnosed infant ALL (*active*)

Continued on next page

Table 1. Continued from previous page.

Agent	Class	Mechanism of action	Target	Clinical trials/ development status in pediatrics
Sorafenib	Signal transduction inhibitor	Receptor tyrosine kinase inhibition	FLT3, RAF, VEGFR	COG: phase III multi-agent reinduction trial in newly diagnosed pediatric AML (*active*)
Quizartinib (AC220)	Signal transduction inhibitor	Receptor tyrosine kinase inhibition	FLT3, c-Kit	TACL: phase I combination reinduction trial in relapsed pediatric ALL and AML (*active*)
Ruxolinitinib (INCB018424)	Signal transduction inhibitor	Janus kinase inhibition	JAK1, JAK2	COG: phase I single-agent trial in relapsed malignancies (*active*)
Decitabine/ vorinostat	Epigenetic modifiers	Demethylation/ histone deacetylase (HDAC) inhibition	Non-specific	TACL: phase I combination reinduction trial in relapsed pediatric ALL (*active*)
Panobinostat (LBH589)	Epigenetic modifier	Histone deacetylase (HDAC) inhibition	Non-specific	TACL: phase I single-agent trial in relapsed pediatric ALL, AML, HD, NHL (*active*)
Plerixafor	Stem cell mobilization	Disruption of CXCL12/ CXCR4 interaction	CXCR4	POETIC: phase I combination reinduction trial in relapsed pediatric ALL and AML (*active*)

COG: Children's Oncology Group (North America, Australasia, Europe); DFCI: Dana-Farber Cancer Institute (United States); EsPhALL: European intergroup study on post-induction treatment of Philadelphia positive ALL (Europe); IBFM SG: International BFM Study Group (International); IntReALL: International Study for treatment of childhood relapsed ALL (Europe); ITCC: Innovative Therapies for Children with Cancer Consortium (Europe); POB: Pediatric Oncology Branch, National Cancer Institute (United States); POETIC: Pediatric Oncology Experimental Therapeutics Investigators' Consortium (North America); TACL: Therapeutic Advances in Childhood Leukemia and Lymphoma (North America, Australia); NOPHO: Nordic Society of Pediatric Hematology and Oncology (Scandinavia, Iceland).

relapsed/refractory pediatric acute leukemia patients available for participation in clinical trials of novel agents, together with the plethora of novel agents awaiting evaluation, means that utilizing only evaluations which rely on survival as the primary efficacy end point would severely restrict our ability to interrogate many promising new drugs.

Single agent *versus* combination?

The traditional development path for new agents in solid tumors is through the sequential conduct of single-agent phase I and phase II trials to establish safety and efficacy, respectively. Drugs with promising single agent activity are then often evaluated in combination with standard therapies in sequential phase I, II and III trials. This development strategy, while rational, can often be ineffective in childhood leukemia for a number of reasons. Due to the aggressive nature of some recurrent acute leukemias, patients treated with a single agent in a phase I leukemia trial may rapidly progress at sub-therapeutic dose levels or when receiving an ineffective agent, thus becoming inevaluable and bringing the phase I trial to a halt. The mechanisms of action of some agents, such as proteosome inhibitors and mTOR inhibitors, suggest that they are likely to have significant efficacy only when administered in combination with traditional chemotherapeutic agents, thus rendering single-agent evaluations less relevant. And finally, the ability of many traditional regimens to attain remissions even in multiply relapsed patients with ALL means that parents and treating physicians may be reluctant to consider participation in new agent trials, with their inherent uncertainties of toxicity and efficacy, when conventional therapies are readily available. Thus, COG and TACL have chosen to develop novel agents in relapsed ALL and AML within the context of standard chemotherapy reinduction platforms whenever possible, so as to accelerate the pace of drug development by providing parents and treating physicians with the 'safety net' of standard therapy, and also by evaluating the toxicities of a novel agent in the context of standard combination therapy. Although evaluation of the toxicities of a novel agent can be challenging when it is administered in combination with conventional agents which are also toxic, methodologies to accomplish such evaluations have been developed and applied to the study of novel agents in both the relapse and newly diagnosed setting in COG.[62]

A summary of agents and clinical trials/development status in pediatrics is available in Table 1.

How can we possibly evaluate all of these novel agents in childhood leukemias?

The pediatric oncology community faces significant challenges if it is to seize the opportunity to quickly and efficiently incorporate novel agents into the treatment of childhood leukemias. For example, the identification of druggable molecular targets that occur in only a small proportion of patients (*i.e,* JAK mutations) make it difficult to attain adequate accrual for evaluation by any single cooperative group. However, a number of positive steps have been taken in the past few years which should provide hope to those engaged in this endeavor, including:

- improving communication and coordination, rather than competition, among the various groups which design and conduct clinical trials of novel agents in pediatric hematologic malignancies through increased exchanges of ideas and results at group meetings and at annual pro-

fessional society meetings;
- increasing collaboration between European and North American groups in the conduct of clinical trials involving novel agents for uncommon subsets of childhood hematologic malignancies, ie, IBFM and COG for blinatumomab and dasatinib, TACL and ITCC for nelarabine, inotuzumab and SAR3419;
- the recent selection of the UK ALL-R3 chemotherapy platform for future drug development in ALL by COG, thereby facilitating the rapid integration of novel agents developed by COG into the European as well as North American communities;
- increasing collaboration between academic investigators and pharmaceutical companies to meet the regulatory requirements of the EMEA and FDA in order to obtain approval of the most promising novel agents;
- the recognition that each research community has unique strengths: the North American community in the integration and evaluation of novel agents in multi-agent chemotherapy regimens, and the European community in the conduct of randomized clinical trials with controlled post-remission interventions, thus allowing for meaningful evaluation of important endpoints such as EFS. For example, the initial phase I/II assessment of epratuzumab in relapsed ALL by COG is being followed by a randomized evaluation of epratuzumab in the International Study for Treatment of Childhood Relapsed ALL (IntReALL) 2010 conducted by the Resistant Disease Committee of the International BFM Study Group.

These and other important steps to align investigative efforts will maximize the ability of the pediatric cancer community to efficiently evaluate promising new agents for hematologic malignancies, thus contributing to continued improvements in both survival and quality of life for our patients.

References

1. Pui CH, Campana D, Pei D, Bowman WP, Sandlund JT, Kaste SC, et al. Treating childhood acute lymphoblastic leukemia without cranial irradiation. N Engl J Med. 2009;360(26):2730-41.
2. Moricke A, Zimmermann M, Reiter A, Henze G, Schrauder A, Gadner H, et al. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia. 2010;24(2): 265-84.
3. Schmiegelow K, Forestier E, Hellebostad M, Heyman M, Kristinsson J, Soderhall S, et al. Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. Leukemia. 2010;24(2):345-54.
4. Hunger SP, Lu X, Devidas M, Camitta BM, Gaynon PS, Winick NJ, et al. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. J Clin Oncol. 2012;30(14): 1663-9.
5. Gaynon PS. Childhood acute lymphoblastic leukaemia and relapse. Br J Haematol. 2005;131(5):579-87.
6. Jeha S, Gandhi V, Chan KW, McDonald L, Ramirez I, Madden R, et al. Clofarabine, a novel nucleoside analog, is active in pediatric patients with advanced leukemia. Blood. 2004;103 (3):784-9.
7. Jeha S, Gaynon PS, Razzouk BI, Franklin J, Kadota R, Shen V, et al. Phase II study of clofarabine in pediatric patients with refractory or relapsed acute lymphoblastic leukemia. J Clin Oncol. 2006;24(12):1917-23.
8. Cooper TM, Razzouk BI, Gerbing R, Alonzo TA, Adlard K, Raetz E, et al. Phase I/II trial of clofarabine and cytarabine in children with relapsed/refractory acute lymphoblastic leukemia (AAML0523): A report from the Children's Oncology Group. Pediatr Blood Cancer. 2013 Jan 17. Epub ahead of print].
9. Hijiya N, Gaynon P, Barry E, Silverman L, Thomson B, Chu R, et al. A multi-center phase I study of clofarabine, etoposide and cyclophosphamide in combination in pediatric patients with refractory or relapsed acute leukemia. Leukemia. 2009;23(12):2259-64.
10. Locatelli F, Testi AM, Bernardo ME, Rizzari C, Bertaina A, Merli P, et al. Clofarabine, cyclophosphamide and etoposide as single-course re-induction therapy for children with refractory/multiple relapsed acute lymphoblastic leukaemia. Br J Haematol. 2009;147(3):371-8.
11. Hijiya N, Thomson B, Isakoff MS, Silverman LB, Steinherz PG, Borowitz MJ, et al. Phase 2 trial of clofarabine in combination with etoposide and cyclophosphamide in pediatric patients with refractory or relapsed acute lymphoblastic leukemia. Blood. 2011;118(23):6043-9.
12. Kurtzberg J, Ernst TJ, Keating MJ, Gandhi V, Hodge JP, Kisor DF, et al. Phase I study of 506U78 administered on a consecutive 5-day schedule in children and adults with refractory hematologic malignancies. J Clin Oncol. 2005;23(15):3396-403.
13. Berg SL, Blaney SM, Devidas M, Lampkin TA, Murgo A, Bernstein M, et al. Phase II study of nelarabine (compound 506U78) in children and young adults with refractory T-cell malignancies: a report from the Children's Oncology Group. J Clin Oncol. 2005;23(15):3376-82.
14. Fraser C, Brown PA, Megason GC, Ahn HS, Cho B, Kirov II, et al. Open-Label Bendamustine for Pediatric Patients with Relapsed or Refractory Acute Leukemia: Safety and Efficacy Outcomes. ASH Annual Meeting Abstracts. 2011;118 (21):3602.
15. O'Brien SM, Aulitzky W, Ben Yehuda D, Lister J, Schiller GJ, Seiter K, et al. Phase II study of marqibo in adult patients with refractory or relapsed philadelphia chromosome negative (Ph) acute lymphoblastic leukemia (ALL). ASCO Meeting Abstracts. 2010;28(Suppl 15):6507.
16. Shah NN, Merchant M, Cole D, Richards K, Delbrook C, Widemann BC, et al. Vincristine Sulfate Liposomes Injection (VSLI, Marqibo): Interim Results From a Phase I Study in Children and Adolescents with Refractory Cancer. ASH Annual Meeting Abstracts. 2012;120(21):1497.
17. Schultz KR, Bowman WP, Aledo A, Slayton WB, Sather H, Devidas M, et al. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. J Clin Oncol. 2009;27(31):5175-81.
18. Kantarjian H, Shah NP, Hochhaus A, Cortes J, Shah S, Ayala M, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med. 2010; 362(24):2260-70.
19. Saglio G, Kim DW, Issaragrisil S, le Coutre P, Etienne G, Lobo C, et al. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. N Engl J Med. 2010;362(24):2251-9.
20. Cortes JE, Kim D-W, Pinilla-Ibarz J, Paquette R, le Coutre PD, Chuah C, et al. PACE: A pivotal phase II trial of ponatinib in patients with CML and Ph+ALL resistant or intolerant to dasatinib or nilotinib, or with the T315I mutation. ASCO Meeting Abstracts. 2012;30(Suppl 15):6503.
21. Raetz EA, Cairo MS, Borowitz MJ, Blaney SM, Krailo MD, Leil TA, et al. Chemoimmunotherapy reinduction with epratuzumab in children with acute lymphoblastic leukemia in marrow relapse: a Children's Oncology Group Pilot Study. J Clin Oncol. 2008;26(22):3756-62.
22. Raetz EA, Cairo MS, Borowitz MJ, Lu X, Devidas M, Reid JM, et al. Reinduction Chemoimmunotherapy with Epratuzumab in Relapsed Acute Lymphoblastic Leukemia (ALL) in Children, Adolescents and Young Adults: Results From Children's Oncology Group (COG) Study ADVL04P2. ASH Annual Meeting Abstracts. 2011;118(21):573.
23. Arceci RJ, Sande J, Lange B, Shannon K, Franklin J, Hutchinson R, et al. Safety and efficacy of gemtuzumab ozogamicin in pediatric patients with advanced CD33+ acute myeloid leukemia. Blood. 2005;106(4):1183-8.
24. Aplenc R, Alonzo TA, Gerbing RB, Lange BJ, Hurwitz CA, Wells RJ, et al. Safety and efficacy of gemtuzumab ozogamicin in combination with chemotherapy for pediatric acute myeloid leukemia: a report from the Children's Oncology Group. J Clin Oncol. 2008;26(14):2390-3295.
25. Gamis AS, Alonzo TA, Gerbing RB, Aplenc R, Sung L, Meshinchi S, et al. Remission Rates In Childhood Acute Myeloid Leukemia (AML) Utilizing a Dose-Intensive Induction Regimen with or without Gemtuzumab Ozogamicin (GO): Initial Results From the Children's Oncology Group Phase III Trial, AAML0531. ASH Annual Meeting Abstracts. 2010;116(21):182.
26. Hasle H, Abrahamsson J, Forestier E, Ha SY, Heldrup J,

Jahnukainen K, et al. Gemtuzumab ozogamicin as postconsolidation therapy does not prevent relapse in children with AML: results from NOPHO-AML 2004. Blood. 2012;120(5):978-84.
27. Ravandi F, Estey EH, Appelbaum FR, Lo-Coco F, Schiffer CA, Larson RA, et al. Gemtuzumab ozogamicin: time to resurrect? J Clin Oncol. 2012;30(32):3921-3.
28. FitzGerald DJ, Wayne AS, Kreitman RJ, Pastan I. Treatment of hematologic malignancies with immunotoxins and antibody-drug conjugates. Cancer Res. 2011;71(20):6300-9.
29. Wayne AS, Bhojwani D, Silverman LB, Richards K, Stetler-Stevenson M, Shah NN, et al. A Novel Anti-CD22 Immunotoxin, Moxetumomab Pasudotox: Phase I Study in Pediatric Acute Lymphoblastic Leukemia (ALL). ASH Annual Meeting Abstracts. 2011;118(21):248.
30. Nagorsen D, Bargou R, Ruttinger D, Kufer P, Baeuerle PA, Zugmaier G. Immunotherapy of lymphoma and leukemia with T-cell engaging BiTE antibody blinatumomab. Leuk Lymphoma. 2009;50(6):886-91.
31. Topp MS, Kufer P, Gokbuget N, Goebeler M, Klinger M, Neumann S, et al. Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. J Clin Oncol. 2011;29(18):2493-8.
32. Handgretinger R, Zugmaier G, Henze G, Kreyenberg H, Lang P, von Stackelberg A. Complete remission after blinatumomab-induced donor T-cell activation in three pediatric patients with post-transplant relapsed acute lymphoblastic leukemia. Leukemia. 2011;25(1):181-4.
33. Topp MS, Gokbuget N, Zugmaier G, Degenhard E, Goebeler ME, Klinger M, et al. Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. Blood. 2012; 120(26):5185-7.
34. Kantarjian H, Thomas D, Jorgensen J, Jabbour E, Kebriaei P, Rytting M, et al. Inotuzumab ozogamicin, an anti-CD22-calecheamicin conjugate, for refractory and relapsed acute lymphocytic leukaemia: a phase 2 study. Lancet Oncol. 2012; 13(4):403-11.
35. Blanc V, Bousseau A, Caron A, Carrez C, Lutz RJ, Lambert JM. SAR3419: an anti-CD19-Maytansinoid Immunoconjugate for the treatment of B-cell malignancies. Clin Cancer Res. 2011;17(20):6448-58.
36. Coiffier B, Morschhauser F, Dupuis J, Haioun C, Laine F, Houot R, et al. Phase I study cohort evaluating an optimized administration schedule of SAR3419, an anti-CD19 DM4-loaded antibody drug conjugate (ADC), in patients (pts) with CD19 positive relapsed/refractory b-cell non-Hodgkin's lymphoma (NCT00796731). ASCO Meeting Abstracts. 2012;30 (Suppl 15):8057.
37. Lock RB, Carol H, Szymanska B, Boehm I, Evans K, Houghton PJ, et al. Abstract C107: The anti-CD19 antibody-drug conjugate SAR3419 prevents hematolymphoid relapse post induction therapy in preclinical models of pediatric acute lymphoblastic leukemia. Molecular Cancer Therapeutics. 2011;10(Suppl1):C107.
38. Horton TM, Gannavarapu A, Blaney SM, D'Argenio DZ, Plon SE, Berg SL. Bortezomib interactions with chemotherapy agents in acute leukemia in vitro. Cancer Chemother Pharmacol. 2006;58(1):13-23.
39. Messinger Y, Gaynon P, Raetz E, Hutchinson R, Dubois S, Glade-Bender J, et al. Phase I study of bortezomib combined with chemotherapy in children with relapsed childhood acute lymphoblastic leukemia (ALL): a report from the therapeutic advances in childhood leukemia (TACL) consortium. Pediatr Blood Cancer. 2010;55(2):254-9.
40. Messinger YH, Gaynon PS, Sposto R, van der Giessen J, Eckroth E, Malvar J, et al. Bortezomib with chemotherapy is highly active in advanced B-precursor acute lymphoblastic leukemia: Therapeutic Advances in Childhood Leukemia & Lymphoma (TACL) Study. Blood. 120(2):285-90.
41. Horton TM, Perentesis J, Gamis AS, Alonzo TA, Gerbing RB, Ballard J, et al. A Phase 2 Study of Bortezomib Combined with Reinduction Chemotherapy in Children and Young Adults with Recurrent, Refractory or Secondary Acute Myeloid Leukemia: A Children's Oncology Group (COG) Study. ASH Annual Meeting Abstracts. 2012;120(21):3580.
42. Teachey DT, Grupp SA, Brown VI. Mammalian target of rapamycin inhibitors and their potential role in therapy in leukaemia and other haematological malignancies. Br J Haematol. 2009;145(5):569-80.
43. Pulsipher MA, Wall DA, Grimley M, Goyal RK, Boucher KM, Hankins P, et al. A phase I/II study of the safety and efficacy of the addition of sirolimus to tacrolimus/methotrexate graft versus host disease prophylaxis after allogeneic haematopoietic cell transplantation in paediatric acute lymphoblastic leukaemia (ALL). Br J Haematol. 2009;147 (5):691-9.
44. Pulsipher MA, Langholz B, Wall DA, Schultz KR, Bunin N, Carroll WL, et al. A Randomized Trial of Sirolimus-Based Graft Versus Host Disease (GVHD) Prophylaxis After Hematopoietic Stem Cell Transplantation (HSCT) in Selected Patients with CR1 and CR2 ALL: Results From Children's Oncology Group Study ASCT0431. ASH Annual Meeting Abstracts. 2011;118(21):837.
45. Meshinchi S, Alonzo TA, Stirewalt DL, Zwaan M, Zimmerman M, Reinhardt D, et al. Clinical implications of FLT3 mutations in pediatric AML. Blood. 2006;108 (12):3654-61.
46. Brown P, Levis M, Shurtleff S, Campana D, Downing J, Small D. FLT3 inhibition selectively kills childhood acute lymphoblastic leukemia cells with high levels of FLT3 expression. Blood. 2005;105(2):812-20.
47. Stam RW, den Boer ML, Schneider P, Nollau P, Horstmann M, Beverloo HB, et al. Targeting FLT3 in primary MLL-gene-rearranged infant acute lymphoblastic leukemia. Blood. 2005;106(7):2484-90.
48. Brown P, Meshinchi S, Levis M, Alonzo TA, Gerbing R, Lange B, et al. Pediatric AML primary samples with FLT3/ITD mutations are preferentially killed by FLT3 inhibition. Blood. 2004;104(6):1841-9.
49. Ravandi F, Cortes JE, Jones D, Faderl S, Garcia-Manero G, Konopleva MY, et al. Phase I/II study of combination therapy with sorafenib, idarubicin, and cytarabine in younger patients with acute myeloid leukemia. J Clin Oncol. 2010;28(11): 1856-62.
50. Zarrinkar PP, Gunawardane RN, Cramer MD, Gardner MF, Brigham D, Belli B, et al. AC220 is a uniquely potent and selective inhibitor of FLT3 for the treatment of acute myeloid leukemia (AML). Blood. 2009;114(14):2984-92.
51. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352(17): 1779-90.
52. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434(7037):1144-8.
53. Mullighan CG, Zhang J, Harvey RC, Collins-Underwood JR, Schulman BA, Phillips LA, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. Proc Natl Acad Sci USA. 2009;106(23):9414-8.
54. Verstovsek S, Kantarjian H, Mesa RA, Pardanani AD, Cortes-Franco J, Thomas DA, et al. Safety and Efficacy of INCB018424, a JAK1 and JAK2 Inhibitor, in Myelofibrosis. New Eng J Med. 2010;363(12):1117-27.
55. Plass C, Oakes C, Blum W, Marcucci G. Epigenetics in acute myeloid leukemia. Semin Oncol. 2008;35(4):378-87.
56. Garcia-Manero G, Yang H, Kuang SQ, O'Brien S, Thomas D, Kantarjian H. Epigenetics of acute lymphocytic leukemia. Semin Hematol. 2009;46(1):24-32.
57. Garcia-Manero G, Tambaro FP, Bekele NB, Yang H, Ravandi F, Jabbour E, et al. Phase II trial of vorinostat with idarubicin and cytarabine for patients with newly diagnosed acute myelogenous leukemia or myelodysplastic syndrome. J Clin Oncol. 2012;30 (18):2204-10.
58. Atadja P. Development of the pan-DAC inhibitor panobinostat (LBH589): successes and challenges. Cancer Lett. 2009;280 (2):233-41.
59. Burger JA, Burkle A. The CXCR4 chemokine receptor in acute and chronic leukaemia: a marrow homing receptor and potential therapeutic target. Br J Haematol. 2007;137(4):288-96.
60. Nervi B, Ramirez P, Rettig MP, Uy GL, Holt MS, Ritchey JK, et al. Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. Blood. 2009;113(24):6206-14.
61. Parker C, Waters R, Leighton C, Hancock J, Sutton R, Moorman AV, et al. Effect of mitoxantrone on outcome of children with first relapse of acute lymphoblastic leukaemia (ALL R3): an open-label randomised trial. Lancet. 2010;376 (9757):2009-17.
62. Horton TM, Sposto R, Brown P, Reynolds CP, Hunger SP, Winick NJ, et al. Toxicity assessment of molecularly targeted drugs incorporated into multiagent chemotherapy regimens for pediatric acute lymphocytic leukemia (ALL): review from an international consensus conference. Pediatr Blood Cancer. 2010;54(7):872-8.

Molecular control of hemoglobin switching

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A B S T R A C T

The human *beta (β)-globin* locus contains five *β-like globin* genes, arranged in the order of their developmental expression pattern 5'-*ε* (embryonic) - *Gγ* - *Aγ* (fetal) - *δ* - *β* (adult). The transition from fetal to adult β-like globin expression, known as hemoglobin switching, has attracted most attention. In patients with β-thalassemia or sickle cell disease, persistent expression of the endogenous *γ-globin* genes ameliorates the clinical manifestation of the disorders. Clinicians, epidemiologists and molecular biologists have studied hemoglobin switching for decades, applying novel tools and knowledge developed by the scientific community. In the 1990s, the field had exhausted the then available technologies without achieving a fundamental understanding of the molecular control of hemoglobin switching. This changed with the advent of the '-omics' era, sparked by the completion of the human genome sequence. Genome-wide association studies, comprehensive gene expression profiling, protein interaction screens and knockdown of genes using short interfering RNAs have propelled the field forward in the first decade of the 21st century. While we are still some way off understanding all the details of hemoglobin switching, there are a number of leads for the development of safe, affordable, off-the-shelf pharmacological compounds targeting the γ-globin suppression pathway.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe developmental regulation of the human *globin* genes and hemoglobin switching;
- understand the challenges to achieve reactivation of γ-globin expression as a means to ameliorate the symptoms of β-thalassemia and sickle cell disease patients;
- discuss recent progress in knowledge of the molecular control of hemoglobin switching.

Thalassemias and sickle cell disease

Diseases affecting red blood cells are by far the most common hereditary disorders in the human population. The symptoms vary from relatively mild, for instance deficiency for the metabolic enzyme glucose-6-phosphate dehydrogenase (G6PD) or erythrocyte structural proteins such as ankyrin (ANK1), α- or β-spectrin (SPTA and SPTB), to lethal when left untreated. The latter is the case in severe forms of the thalassemias, a group of diseases caused by insufficient α- or β-globin production. Hemoglobin, a tetramer composed of 2 α-like and 2 β-like globin proteins, is responsible for the gas transport functions of the erythrocytes, and comprises more than 90% of the soluble protein in these cells. Each erythrocyte contains approximately 250×10^6 hemoglobin molecules, and balanced production of the α-like and β-like globins in terminally differentiating erythroid progenitors is important to achieve a 1:1 ratio. Excess α-like or β-like chains are unable to form functional hemoglobin tetramers and will damage the erythroid cells. Insufficient production of α-globin leads to α-thalassemia. Severely compromised α-globin production results in prenatal lethality, a condition known as hemoglobin Barts hydrops foetalis. The symptoms of β-thalassemia, when β-globin expression is compromised, develop during the first year after birth. Humans have two specialized fetal *-like globin* genes, *HBG1* and *HBG2*, which encode the Aγ- and Gγ-globins, respectively (Figure 1). These fetal genes are gradually silenced while expression of the adult - and *-globin* genes is activated (Figure 2). Malfunction of the *HBB* gene resulting in β-thalassemia becomes manifested during the first year of life following the switch from fetal-to-adult globin expression. There is no fetal *α-like* gene, and in severe α-thalassemia cases dysfunctional γ-globin tetramers are formed (hemoglobin Barts) at fetal stages. The presence of γ-globin protects the developing fetus from the effects of pathological β-globin variants. The most common mutation substitutes glutamic acid for valine (Glu6Val or E6V) in the 6th codon of β-globin and is the cause of sickle cell disease (SCD). The substitution of a glutamic acid by a valine residue (HbS) affects the biophysical properties of the hemoglobin tetramers. The change from a charged (E) to a non-polar (V) side chain enables polymerization of hemoglobin molecules under low oxygen conditions. This deforms the erythrocytes into the characteristic sickled shape. This has profound effects on the physiological properties of the

cells. Abnormal adhesion to blood vessel walls and microvascular occlusion result in local hypoxia aiding further sickling that leads to lasting organ damage. The lifespan of sickle erythrocytes is reduced from approximately 120 days to 16-20 days, placing the erythroid system under constant stress. Even with the best medical care, the life expectancy of SCD patients is still reduced by 2-3 decades. Hemoglobinopathies are a worldwide burden on the human population, with over 300,000 new patients born every year. The majority of these (83%) are SCD patients.[2]

Potential cures for hemoglobinopathies

Current standard medical care for thalassemia and SCD patients is aimed at alleviating the symptoms as much as possible. Progress has been impressive and has improved the quality of life for many patients, allowing them, to a large extent, to participate in society like any other individual. Carefully timed transfusion regimens, chelation therapy to remove excess iron from vital organs such as the heart and the liver, and early detection of potential adverse events have greatly contributed to the management of these diseases. However, this level of care is not available to the large majority of patients who live in parts of the world lacking such a sophisticated health care system. Furthermore, the sheer number of patients would be an unbearable burden on any health care system. For example, Nigeria, with a population of 90 million, has an estimated 1-1.5 million SCD patients. Alternative treatments are, therefore, urgently needed. A limited number of patients have been cured by bone marrow transplantation. Clearly, this costly procedure is not available to the large majority of patients, since it requires a compatible donor and life-long follow up after transplantation.

Gene therapy

An attractive alternative would be to correct the defective gene in the patients' cells through the intermediary action of induced pluripotent stem cells (iPS cells). Proof of principle for this approach has already been obtained in a mouse model for SCD.[3] A more straightforward approach is gene addition, in which a missing or corrected globin gene is added to the hematopoietic stem cells of the patients. In the past 25 years, formidable obstacles have been overcome to make gene therapy of hemoglobinopathies a realistic option. Globins must be expressed at very high levels in the erythroid cells, and this requires the addition of carefully selected elements of the *LCR* to the gene therapy vectors. The globin gene itself was also fine-tuned for use in this specific context. For instance,

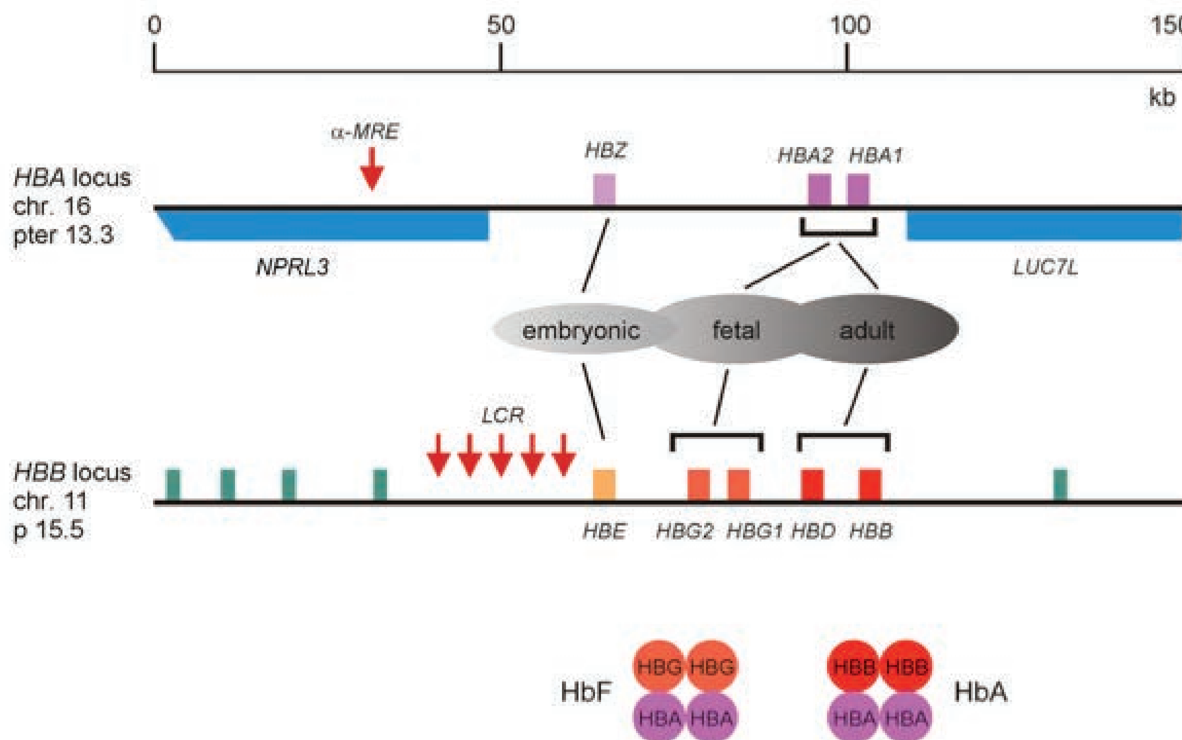
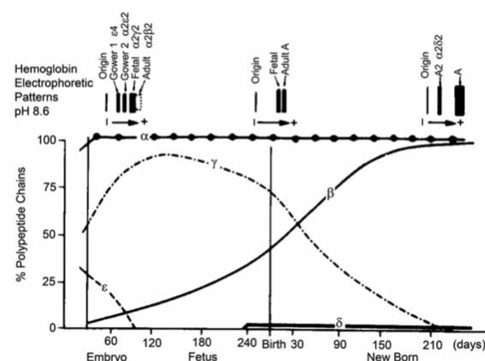


Figure 1. The human hemoglobin *loci*. The *α-like globin* genes (*HBA1* and *HBA2*) are flanked by ubiquitously expressed genes (*NPRL3* and *LUC7L*). The major regulatory element (*α-MRE*) is located upstream of the embryonic ζ-gene (*HBZ*). The *β-like globin* genes are flanked by putative odorant receptor genes (green). The Locus Control Region (*LCR*), comprising 5 DNaseI hypersensitive sites required for high-level expression of the *β-like globin* genes, is located upstream of the embryonic *ε-globin* gene (*HBE*). The developmentally regulated expression patterns and the composition of fetal (HbF, $\alpha_2\gamma_2$ (HBA_2HBG_2)) and adult (HbA, $\alpha_2\beta_2$ (HBA_2HBB_2)) hemoglobin are indicated.

splicing is a requirement for efficient expression and the introns of the globin gene were minimized to meet these demands. While this work was going on, it became clear that gene therapy vectors based on lentiviruses were superior to those based on the classically used γ-retroviruses. Unlike γ-retroviruses, lentiviruses are able to transduce non-dividing cells such as hematopoietic stem cells with high efficiency. The synthesis of all these efforts is the first demonstration of globin gene therapy in mice in 2000,[4] and the first report on a patient treated with a globin gene therapy vector in 2010.[5] While cautious optimism is justified, there are still a number of issues to be solved. Achieving globin expression at therapeutic levels is still challenging, and will require further optimization of the gene therapy vectors. An immediate concern is integration of the gene therapy construct in the genome of the patient's cells, which may interfere with the expression of neighboring genes. In a gene therapy trial for X-SCID, the γ-retroviral vector had integrated in proximity to the promoter of the *LMO2* proto-oncogene, leading to aberrant expression of LMO2 and uncontrolled exponential clonal proliferation of mature T cells.[6] The patient who received globin gene therapy displayed outgrowth of a hematopoietic clone in which the lentiviral gene therapy vector had integrated close to the *HMGA2* gene leading to deregulated expression of this gene.[5] Although no adverse effects of this integration have been reported to date, it raises long-term safety issues. Targeted integration to 'safe harbors', which are apparently genetically empty areas of the genome, might be a step towards solving this problem.[7] It is encouraging that targeted site-specific integration of a globin transgene has recently been demonstrated.[8] This was achieved using zinc finger nucleases engineered to generate a double strand break at a specific location in the genome.[9] In conclusion, significant progress has been made in the development of cellular therapies involving gene correction or addition, and clinical trials of globin gene therapy have started. It is unlikely that such cellular therapies will become available to the large majority of patients within a reasonable time frame.

Reactivation of fetal globin expression

Almost all β-thalassemia and SCD patients will have normal fetal *-globin* genes, which were expressed before birth but switched off during the first year after birth. This gradual change from γ- to β-globin expression is referred to as hemoglobin switching, and 'reversing the switch' is thought to ameliorate the symptoms of β-thalassemia and in particular SCD patients. Higher levels of γ-globin correlate positively with reduced pathology and clinical events in SCD patients. SCD patients with γ-globin levels higher than 20% of total β-like chains are often virtually event free and require little hospital care. The reason is that γ-globin acts as a chain breaker, stopping sickle hemoglobin from forming long polymers under low oxygen conditions. For β-thalassemia patients, reactivation of γ-globin would have to be more quantitative, since it needs to compensate for the absence of β-globin chains. In both cases, γ-globin is a well-known disease modifier and understanding hemoglobin switching at the molecular level has, therefore, been the subject of intense research efforts since the 1970s.



Kitchen, H. and Brett, I. *Annals New York Academy of Sciences* **241**, 653-671 (1974)

Figure 2. Expression of hemoglobins during human development. The developmental expression patterns of the human hemoglobins are shown. (Modified from Kitchen and Brett.[1])

Hereditary persistence of fetal hemoglobin (HPFH)

Sustained expression of HbF in otherwise healthy individuals is termed hereditary persistence of fetal hemoglobin (HPFH). Initially, mutations in the *-globin* locus were found that correlated with increased γ-globin levels. In most cases, this involved deletions of sequences 3' to the *-globin* genes, suggesting removal of repressor elements. Such a function was specifically allocated to the intergenic region between the *A* - and *-globin* genes.[10,11] In some cases, very large deletions were found which are thought to bring novel enhancer elements in close proximity to the *-globin* genes.[12] Of particular interest are point mutations and small deletions that affect γ-globin levels in adults, since these potentially provide mechanistic insight into the hemoglobin switching mechanism. These are confined to the *-globin* promoters and alter expression of the linked gene only, suggesting a direct effect on promoter accessibility in adult erythroid cells. The most common variant is a single nucleotide polymorphism (SNP) C>T at position -158 in the *G* promoter, known as the *XmnI* polymorphism.[13] This is a common sequence variant in all population groups, found at a frequency of 0.32-0.35.[14] Presence of the T allele is associated with increased HbF levels. Quantitatively, a rare variant in the *A* promoter, the -117 G to A mutation, has the most dramatic effect. Heterozygotes display 10-20% HbF, containing only Aγ chains.[15] The HPFH condition was mimicked in mice carrying a human *-globin* locus transgene in which the -117 G to A mutation was introduced.[16] This provided unambiguous evidence that the HPFH phenotype was caused by the point mutation in the *A* promoter. This raised the hypothesis that the -117 G to A mutation would alter direct binding of a regulatory protein to the *A* promoter, either allowing binding of an activator or preventing binding of a repressor, or both. Unfortunately, systematic testing of this hypothesis through the combination of *in vitro* DNA binding assays, introduction of novel point mutations in the -117 area, and functional analysis of these engineered promoters in the transgenic mouse assay consistently failed to identify the factor(s) involved.[17] Similarly, very

interesting models were developed for the -202 C>G or C>T HPFH mutations. This area is capable of forming an intra-molecular triplex termed H-DNA, and the HPFH mutations were shown to destabilize this structure. A 'cold shock' domain protein YBX1 binds specifically to the -202 region, and binding affinity is reduced by the HPFH mutations.[18] However, an impact on globin regulation was not found in *Ybx1* knockout mice,[19] possibly due to functional redundancy with the closely related MSY4 factor. Thus, while the HPFH phenotype could be reproduced in transgenic mice carrying the human β-globin locus and biochemical assays revealed potential molecular mechanisms, unambiguous identification of proteins directly involved in globin switching remained elusive.

The dawn of the "-omics" era

Not all variation in γ-globin expression is due to mutations in the β-globin locus. In rare HPFH families, the phenotype is not linked to the *-globin* cluster or chromosome 11. An early example is provided by a large Indian kindred. Linkage analysis showed that the genetic determinant for HPFH segregated independently from the *-globin* cluster,[20] suggesting involvement of a *trans*-acting factor. Via a painstaking mapping exercise the genomic location of this factor was mapped to chr6q23,[21] and could eventually be pinpointed to variants in the region between the *HBS1L* and *MYB* genes.[22] Recent work has shown that this intergenic region contains distal enhancers required for *MYB* gene activation.[23] The completion of the human genome sequence in 2001[24,25] enabled the development of genome-wide association analysis in population studies (GWAS). Application of GWAS led to the identification of *BCL11A* on chromosome 2p15 as a potential modifier of γ-globin levels.[26,27] Functional studies in primary human erythroid progenitors[28] and mice[29] demonstrated that BCL11A is a transcriptional repressor protein essential for the timing of the transition of fetal-to-adult globin expression. These discoveries have sparked an enormous interest in BCL11A as a target for γ-globin reactivation. This interest was further boosted by the observation that inactivation of BCL11A in the adult erythroid system corrects hematologic and pathological defects in a mouse model of SCD through induction of γ-globin expression.[30] Thus, within the time frame of a few years, BCL11A has been firmly established as the first realistic molecular target for reactivation of γ-globin expression in adults. It should be realized that tinkering with MYB is likely to adversely affect hematopoiesis and erythroid differentiation.[31] Unfortunately, both MYB[32] and BCL11A[33] potentially have been implicated in human malignancy, and mice transplanted with Bcl11a-deficient cells died from T-cell leukemia derived from the host.[34] Erythroid-specific ablation of BCL11A in mice did not result in any oncogenic events,[30,35] indicating that erythroid-specific inactivation of BCL11A in humans might be safe. To achieve this will be challenging; transcription factors such as BCL11A are viewed as highly unattractive drug targets *per se*, even without the confounding requirement for cell type-specific targeting. However, transcription factors perform their functions as part of multi-protein complexes, and BCL11A is known to interact with several other nuclear factors.[28] Identification of an essential partner in erythroid cells may provide a handle on developing pharmacological compounds blocking these protein-protein interactions. Cell type-specific delivery might be achieved through erythroid-specific receptors.[36] Clearly, development of such novel therapeutic approaches will be the topic of intense research efforts in the coming years.

Variants in the *HBB*, *BCL11A* and *HBS1L-MYB* loci together account for approximately 50% of the variation in γ-globin expression.[37] The remaining variation could be accounted for by loci with relatively small impact, and by rare variants with significant quantitative effects on γ-globin expression that are typically missed by GWAS population studies. An example of the latter is provided by the identification of the *KLF1* gene as a γ-globin modifier through the study of a Maltese family in whom HPFH was found in 10 of 27 members.[38] A genome-wide SNP scan followed by linkage analysis identified a candidate region on chromosome 19p13.12–13. Sequencing revealed a nonsense mutation in the *KLF1* gene, p.K288X, which ablated the DNA-binding domain of this key erythroid transcriptional regulator.[39] Only family members with HPFH were heterozygous carriers of this mutation, suggesting that haploinsufficiency for *KLF1* was the cause of the HPFH phenotype. The *KLF1* p.K288X carriers displayed high HbF levels, although with considerable variation (mean 8.4%; range 3.3-19.5%). Part of this variability could be explained by SNP haplotypes at the *BCL11A* locus. Importantly, BCL11A expression was reduced in the *KLF1* p.K288X carriers and KLF1 was shown to be a direct activator of BCL11A expression.[38,40] Knockout studies in mice had previously established that KLF1 is essential to activation of β-globin expression.[41,42] Remarkably, expression of embryonic and fetal *-like globin* genes was fully activated in the absence of KLF1.[43,44] Collectively, this has led to the proposal of the 'double whammy' model[38] (Figure 3). Firstly, KLF1 acts on the *HBB* locus as a preferential activator of the *-globin* gene.[43] Secondly, it activates expression of BCL11A, which in turn represses the *-globin* genes.[28,38,40] This dual activity ensures that, in most adults, HbF levels are less than 1% of total Hb. Notably, MYB is thought to be an activator of KLF1 expression in human adult erythroid progenitors[45] and, therefore, the rough contours of a γ-globin suppression network are appearing.

Mutations in *KLF1* were first described to cause the rare 'inhibitor of Lutheran antigens' (In(Lu)) blood group phenotype,[46] and more recently a steady stream of novel mutations found across different populations has been reported.[47-52] Similar to the observations in the Maltese families, HbF levels associated with *KLF1* mutations were found to be highly variable.[53] This may be explained by the KLF1 expression level derived from the remaining intact *KLF1* allele, but also suggests interplay with other modifier loci such as *BCL11A*.[38] Remarkably, all the mutations reported to date affect the DNA binding domain of KLF1. In many cases, a premature stop codon completely ablates the DNA binding domain, as was the case in the Maltese family. In other cases, amino acid substitutions are found in critical residues of the three zinc fingers comprising the DNA binding domain. These either interfere with the three dimensional structure of the zinc finger domains, or directly affect specificity of DNA target site recognition.[53,54] The latter mutations result in most cases in partially functional proteins. This is illustrated by the

remarkable discovery of two compound heterozygotes for the p.S270X nonsense and p.K332Q missense mutations in a Sardinian family[51] who displayed 22.1% and 30.9% HbF, respectively. HbF levels in the parents were unremarkable. The same study noticed that zinc protoporphyrin levels were increased in these 2 individuals, consistent with the notion that KLF1 regulates several enzymes in the heme synthesis pathway.[55] In separate studies, delayed hemoglobin switching[49] and increased HbA2[50] were reported in heterozygous carriers of *KLF1* mutations. This is clinically important since increased HbA2 (>3.9%) is a distinguishing feature of β-thalassemia carriers. Borderline cases require an extensive laboratory workup to exclude β-thalassemia carrier status; screening for *KLF1* mutations in such cases will aid the identification of couples at risk. One mutation with a dominant phenotype has been reported. The p.E325K missense mutation causes congenital dyserythropoietic anemia.[52] It changes a negatively charged (E-glutamic acid) into a positively charged amino acid (K-lysine) of an absolutely conserved residue in the second zinc finger which is directly involved in DNA sequence recognition.[54] The p.E325K KLF1 mutant has a dominant-negative effect on the transcriptional activation properties of wild-type KLF1. This affected globin expression but also expression of other KLF1 target genes, such as the water channel *AQP1* and the adhesion molecule *CD44*. Remarkably, HbF levels were 37.3% in one patient, who also expressed detectable levels of embryonic hemoglobin (Hb Portland, $\zeta_2\gamma_2$). There are remarkable parallels with the phenotype of the mouse *Nan* mutant, which carries a missense mutation in the corresponding residue in mouse KLF1,[56,57] even though the *Nan* mutation p.E339D does not introduce a positively charged amino acid but leaves the negative charge intact (D-aspartic acid).

Clearly, it will be of great interest to investigate the impact of *KLF1* missense mutations on erythroid gene expression and terminal differentiation at the molecular level. Unlike BCL11A, expression of KLF1 is largely, although not exclusively,[58,59] restricted to erythroid cells and no association of KLF1 mutations with malignancy has been reported. In principle, attenuating KLF1 activity would, therefore, provide a safe approach to raise HbF levels in individuals with β-type hemoglobinopathies. Our recent analysis of compound *KLF1::BCL11A* mouse mutants showed that erythroid-specific ablation of BCL11A, alone or in combination with *KLF1* haploinsufficiency, only mildly affected steady-state erythropoiesis.[35] Furthermore, expression of γ-globin from a single-copy human *-globin* locus was markedly increased in adult mice, lending further support to the role of the KLF1-BCL11A axis in globin switching. An important observation from the mouse studies is that in the complete absence of BCL11A, even in combination with *KLF1* haploinsufficiency, the *-globin* genes are not expressed to the full extent.[30,35] I propose that the tight repression of the *-globin* genes in mice provides a window of opportunity for identification of additional factors involved in the silencing mechanism at the adult stage. Enforcement of repression of the embryonic/fetal program in adult erythropoiesis may be executed by, for instance, the transcription factors MYB[22] and SOX6,[60] the chromatin-bound FOP/CHTOP protein[61] and NuRD complex,[62,63] the orphan nuclear receptors TR2/TR4[64] and the protein arginine methyl transferase PRMT5,[65] and is likely to include additional epigenetic mechanisms such as polycomb group (PcG) complex recruitment and DNA methylation. Future work should, therefore, be aimed at further clarifying the multi-layered repressive network of the embryonic/fetal program in the adult erythroid environment. The first steps to clarify the molecular differences between the developmental stages of erythroid cells have been taken in mouse[66] and human,[67] and have revealed many differentially expressed genes. These data have not yet been

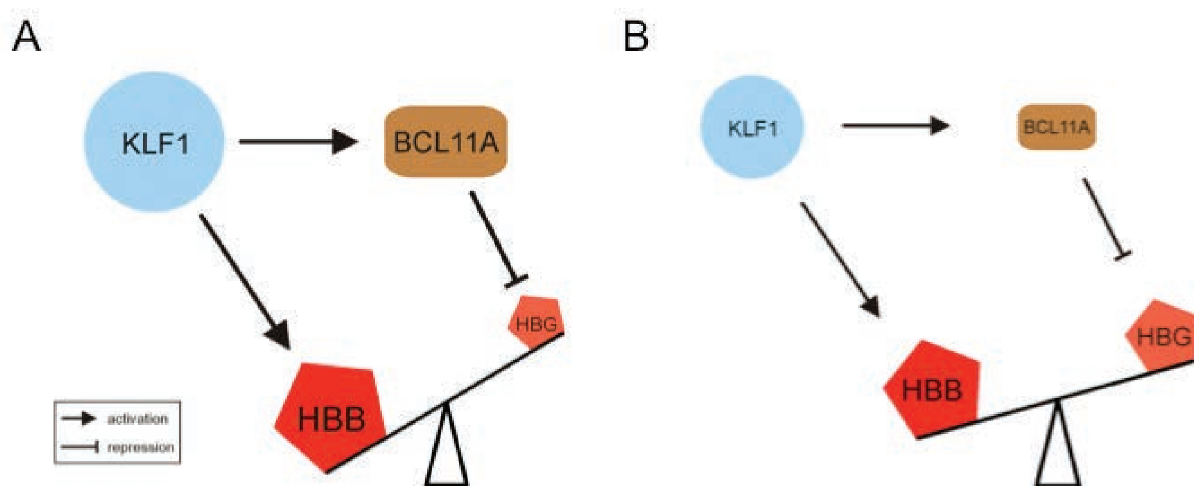


Figure 3. Model for regulation of hemoglobin switching by KLF1 and *BCL11A*. (A) KLF1 preferentially activates the adult *HBB* gene. It also activates the *BCL11A* gene, and the BCL11A protein silences the fetal *HBG1/HBG2* (*HBG*) genes. (B) *KLF1* activity is reduced in member of the Maltese family carrying the KLF1 K288X mutation. This decreases expression of BCL11A and the diminished amount of *BCL11A* protein alleviates repression of the *HBG1/HBG2* genes. The combined reduction of KLF1 and BCL11A activity shifts the balance towards expression of the *HBG1/HBG2* genes.

explored in the context of globin switching. Another recent development is the potential role of micro-RNAs, more specifically micro-RNA-15a and 16-1 which are believed to exert their function via MYB.[68] There are, therefore, many leads that need to be followed up.

Therapeutic reactivation of fetal hemoglobin

The ultimate goal of all these efforts is to develop safe pharmacological compounds targeting the γ-globin suppression pathway specifically. Currently, hydroxyurea,[69] 5-azacytidine[70] and short-chain fatty acids (butyrates)[71] go some way in increasing HbF levels, but none of these agents are specific and long-term safety is a concern. Of these, only hydroxyurea has FDA approval for treatment of SCD patients and it is used with considerable success.[69] Its beneficial effects are only partly due to increased HbF levels; it also reduces cell deformability and improves hydration status of sickle erythrocytes.[72] The majority of patients increase HbF production upon HU treatment;[73] however, HbF baseline and response magnitude among the patients is highly variable. In the 1990s, a screen of pharmacological compounds was conducted by OSI Pharmaceuticals, in which approximately 186,000 defined chemicals and fungal extracts were evaluated.[74] Eleven distinct classes of compounds were identified, many of which activated the stress response suggesting this was part of the mechanism of γ-globin induction. An activated stress response also appears to have a part in the distinction of responders and non-responders to hydroxyurea treatment.[75] Many other compounds with γ-globin inducing properties have been reported in the literature. Resveratrol[76] (a compound found in red wine), anthracyclines,[77] statins[78] and thalidomide derivatives[79] are just a few examples. Drugs already in use for treatment of other conditions, such as statins and thalidomide, are particularly attractive since these could in principle be adapted relatively quickly for therapy for β-thalassemia and SCD. Unfortunately, HbF levels are not routinely determined in patients receiving long-term medication, and hence HbF induction as a side effect is essentially a chance discovery. Collectively, it can be concluded that random screening of compounds has as yet not yielded any alternative to hydroxyurea. It, therefore, appears that better screening systems integrated with approaches designed to directly target the γ-globin suppression pathway should be developed.

Challenges for the future

Recent progress has been fuelled by the application of "-omics" technologies, but seemingly incremental improvements in other laboratory techniques have been equally important. Culture of primary erythroid cells, initially pioneered by Eitan Fibach,[80] were adapted to completely defined synthetic media by the late Hartmut Beug[81,82] (Figure 4). Using a buffy coat from as little as 15 mL of peripheral blood, we can now expand erythroid progenitors from healthy individuals[38] and patients[61] and use these cells for functional experiments. There is no need to select CD34-positive cells, since the majority of the *in vitro* erythroid expansion potential resides in CD34-negative cells.[83] The development of recombinant lentiviruses enabled efficient transduction of these cells, allowing shRNA-mediated knockdown and expression of exogenous proteins.[38,61] Lentiviral shRNA libraries targeting

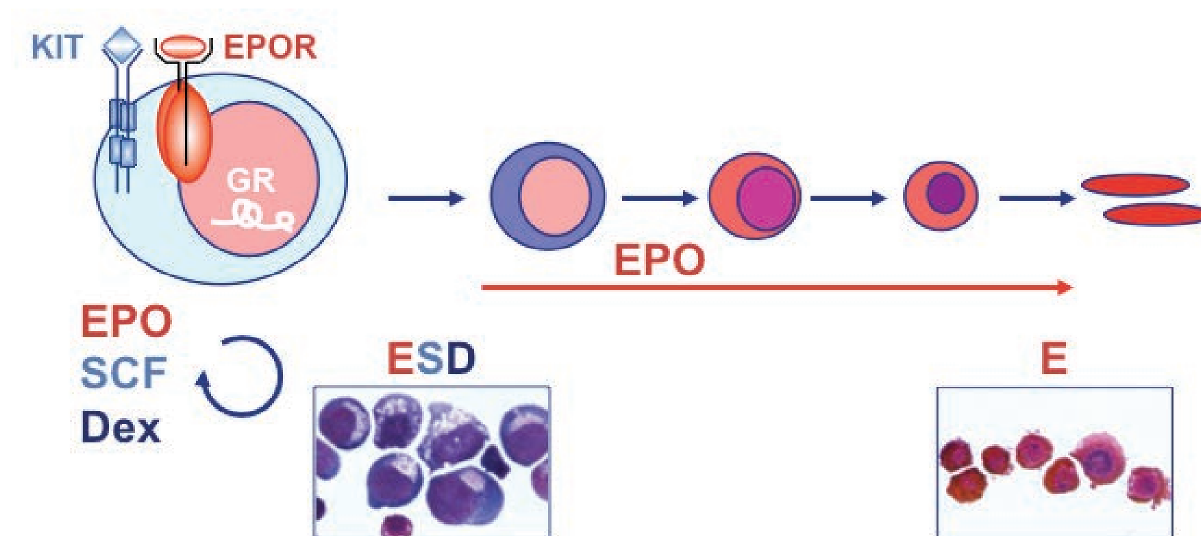


Figure 4. Culture of primary human erythroid progenitors. Erythroid progenitors can be expanded from human peripheral blood mononuclear cells in synthetic medium containing erythropoietin (EPO, ligand for the erythropoietin receptor (EPOR)), stem cell factor (SCF, ligand for KIT) and dexamethasone (Dex, ligand for the glucocorticoid receptor (GR)) as survival and growth factors. After approximately seven days under these conditions (ESD) the cultures are composed predominantly of proerythroblasts. Transfer to medium containing EPO but lacking SCF and Dex (E conditions) forces the proerythroblasts into terminal differentiation, leading to hemoglobinization (brown staining) and enucleation.

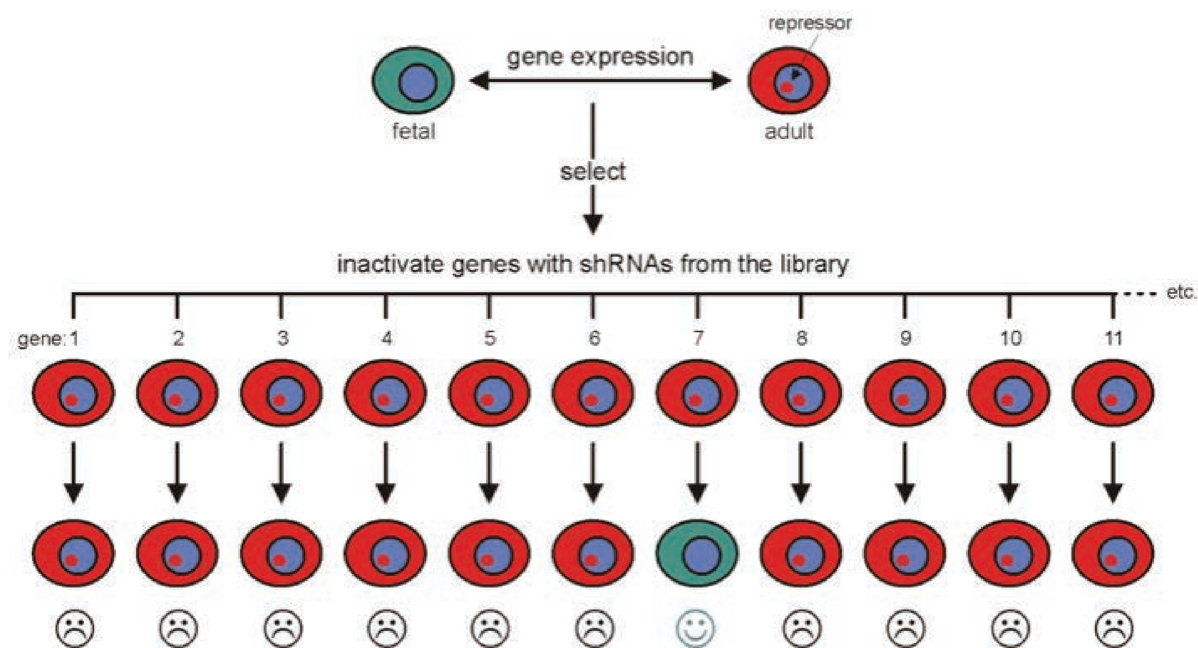


Figure 5. Propsosed shRNA screen for identification of γ-globin repressors. Genome-wide gene expression profiles of cultured human fetal liver- and adult blood-derived proerythroblasts are compared, and genes over-expressed in adult cells (which should include γ-globin suppressors) are selected for lentiviral shRNA-mediated knockdown. Knockdown of a γ-globin repressor should result in increased γ-globin expression.

every protein-encoding gene in the human genome are available[84] and these can be used to identify γ-globin suppressors[28,38,61] (Figure 5). But there is a catch: *in vitro* cultures mimic stress conditions, and human erythroid progenitors respond to this by increasing γ-globin expression. HbF levels of approximately 5% are observed with most cells cultured from healthy donors. A further increase in HbF is easily achieved by applying additional stress to the cells, for instance lentiviral transduction. Typically, HbF levels may reach 30%. It is, therefore, highly recommended to rescue knockdown experiments by expression of shRNA-resistant versions of the genes of interest.[61] In adult mouse erythroid progenitors containing a complete human *-globin* locus, transgene silencing of the *-globin* genes is much tighter.[35] This will, therefore, likely provide a much more stringent system for screening purposes. Building reporter loci in which expression of fluorescent proteins is dependent on activation of the *-globin* genes, and creation of immortalized erythroid progenitor lines[81] from mice carrying such reporter loci, will provide much improved tools for high-throughput screening of γ-globin activating molecules. Such systems are currently being developed.[85,86]

It will remain important to investigate HbF variation in the human population. Currently, no mutations affecting the MYB or BCL11A proteins have been reported, but it would be very interesting to know what the consequences of haploinsufficiency for these factors are. In addition, we have only begun to identify the players in the γ-globin suppression pathway. A locus on chr8q has been associated with HbF in the context of the *XmnI-G* polymorphism,[87] but no causative link to a specific gene on chr8q has been made. We have also largely ignored the potential stromal contribution to hemoglobin switching, even though hemoglobin switching is paralleled by the transition of the site of erythropoiesis from the fetal liver to the bone marrow. More likely than not, the erythroid progenitors will be exposed to very different microenvironments in these two tissues. Identification of extracellular factors that promote the transition from fetal to adult erythropoiesis could be a major step forward in globin switching research. A recent paper implicating reduction of hedgehog signaling with developmental progression of hematopoiesis throughout human ontogeny might provide an example of such a factor.[88] Any factor identified as potentially involved in hemoglobin switching will have to be rigorously tested using conditional knockouts and well-established mouse models for human globin switching[19,29,30,34,35,40,62,63] before they are taken forward as targets for γ-globin reactivation in adults.

References

1. Kitchen H, Brett I. Embryonic and fetal hemoglobin in animals. Ann N Y Acad Sci. 1974;241:653-71.
2. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ. 2008;86(6):480-7.
3. Hanna J, Wernig M, Markoulaki S, et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science. 2007;318(5858):1920-3.
4. May C, Rivella S, Callegari J, et al. Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. Nature. 2000;406(6791):82-6.
5. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature. 2010;467(7313):318-22.
6. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science. 2003;302(5644):415-9.

7. Papapetrou EP, Lee G, Malani N, et al. Genomic safe harbors permit high beta-globin transgene expression in thalassemia induced pluripotent stem cells. Nat Biotechnol. 2011;29 (1):73-8.
8. Chang CJ, Bouhassira EE. Zinc-finger nuclease-mediated correction of alpha-thalassemia in iPS cells. Blood. 2012;120(19):3906-14.
9. Urnov FD, Miller JC, Lee YL, et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature. 2005;435(7042):646-51.
10. Huisman TH, Schroeder WA, Efremov GD, et al. The present status of the heterogeneity of fetal hemoglobin in beta-thalassemia: an attempt to unify some observations in thalassemia and related conditions. Ann NY Acad Sci. 1974;232(0):107-24.
11. Gazouli M, Katsantoni E, Kosteas T, Anagnou NP. Persistent fetal gamma-globin expression in adult transgenic mice following deletion of two silencer elements located 3' to the human Agamma-globin gene. Mol Med. 2009;15(11-12):415-24.
12. Anagnou NP, Perez-Stable C, Gelinas R, et al. Sequences located 3' to the breakpoint of the hereditary persistence of fetal hemoglobin-3 deletion exhibit enhancer activity and can modify the developmental expression of the human fetal A gamma-globin gene in transgenic mice. J Biol Chem. 1995;270(17):10256-63.
13. Gilman JG, Huisman TH. DNA sequence variation associated with elevated fetal G gamma globin production. Blood. 1985;66(4):783-7.
14. Garner C, Tatu T, Game L, Cardon LR, Spector TD, Farrall M, et al. A candidate gene study of F cell levels in sibling pairs using a joint linkage and association analysis. GeneScreen. 2000;1:9-14.
15. Huisman TH, Schroeder WA, Stamatoyannopoulos G, et al. Nature of fetal hemoglobin in the Greek type of hereditary persistence of fetal hemoglobin with and without concurrent beta-thalassemia. J Clin Invest. 1970;49(5):1035-40.
16. Berry M, Grosveld F, Dillon N. A single point mutation is the cause of the Greek form of hereditary persistence of fetal haemoglobin. Nature. 1992;358(6386):499-502.
17. Ronchi A, Berry M, Raguz S, et al. Role of the duplicated CCAAT box region in gamma-globin gene regulation and hereditary persistence of fetal haemoglobin. EMBO J. 1996;15(1):143-9.
18. Horwitz EM, Maloney KA, Ley TJ. A human protein containing a "cold shock" domain binds specifically to H-DNA upstream from the human gamma-globin genes. J Biol Chem. 1994;269(19):14130-9.
19. Lu ZH, Books JT, Ley TJ. YB-1 is important for late-stage embryonic development, optimal cellular stress responses, and the prevention of premature senescence. Mol Cell Biol. 2005;25(11):4625-37.
20. Thein SL, Weatherall DJ. A non-deletion hereditary persistence of fetal hemoglobin (HPFH) determinant not linked to the beta-globin gene complex. Prog Clin Biol Res. 1989;316B:97-111.
21. Garner C, Mitchell J, Hatzis T, Reittie J, Farrall M, Thein SL. Haplotype mapping of a major quantitative-trait locus for fetal hemoglobin production, on chromosome 6q23. Am J Hum Genet. 1998;62(6):1468-74.
22. Thein SL, Menzel S, Peng X, et al. Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. Proc Natl Acad Sci USA. 2007;104(27):11346-51.
23. Stadhouders R, Thongjuea S, Andrieu-Soler C, et al. Dynamic long-range chromatin interactions control Myb proto-oncogene transcription during erythroid development. EMBO J. 2012;31(4):986-99.
24. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. Science. 2001;291(5507):1304-51.
25. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409(6822):860-921.
26. Menzel S, Garner C, Gut I, et al. A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nat Genet. 2007;39(10):1197-9.
27. Uda M, Galanello R, Sanna S, et al. Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. Proc Natl Acad Sci USA. 2008;105(5):1620-5.
28. Sankaran VG, Menne TF, Xu J, et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. Science. 2008;322(5909):1839-42.
29. Sankaran VG, Xu J, Ragoczy T, et al. Developmental and species-divergent globin switching are driven by BCL11A. Nature. 2009;460(7259):1093-7.
30. Xu J, Peng C, Sankaran VG, et al. Correction of sickle cell disease in adult mice by interference with fetal hemoglobin silencing. Science. 2011;334(6058):993-6.
31. Emambokus N, Vegiopoulos A, Harman B, Jenkinson E, Anderson G, Frampton J. Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. EMBO J. 2003;22(17):4478-88.
32. Lahortiga I, De Keersmaecker K, Van Vlierberghe P, et al. Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. Nat Genet. 2007;39(5):593-5.
33. Satterwhite E, Sonoki T, Willis TG, et al. The BCL11 gene family: involvement of BCL11A in lymphoid malignancies. Blood. 2001;98(12):3413-20.
34. Liu P, Keller JR, Ortiz M, et al. Bcl11a is essential for normal lymphoid development. Nat Immunol. 2003;4(6):525-32.
35. Esteghamat F, Gillemans N, Bilic I, et al. Erythropoiesis and globin switching in compound Klf1::Bcl11a mutant mice. Blood. 2013. In press.
36. Kasahara N, Dozy AM, Kan YW. Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. Science. 1994;266(5189):1373-6.
37. Thein SL, Menzel S, Lathrop M, Garner C. Control of fetal hemoglobin: new insights emerging from genomics and clinical implications. Hum Mol Genet. 2009;18(R2):R216-223.
38. Borg J, Papadopoulos P, Georgitsi M, et al. Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. Nat Genet. 2010;42(9):801-5.
39. Siatecka M, Bieker JJ. The multifunctional role of EKLF/KLF1 during erythropoiesis. Blood. 2011;118(8):2044-54.
40. Zhou D, Liu K, Sun CW, Pawlik KM, Townes TM. KLF1 regulates BCL11A expression and gamma- to beta-globin gene switching. Nat Genet. 2010;42(9):742-4.
41. Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosveld F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. Nature. 1995;375(6529):316-8.
42. Perkins AC, Sharpe AH, Orkin SH. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. Nature. 1995;375(6529):318-22.
43. Wijgerde M, Gribnau J, Trimborn T, et al. The role of EKLF in human beta-globin gene competition. Genes Dev. 1996;10(22):2894-902.
44. Perkins AC, Gaensler KM, Orkin SH. Silencing of human fetal globin expression is impaired in the absence of the adult beta-globin gene activator protein EKLF. Proc Natl Acad Sci USA. 1996;93(22):12267-71.
45. Bianchi E, Zini R, Salati S, et al. c-myb supports erythropoiesis through the transactivation of KLF1 and LMO2 expression. Blood. 2010;116(22):e99-110.
46. Singleton BK, Burton NM, Green C, Brady RL, Anstee DJ. Mutations in EKLF/KLF1 form the molecular basis of the rare blood group In(Lu) phenotype. Blood. 2008;112(5):2081-8.
47. Radmilovic M, Zukic B, Petrovic MS, et al. Functional analysis of a novel KLF1 gene promoter variation associated with hereditary persistence of fetal hemoglobin. Ann Hematol. 2013;92(1):53-8.
48. Gallienne AE, Dreau HM, Schuh A, Old JM, Henderson S. Ten novel mutations in the erythroid transcription factor KLF1 gene associated with increased fetal hemoglobin levels in adults. Haematologica. 2012;97(3):340-3.
49. Satta S, Perseu L, Maccioni L, Giagu N, Galanello R. Delayed fetal hemoglobin switching in subjects with KLF1 gene mutation. Blood Cells Mol Dis. 2012;48(1):22-4.
50. Perseu L, Satta S, Moi P, et al. KLF1 gene mutations cause borderline HbA(2). Blood. 2011;118(16):4454-8.
51. Satta S, Perseu L, Moi P, et al. Compound heterozygosity for KLF1 mutations associated with remarkable increase of fetal hemoglobin and red cell protoporphyrin. Haematologica. 2011;96(5):767-70.
52. Arnaud L, Saison C, Helias V, et al. A dominant mutation in the gene encoding the erythroid transcription factor KLF1 causes a congenital dyserythropoietic anemia. Am J Hum Genet. 2010;87(5):721-7.
53. Borg J, Patrinos GP, Felice AE, Philipsen S. Erythroid phenotypes associated with KLF1 mutations. Haematologica. 2011;96(5):635-8.
54. Suske G, Bruford E, Philipsen S. Mammalian SP/KLF transcription factors: bring in the family. Genomics. 2005;85(5):551-6.
55. Drissen R, von Lindern M, Kolbus A, et al. The erythroid phenotype of EKLF-null mice: defects in hemoglobin metabolism

and membrane stability. Mol Cell Biol. 2005;25(12):5205-14.
56. Siatecka M, Sahr KE, Andersen SG, Mezei M, Bieker JJ, Peters LL. Severe anemia in the Nan mutant mouse caused by sequence-selective disruption of erythroid Kruppel-like factor. Proc Natl Acad Sci USA. 2010;107(34):15151-6.
57. Heruth DP, Hawkins T, Logsdon DP, et al. Mutation in erythroid specific transcription factor KLF1 causes Hereditary Spherocytosis in the Nan hemolytic anemia mouse model. Genomics. 2010;96(5):303-7.
58. Porcu S, Manchinu MF, Marongiu MF, et al. Klf1 affects DNase II-alpha expression in the central macrophage of a fetal liver erythroblastic island: a non-cell-autonomous role in definitive erythropoiesis. Mol Cell Biol. 2011;31(19):4144-54.
59. Luo Q, Ma X, Wahl SM, Bieker JJ, Crossley M, Montaner LJ. Activation and repression of interleukin-12 p40 transcription by erythroid Kruppel-like factor in macrophages. J Biol Chem. 2004;279(18):18451-6.
60. Xu J, Sankaran VG, Ni M, et al. Transcriptional silencing of {gamma}-globin by BCL11A involves long-range interactions and cooperation with SOX6. Genes Dev. 2010;24(8):783-98.
61. van Dijk TB, Gillemans N, Pourfarzad F, et al. Fetal globin expression is regulated by Friend of Prmt1. Blood. 2010;116(20):4349-52.
62. Gnanapragasam MN, Scarsdale JN, Amaya ML, et al. p66Alpha-MBD2 coiled-coil interaction and recruitment of Mi-2 are critical for globin gene silencing by the MBD2-NuRD complex. Proc Natl Acad Sci USA. 2011;108(18):7487-92.
63. Costa FC, Fedosyuk H, Chazelle AM, Neades RY, Peterson KR. Mi2beta Is Required for gamma-Globin Gene Silencing: Temporal Assembly of a GATA-1-FOG-1-Mi2 Repressor Complex in beta-YAC Transgenic Mice. PLoS Genet. 2012;8(12):e1003155.
64. Cui S, Kolodziej KE, Obara N, et al. Nuclear receptors TR2 and TR4 recruit multiple epigenetic transcriptional corepressors that associate specifically with the embryonic beta-type globin promoters in differentiated adult erythroid cells. Mol Cell Biol. 2011;31(16):3298-311.
65. Rank G, Cerruti L, Simpson RJ, Moritz RL, Jane SM, Zhao Q. Identification of a PRMT5-dependent repressor complex linked to silencing of human fetal globin gene expression. Blood. 2010;116(9):1585-92.
66. Kingsley PD, Greenfest-Allen E, Frame JM, et al. Ontogeny of erythroid gene expression. Blood. 2012 Dec 12 [Epub ahead of print].
67. Xu J, Shao Z, Glass K, et al. Combinatorial assembly of developmental stage-specific enhancers controls gene expression programs during human erythropoiesis. Dev Cell. 2012;23(4):796-811.
68. Sankaran VG, Menne TF, Scepanovic D, et al. MicroRNA-15a and -16-1 act via MYB to elevate fetal hemoglobin expression in human trisomy 13. Proc Natl Acad Sci USA. 2011;108(4):1519-24.
69. Steinberg MH, McCarthy WF, Castro O, et al. The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: A 17.5 year follow-up. Am J Hematol. 2010;85(6):403-8.
70. Ley TJ, DeSimone J, Anagnou NP, et al. 5-azacytidine selectively increases gamma-globin synthesis in a patient with beta+ thalassemia. N Engl J Med. 1982;307(24):1469-75.
71. Garbutt GJ, Abraham EC. Stimulation of minor fetal hemoglobin synthesis in cord blood reticulocytes by butyrate. Biochem Biophys Res Commun. 1981;98(4):1051-6.
72. Ballas SK, Dover GJ, Charache S. Effect of hydroxyurea on the rheological properties of sickle erythrocytes in vivo. Am J Hematol. 1989;32(2):104-11.
73. Akinsheye I, Alsultan A, Solovieff N, et al. Fetal hemoglobin in sickle cell anemia. Blood. 2011;118(1):19-27.
74. Haley JD, Smith DE, Schwedes J, et al. Identification and characterization of mechanistically distinct inducers of gamma-globin transcription. Biochem Pharmacol. 2003;66(9):1755-68.
75. Pourfarzad F, von Lindern M, Azarkeivan A, et al. Hydroxyurea responsiveness in beta-thalassemic patients is determined by the stress response adaptation of erythroid progenitors and their differentiation propensity. Haematologica. Dec 14 2012. [Epub ahead of print].
76. Fibach E, Prus E, Bianchi N, et al. Resveratrol: Antioxidant activity and induction of fetal hemoglobin in erythroid cells from normal donors and beta-thalassemia patients. Int J Mol Med. 2012;29(6):974-82.
77. Spyrou P, Phylactides M, Lederer CW, et al. Compounds of the anthracycline family of antibiotics elevate human gamma-globin expression both in erythroid cultures and in a transgenic mouse model. Blood Cells Mol Dis. 2010;44(2):100-6.
78. Macari ER, Schaeffer EK, West RJ, Lowrey CH. Simvastatin and t-butylhydroquinone suppress KLF1 and BCL11A gene expression and additively increase fetal hemoglobin in primary human erythroid cells. Blood. 2012 Dec 6 [Epub ahead of print].
79. Moutouh-de Parseval LA, Verhelle D, Glezer E, et al. Pomalidomide and lenalidomide regulate erythropoiesis and fetal hemoglobin production in human CD34+ cells. J Clin Invest. 2008;118(1):248-58.
80. Fibach E, Manor D, Oppenheim A, Rachmilewitz EA. Proliferation and maturation of human erythroid progenitors in liquid culture. Blood. 1989;73(1):100-3.
81. Dolznig H, Kolbus A, Leberbauer C, et al. Expansion and differentiation of immature mouse and human hematopoietic progenitors. Methods Mol Med. 2005;105:323-44.
82. Leberbauer C, Boulme F, Unfried G, Huber J, Beug H, Mullner EW. Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. Blood. 2005;105(1):85-94.
83. van den Akker E, Satchwell TJ, Pellegrin S, Daniels G, Toye AM. The majority of the in vitro erythroid expansion potential resides in CD34(-) cells, outweighing the contribution of CD34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. Haematologica. 2010;95(9):1594-8.
84. Moffat J, Grueneberg DA, Yang X, et al. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell. 2006;124(6):1283-98.
85. Chan KS, Xu J, Wardan H, McColl B, Orkin S, Vadolas J. Generation of a genomic reporter assay system for analysis of gamma- and beta-globin gene regulation. FASEB J. 2012;26(4):1736-44.
86. Papadopoulos P, Gutierrez L, van der Linden R, et al. A Dual Reporter Mouse Model of the Human beta-Globin Locus: Applications and Limitations. PLoS One. 2012;7(12):e51272.
87. Garner CP, Tatu T, Best S, Creary L, Thein SL. Evidence of genetic interaction between the beta-globin complex and chromosome 8q in the expression of fetal hemoglobin. Am J Hum Genet. 2002;70(3):793-9.
88. McIntyre BA, Ramos-Mejia V, Rampalli S, et al. Gli3-mediated hedgehog inhibition in human pluripotent stem cells initiates and augments developmental programming of adult hematopoiesis. Blood. 2013 Jan 4. [Epub ahead of print].

Alpha-thalassemia syndromes: from clinical and molecular diagnosis to bedside management

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A B S T R A C T

Heterozygotes for α^0-and α^+-thalassemias are usually asymptomatic or have microcytic-hypochromic red blood cells. Interactions of α^0-and α^+-thalassemias result in a non-fatal form of alpha-thalassemia syndrome; hemoglobin H (Hb H) disease. Patients with this condition present with a diverse clinical severity, from mild to moderate severity, included in the broader syndrome of non-transfusion dependent thalassemia (NTDT). In general, patients with non-deletional (--/$\alpha^T\alpha$) Hb H are usually more severe than deletional Hb H (--/-α) types. Moreover, certain non-deletional Hb H patients have the most severe phenotype, referred to as Hb H hydrops fetalis. In these rare cases, intrauterine and neonatal complications develop with hydropic features. These patients require regular blood transfusion for survival similar to patients with beta(β)-thalassemia major. Other mechanisms beside imbalanced globin synthesis might influence the Hb H disease pathophysiology resulting in heterogeneous clinical phenotypes. Hb Bart's hydrops fetalis characterized by a complete loss of all α globin loci (--/--) usually leads to death *in utero* or soon after birth. Due to advanced perinatal and neonatal care, the number of surviving Hb Bart's hydrops is increasing, raising concerns regarding the long-term outcome, in particular cognitive and neurological development. Although stem cell transplantation offers a curative measure for these severe α-thalassemia syndromes, its application has been limited by donor availability. Management guidelines for α-thalassemia syndromes are proposed here.

Learning goals

At the conclusion of this activity, participants should be able to:

- provide an understanding of the molecular basis underlying α-thalassemia and how interactions of α-thalassemia genes give rise to syndromes with different clinical phenotypes;
- acquire the ability to clinically diagnose and interpret relevant hematology laboratory and molecular studies in order to correctly identify the different types of α-thalassemia syndromes;
- provide an appropriate management plan, from supportive care, blood transfusion, iron chelation, up to stem cell transplantation, for patients with α-thalassemia syndromes, with special emphasis on Hb H disease.

Introduction

Hemoglobin (Hb) is a tetramer of two α-like and two β-like globin chains which are covalently linked to heme, the oxygen-binding molecule.[1,2] In normal adult erythropoiesis, approximately 95% of the Hb produced is Hb A (α2β2), followed by approximately 2%-3% Hb A2 (α2δ2) and less than 1% fetal Hb (Hb F, α2γ2).[1] The α globin gene cluster is located on the subtelomeric region of the short arm of chromosome 16 (16p 13.3) and consists of four functional genes, from 5' to 3': *HBZ* (OMIM 142310), *HBA2* (OMIM 141850), *HBA1* (OMIM 141800), and *HBQ1* (OMIM 142240).[3] These genes produce ζ, α and θ globin chains, respectively, and are under the control of the upstream regulatory sequences, a multispecies conserved, non-coding regulatory sequence (MCS-R 1 and 2) (see Figure 1 and review in Higgs[4]). The β globin gene cluster is located on chromosome 11 p15.4 and composed of five functional genes, from 5' to 3': *HBE1* (OMIM 142100), *HBG2* (OMIM 142250), *HBG1* (OMIM 142200), *HBD* (OMIM 142000), and *HBB* (OMIM 141900).[1] These genes encode ε, Gγ, Aγ, δ, and β globin chains, respectively. Similar to the α-globin gene cluster, a stage-specific temporal expression of these β-like globin chains is under the control of an upstream regulatory region, known as the β globin locus control region (LCR).[5] During erythroid development, from embryonic to fetal and adult hematopoiesis, a precise co-ordinated expression of both globin clusters is required to generate a balanced and adequate amount of stage-specific hemoglobins required for the red blood cell function. This process is highly critical since 200 billion red blood cells are produced daily to support continuous oxygen flow and supply.[1]

A great deal has been learnt about the normal regulation of globin gene expression from the analysis of naturally occurring mutations of the globin clusters, which cause α and β thalassemia.[6] Over the last 40 years, more than 120 mutations that cause α thalassemia and over 270 mutations that cause β thalassemia

have been characterized.[1] Thalassemia most frequently results from deletions or point mutations which affect the normal structures of the α and β globin genes.[2] These mutations fall into three main groups. First, there are deletions of the structural genes that are a particularly common cause of α thalassemia and, in a few cases, of β thalassemia. Second, point mutations of the structural genes and their critical elements, which in contrast are extremely common in β thalassemia (>220 different mutations) and less common in α thalassemia. Third, rare deletions involving the regulatory elements (MCS-R 1 and 2 and β-LCR, see below).[2] Studies of such natural mutations that can inactivate or severely down-regulate gene expression provide important insights into all aspects of gene structure regulation, including transcription and mRNA processing. The importance of promoter and enhancer elements, the role of upstream and downstream untranslated region (UTR) in mRNA transcription, stabilizing nascent mRNA and the translation process were also derived from studies of thalassemia. These mutations thus generate 'natural models' which help our understanding of globin gene expression.[2]

Molecular basis of α-thalassemia

There are two copies of the α globin gene per haploid genome, annotated αα/αα. The α2 gene lies upstream of the α1 gene and is expressed 2-3 fold more than the α1 gene. Alpha-thalassemia syndromes are remarkable for their variable molecular basis and phenotypic diversity depending on the degree of α globin deficit according to the number of the affected α globin genes.[2-4, 7-9]

In α^0 thalassemia (a condition in which α globin expression from one chromosome is completely abolished), both of the linked α globin genes are lost (--/αα) due to deletions that involve part or the entire α globin gene cluster (Figure 1). Heterozygotes for α^0 thalassemia are clinically normal but have a mild hypochromic, microcytic anemia (mean cell volume, MCV, <78 fL; mean corpuscular hemoglobin, MCH, <27 pg).[10,11] Other molecular mechanisms that can result in a similar degree of the α-globin deficit akin to that of α^0-thalassemia include: 1) upstream deletions that remove the major regulatory elements of the α globin cluster;[12-14] 2) an interstitial deletion (>18 kb, ZF deletion) that removes only the α1 gene but causes *de novo* methylation and downregulation of the remaining α2 gene;[15] and 3) large deletions that extend beyond the α globin gene cluster, identified in patients with dysmorphism and alpha-thalassemia mental retardation syndrome (ATR-16)[16] (shown in Figure 1 and comprehensively reviewed by Higgs[4]).

In the less severe condition (α^+-thalassemia), the α globin expression from one chromosome is reduced but not abolished. There are two types of α^+-thalassemia; deletional α^+ and non-deletional α^+-thalassemia.[4] The high

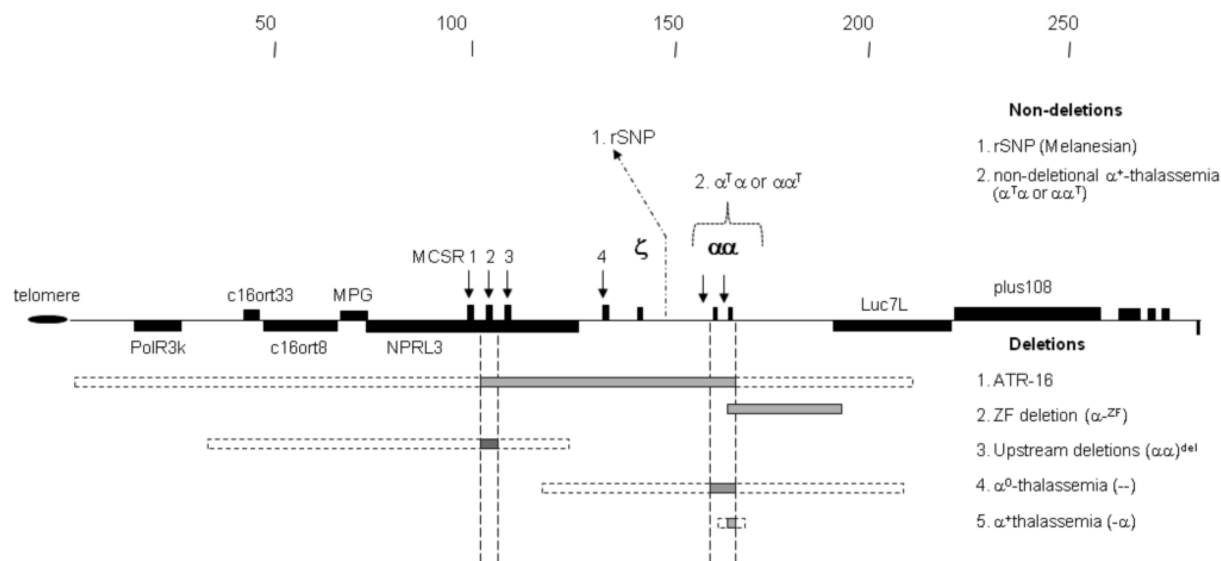


Figure 1. Summary of all reported deletional and non-deletional mutations causing a significant reduction of the α-globin gene expression and α^0- and α^+-thalassemia phenotypes. A schematic diagram of the subtelomeric region (black oval) of chromosome 16 (16p13.3) showing the human α-globin cluster (5'- ζ-α2-α1-3') flanked by ubiquitously expressed genes (as denoted with gene annotations above and below the line by Higgs[4]). Two major molecular mechanisms of α-thalassemia; deletional and non-deletional mutations are demonstrated (see text). Below the line, several gray boxes showed the critical regions removed by different deletions that involved (1). A multispecies conserved non-coding regulatory sequence (MCS-R2) essential for α-globin expression, (2). Single or both linked α-globin genes and (3). A region 3' to the α1 gene that caused epigenetic dysregulation in ZF deletion. ATR-16 is the large deletion that extended beyond the α-globin cluster and removed all critical regions. Above the line, two types of non-deletional mutations are shown; the rSNP; regulatory single nucleotide polymorphism or Melanesian mutation and the conventional nucleotide mutations involving coding sequences of either α2 or α1 genes. Black arrows show erythroid specific hypersensitive sites along the α-globin cluster.

homology of two α globin loci and local chromosomal constraints make the α globin cluster vulnerable to homologous recombination.[17] The majority of α^+-thalassemias results from deletions removing either the α2 gene, leaving the α1 gene intact (-$\alpha^{4.2}$ or 4.2 kb-deletion) or part of both α2 and α1 genes, generating a new hybrid α globin gene (-$\alpha^{3.7}$ or 3.7 kb-deletion) (Figure 1).[4,18] Both types of deletions have been found worldwide with a few others, such as 3.5 kb-deletion, found at a lower incidence.[9]

Less commonly, α^+ thalassemia results from mutations in one or a few nucleotides in critical regions of the α genes usually, but not always, affecting the highly expressed α2 gene ($\alpha^T\alpha$) rather than the α1 gene ($\alpha\alpha^T$). This is called 'non-deletional α thalassemia' and more than 70 different non-deletional mutations have been reported so far (as regularly up-dated at the globin gene server. Available from: http://globin.cse.psu.edu/).[9]

Recently, De Gobbi and Viprakasit *et al.* have described a regulatory single nucleotide polymorphism (rSNP) located in the region in between the ζ and the α2 gene that creates a new GATA binding site as an underlying cause of α-thalassemia in Melanesian population.[14] This rSNP demonstrated a novel mechanism for downregulation of the downstream α globin genes, creation of a GATA site competes with the α-globin promoters in the interaction with the MSC-Rs and has a 'stealing effect' on the associated erythroid specific and basal transcription machinery.[4,14] Heterozygotes for this rSNP have the phenotypes similar to non-deletional α^+-thalassemia. In addition, homozygotes and compound heterozygotes of this rSNP and α^0-or α^+-thalassemia can cause clinical Hb H disease (V Viprakasit, unpublished data, 2008).

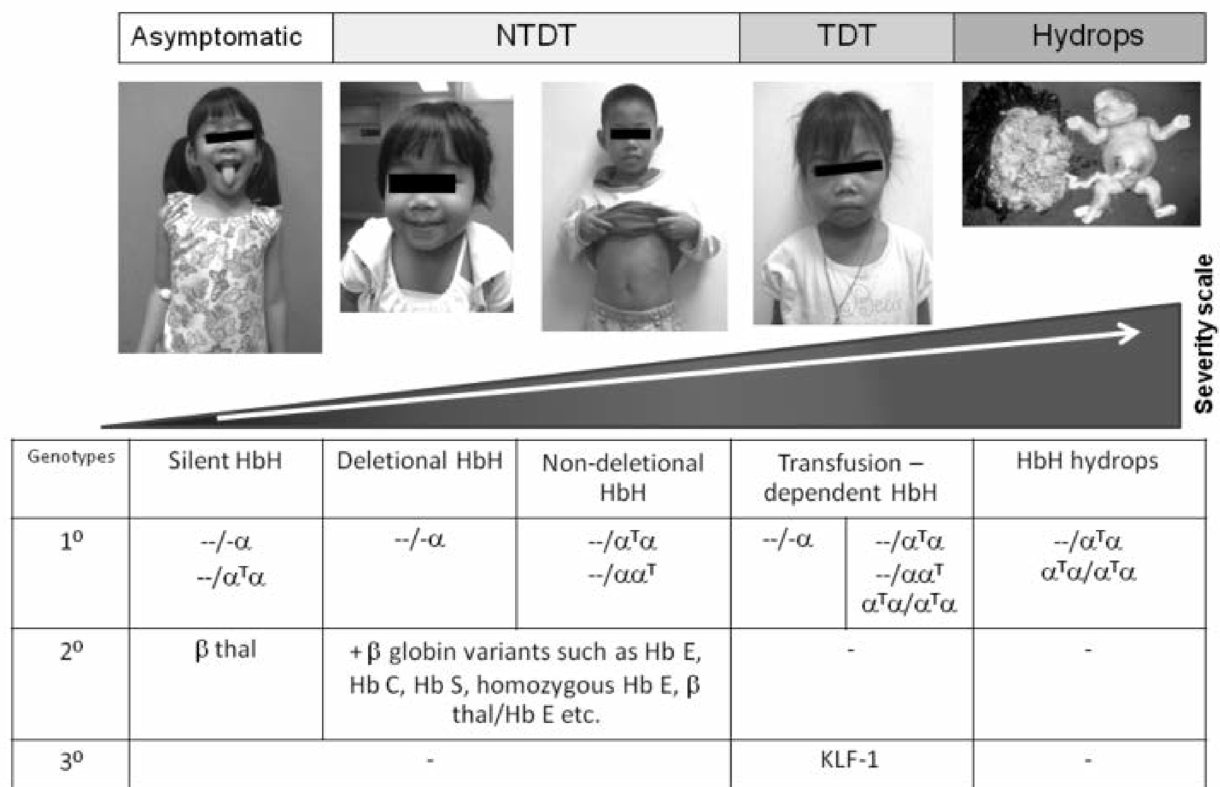
Molecular genotype-phenotype correlation in α-thalassemia syndromes

Hemoglobin (Hb) Bart's hydrops fetalis, characterized by a complete loss of four α-globin genes (--/--), is the most severe form of α-thalassemia syndromes.[8,19,20] The complete deficit of the α-globin chains is caused by deletional loss (homozygote or compound heterozygotes for the different molecular genotypes of α^0 thalassemia. A complete absence of α globin that is critically required for fetal erythropoiesis to produce Hb F (α2γ2) causes intrauterine death of the affected fetus or death soon after birth. The free γ-globin chains in the fetus combine to form tetrameric hemoglobin known as Hb Bart's (γ_4) comprising 100% of total hemoglobin in affected patients. In general, such fetuses survive until the third trimester of pregnancy because they continue to produce small amounts of the embryonic Hbs Portland I (ζ2γ2) and Portland II (ζ2β2). However, at this stage they often have multiple congenital abnormalities and die of heart failure as a result of anemia.[20,21] Moreover, hydropic changes of the fetus can also result in several maternal complications including preeclampsia and hemorrhage.[9] In the past, the majority of Hb Bart's hydrops perished *in utero*. However, there are increasing reports of cases of Hb Bart's hydrops, which, with or without intrauterine intervention, had survived until delivery.[22] These patients require immediate care and effective neonatal resuscitation including blood transfusion during the neonatal period.

A loss of three out of four copies of the α-globin genes (--/-α) due to compound heterozygosity for α^0- and deletional α^+-thalassemia is the most common molecular mechanisms underlying Hb H disease.[2,18,20,23] The excess β-globin chains form tetrameric hemoglobin (β4) called Hb H. This classical form of deletional Hb H disease affects millions of people worldwide due to a high frequency of α-thalassemia alleles.[2,24] However, this condition is quite benign and may require the occasional blood transfusion during hemolytic episodes.[18,25,26] This α-thalassemia syndrome is the most common cause of non-transfusion dependent thalassemia (NTDT) around the world.[27] Interaction of rare mechanisms of α^0-thalassemia described above with α^+-thalassemia can also result in clinical Hb H disease.[13,28] Less commonly, non-deletional Hb H disease (--/$\alpha^T\alpha$ or --/$\alpha\alpha^T$) results from interactions of α^0- and non-deletional α^+-thalassemia. The common non-deletional mutations include; Hb Constant Spring ($\alpha^{CS}\alpha$, termination codon, TAA-CAA),[29] the most prevalent non-deletional α thalassemia identified to date in several countries, Hb Paksé ($\alpha^{PS}\alpha$, another termination codon mutation, TAA-TAT),[30] an initiation codon mutation (ATG to A-G),[31,32] Hb Quong Sze ($\alpha^{QS}\alpha$, codon 125, CTG-CCG)[33] and different types of polyadenylation site of the α 2 gene mutation including the $\alpha^{TSaudi}\alpha$ (AATAAA to AATAAG).[34] Patients with non-deletional Hb H disease have a more severe phenotype than deletional Hb H as demonstrated by the greater degree of anemia and jaundice, earlier presentation, greater degree of hepatosplenomegaly, greater need for blood transfusion, and splenectomy[25,26,35-43] In addition, non-deletional Hb H patients have higher levels of Hb H inclusion bodies and many of the patients with Hb H disease who have transfusion dependent thalassemia (TDT) or are thalassemia major (TM)-like fall into the non-deletional group.[18] The deficit in α globin expression in these patients appears to be greater than in deletional Hb H disease (--/-α). Sometimes, non-deletional mutations have additional deleterious effects on terminal erythroid differentiation and red cell metabolism.[44,45] These effects might include globin instability as in Hb Constant Spring (CS), Hb Paksé (PS), Hb Quong Sze and Hb Adana, $\alpha^{Adana}\alpha$, codon59,GGC-GAC, which results in a more severe phenotype. Other non-deletional mutations, such as those involving the initiation codon or splice site mutation, only reduce α-globin mRNA expression without generating unstable variants, and might not be as severe as the former ones.[46] These findings suggest that a precise molecular characterization will be required to provide appropriate counseling and a management plan for future patients. Nevertheless, there is considerable clinical diversity in both deletional (--/-α) and non-deletional (--/$\alpha^T\alpha$) Hb H disease which remains unexplained.[18,47] Until recently, mutations in erythroid specific transcription factor erythroid krüpple-like factor, EKLF or KLF-1, have been identified in several pedigrees of patients with Hb H disease with unexpectedly severe phenotype.[48] It is plausible that these *trans* acting mutations might play a role as major genetic modifiers in patients with α-thalassemia syndromes. Homozygotes for many types of non-deletional α^+ thalassemia ($\alpha^T\alpha/\alpha^T\alpha$) usually have a mild hypochromic, microcytic anemia with no detectable Hb H on electrophoresis whilst others may have small amounts of Hb H.[49-52] However, homozygotes for a mutation affecting the polyA addition site of the α2 gene, first described

in patients from Saudi Arabia, $\alpha^{TSaudi}\alpha$ consistently have Hb H disease of variable severity and 8.0%-26.6% Hb H detectable on electrophoresis.[53] In one pedigree from Turkey, homozygosity for the $\alpha^{Tsaudi}\alpha$ chromosome led to fetal loss raising questions for the reason for phenotypic discrepancies.[54] Similar unexplained findings were also observed in patients with homozygous Hb CS.[55]

The most severe viable form of α-thalassemia syndromes is Hb H hydrops, a transfusion-dependent Hb H disease that is caused by specific non-deletional α-thalassemia mutations.[34,56-59] The α globin expression is severely reduced but not absent in these rare infants with non-deletional Hb H disease (--/$\alpha^{T}\alpha$), a result being the profound anemia *in utero* (3.4-9.7 g/dL) and hydropic features, with 31-65% Hb Bart's. This clinical syndrome has been seen in patients with rare non-deletional mutations such as; $\alpha^{Cd\ 59Gly\text{-}Asp}\alpha$, $\alpha^{\Delta Cd\ 30}\alpha$,[12] $\alpha^{Cd\ 66\ Leu\text{-}Pro}\alpha$ and $\alpha^{Cd\ 35Ser\text{-}Pro}\alpha$.[57] In multiple affected pedigrees, this interaction resulted in fetal lethality in late gestation or in death in the early neonatal period,[59] whereas the few survivors had a severe transfusion-dependent type of Hb H disease. This suggests that additional environmental and genetic factors may modify the outcome of this clinical syndrome. For example the interaction of Hb Pak Num Po (PNP), a rare α1 gene mutation, with α^{0}-thalassemia (--/) results in transfusion-dependent phenotype[60] while interactions with either deletional (-$\alpha^{4.2}$/) or non-deletional α^{+}-thalassemias ($\alpha^{PS}\alpha$/) causes a non transfusion-dependent phenotype with variable severity.[61] Molecular genotyping of non-deletional α-thalassemia is of clinical importance and should be performed in all severe Hb H patients. Clinical heterogeneity of α-thalassemia syndromes from silent Hb H disease to Hb H hydrops and the associated genetic determinants are summarized in Figure 2.



Genotypes	Silent HbH	Deletional HbH	Non-deletional HbH	Transfusion – dependent HbH		HbH hydrops
1⁰	--/-α --/$\alpha^{T}\alpha$	--/-α	--/$\alpha^{T}\alpha$ --/$\alpha\alpha^{T}$	--/-α	--/$\alpha^{T}\alpha$ --/$\alpha\alpha^{T}$ $\alpha^{T}\alpha/\alpha^{T}\alpha$	--/$\alpha^{T}\alpha$ $\alpha^{T}\alpha/\alpha^{T}\alpha$
2⁰	β thal	+ β globin variants such as Hb E, Hb C, Hb S, homozygous Hb E, β thal/Hb E etc.		-		-
3⁰	-			KLF-1		-

Figure 2. Heterogeneous clinical presentation and severity of patients with α-thalassemia syndromes from Hb H hydrops to silent Hb H disease. The table shows three levels of partially known genetic basis underlying the clinical heterogeneity of Hb H disease. Primary defects are based on the types of α-globin mutations (deletional and non-deletional α-thalassemias) and their interaction. The secondary level of genetic control is the co-inheritance of β-thalassemia[62-64] or β-hemoglobinopathy such as Hb E.[65,66] The presence of β-thalassemia generally causes more balanced globin synthesis resulting in a milder phenotype with possible absence of Hb H (silent Hb H), while inheritance of unstable β-globin variants in particular Hb E or homozygous Hb E causing AE Bart's and EF Bart's disease results in a more severe phenotype than simple deletional Hb H disease.[67] The tertiary level involves other genetic modifiers outside the globin gene clusters. At present, only KLF-1 was found to deteriorate the clinical course of patients with deletional and non-deletional Hb H disease.[48] Other genetic modifiers that might affect other complications such as bone disease (vitamin D receptor gene), iron overload (Hfe and others), jaundice and gall stone formation (UGT1A1 and others) are not shown and were reviewed previously.[18] NTDT: non-transfusion dependent thalassemia; TDT: transfusion-dependent thalassemia.

Diagnosis of α-thalassemia syndromes

Heterozygotes for single α gene deletions (-α/αα) are clinically and hematologically normal and cannot be diagnosed correctly without molecular and DNA studies while α^0-thalassemia traits can be diagnosed using aforementioned MCV and MCH cut offs, but not RBC and RDW values (Figure 3A). Patients with Hb H disease have hypochromic microcytic anemia with reticulocytosis similar to patients with β-thalassemia syndromes. Peripheral blood smear shows numerous target cells, aniso-poikilocytosis with polychromasia mimicking alterations found in β-thalassemia disease (Figure 3B).[18] Of note, patients with Hb H-Hb CS usually have numerous basophilic stippling positive red blood cells.[35] The key diagnostic marker is the presence of Hb H (from <2% to >25% of total Hb) in the peripheral blood that is visualized by using a special staining (brilliant cresyl blue) or by hemoglobin electrophoresis or chromatography.[8] It should be noted that due to the unstable nature of Hb H tetramer, the identification of this Hb species can be jeopardized by the quality and age of the blood samples; old blood or inappropriately stored samples could provide false negative results. Quantitation of Hb H might be problematic on some hemoglobin analysis platforms, such as high performance liquid chromatography (HPLC), since the instrument is not pre-set to detect and quantify Hb H species.[18] A new generation of capillary electrophoresis (CE) is better suited for measuring the amount and detection of Hb H in hemolysate.[68] Ultimately, a molecular diagnosis using DNA testing such as GAP-polymerase chain reaction (GAP-PCR) for common deletional α-thalassemias is

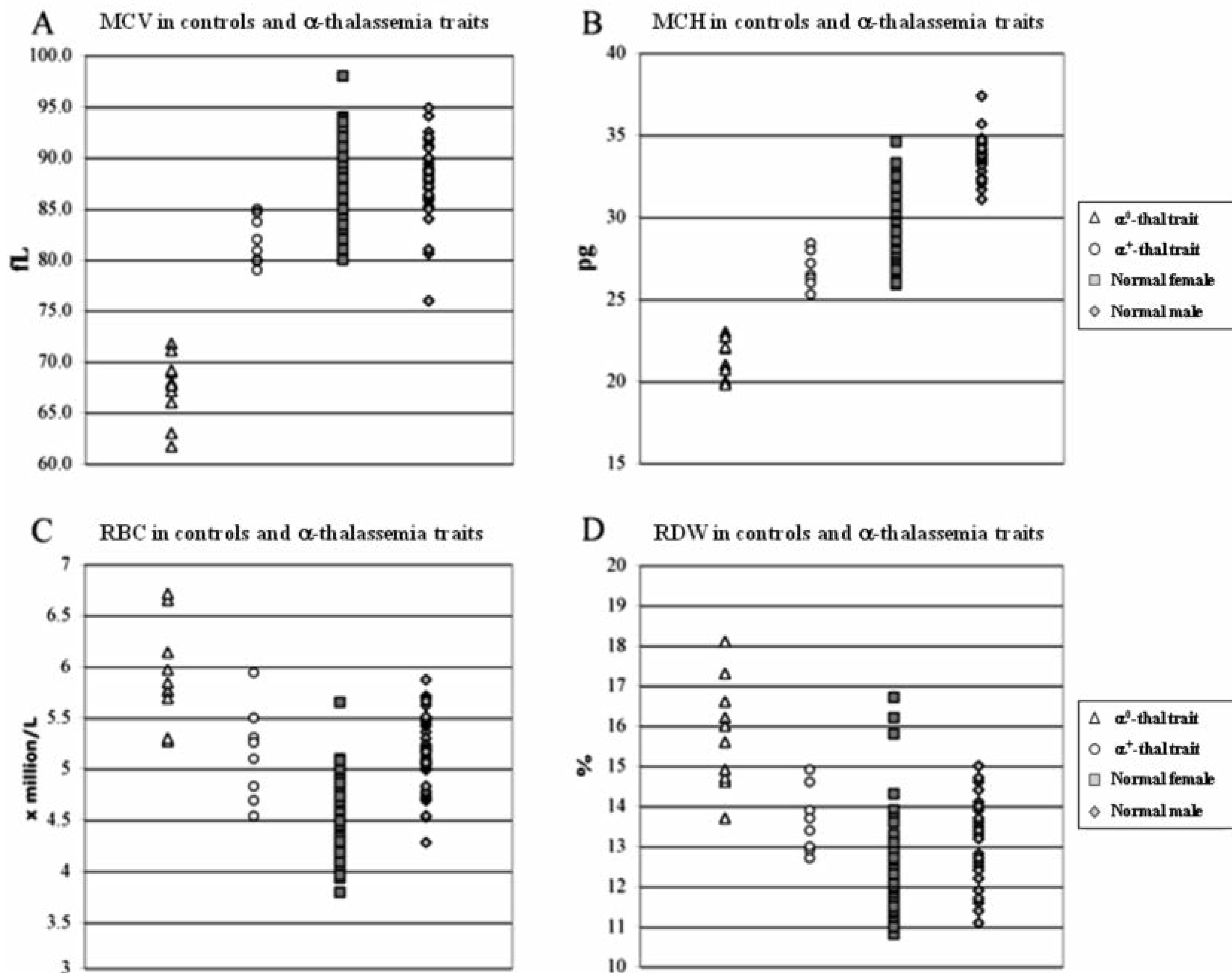


Figure 3A. Red blood cell indexes comparing between α^0-thalassemia traits (--/αα), deletional α^+-thalassemia (-α/αα) traits and normal age-matched controls. Red blood cell indexes composed of mean corpuscular volume (MCV), mean cell hemoglobin (MCH), red blood cell count (RBC), and red cell distribution width (RDW), n=350. Adapted from Viprakasit.[10]

highly recommended[69] in cases that are not easily diagnosed by complete blood count (CBC) and hemoglobin analysis. Detailed molecular techniques to identify α-globin gene defects have recently been reviewed.[9] Moreover, precise molecular characterization of either deletional or non-deletion Hb H disease including the type of the non-deletional mutations can be useful to roughly predict the clinical severity and provide some guidance for clinical management (Figure 4). Patients with non-deletional mutations should be closely followed up every 2-3 months in view of worsening clinical severity with age, while the clinical course in deletional Hb H patients is more stable and a regular follow up on a 4-6 monthly basis may be adequate.

Clinical management of α-thalassemia syndromes

Hb Bart's hydrops fetalis[8,19]

Only a few surviving Hb Bart's hydrops cases have been documented in the literature. It has been suggested that due to marked anemia in early gestation, Hb Bart's hydrops patients could suffer from other physical complications including limb anomalies, abnormal urogenital and most seriously, neurological development.[21,70] Moreover, all rescued Hb Bart's hydrops would be dependent on life-long transfusion. At Siriraj Hospital, Bangkok, Thailand, 5 surviving patients with Hb Bart's

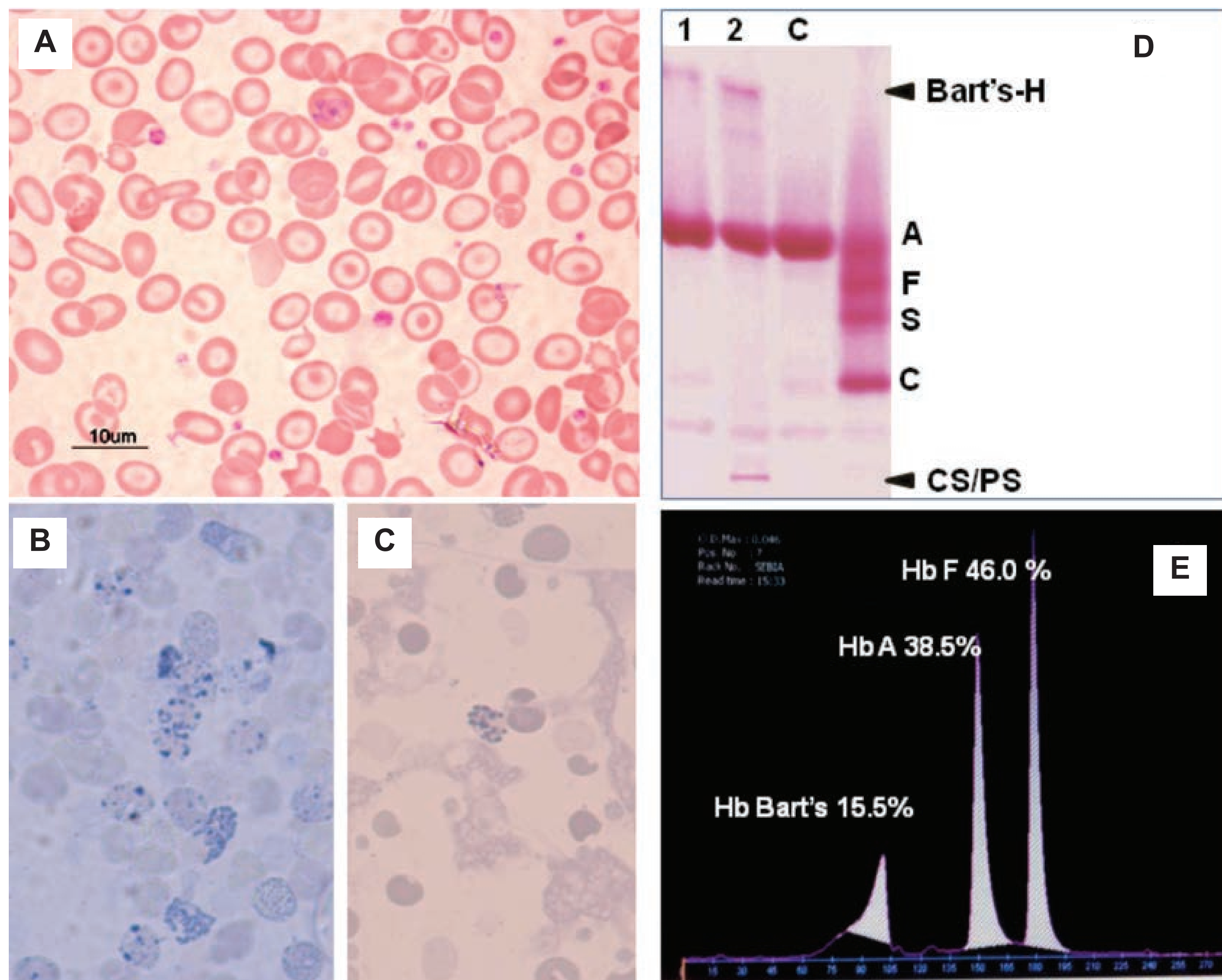


Figure 3B. (A) Peripheral blood smear shows marked hypochromic microcytosis with anisopoikilocytosis and numerous targets and fragmented red blood cells. (B) A supravital staining shows numerous HbH inclusion bodies with a golf-ball appearance in a patient with deletional Hb H ($--^{SEA}/-\alpha^{3.7}$). The presence of Hb H inclusion bodies can be rare in a patient who also co-inherited Hb E (AE Bart's disease; $--^{SEA}/-\alpha^{3.7}/\beta^{E}/\beta^{A}$), as shown in (C). (D) Hemoglobin analysis by cellulose acetate electrophoresis from a patient with deletional Hb H (1; $--^{SEA}/-\alpha^{4.2}$) and non-deletional Hb H (2; $--^{SEA}/\alpha^{CS}\alpha$) with the presence of Hb Bart's and Hb H. Using this analysis, the presence of a slow moving hemoglobin at the end of the strip suggests coinheritance of non-deletional mutation such as Hb Constant Spring or Hb Pakse'.[30] (E) Capillary electrophoresis (CE) of a cord blood sample from a patient with Hb H disease ($--^{SEA}/-\alpha^{3.7}$) at birth. The patient developed severe anemia and neonatal jaundice requiring blood transfusion. Hb Bart's is approximately 15%

hydrops are under regular transfusion. They had a wide range of associated anomalies, in particular of the limbs. However, none had delayed mental and/or neurological development (Vπ Viprakasit, unpublished data, 2013). These possible risks and associated complications must be well known and carefully weighted by physicians and patients' parents when deciding about rescuing affected fetus by intrauterine blood transfusion. Although successful stem cell transplantation in Hb Bart's hydrops has been reported from several centers using different sources of stem cells from matched and mismatched related bone marrow and cord blood to unrelated donors,[71-74] it is important to follow these 'cured' Hb Bart's hydrops patients on a long-term follow up, particularly with regard to their neurological development and cognitive function.

Hb H hydrops/transfusion dependent Hb H disease

Similar to surviving Hb Bart's hydrops patients, patients with Hb H hydrops or severe Hb H disease such as Hb PNP[60,61] or Hb Adana[59] become transfusion-dependent later in life. In contrast to other types of Hb H disease (see below), patients with this severe form of α-thalassemia seldom respond to splenectomy, and surgery should not be provided unless patients show clinical signs of hypersplenism. Regular transfusion with iron chelation therapy similar to protocols used in patients with β-thalassemia major seems a more appropriate treatment for Hb H hydrops. Recently, stem cell transplantation was performed to provide cure in a patient within this category owing to the fact that the transplantation-related morbidity and mortality is rather low, especially when an HLA-matched sibling donor is available.[61]

Hb H disease

In general, patients with Hb H disease have a rather mild anemia. The majority of these patients should receive supplementary folic acid (up to 5 mg/day), multivitamins including vitamin D, antioxidant (vitamin E 10 U/kg/day) and nutritional supplement (calcium and zinc) to support their increased bone marrow activity and increased oxidative stress.[18, 75, 76] However, clinical presentation in patients with Hb H disease can be heterogeneous and some might suffer more from clinical anemia, especially patients with non-deletional Hb H disease.

Patients under six years of age with clinical anemia (Figure 4) should receive regular blood transfusion with appropriate iron chelation therapy similar to those for patients with transfusion-dependent thalassemia (TDT).[77] Splenectomy after six years of age has been proven to be effective in patients with Hb H disease who have moderately severe phenotype,[78] but it is associated with increased risk of thrombosis and vasculopathy in later life. Splenectomy could restore transfusion independence in Hb H disease patients in the long term apart from

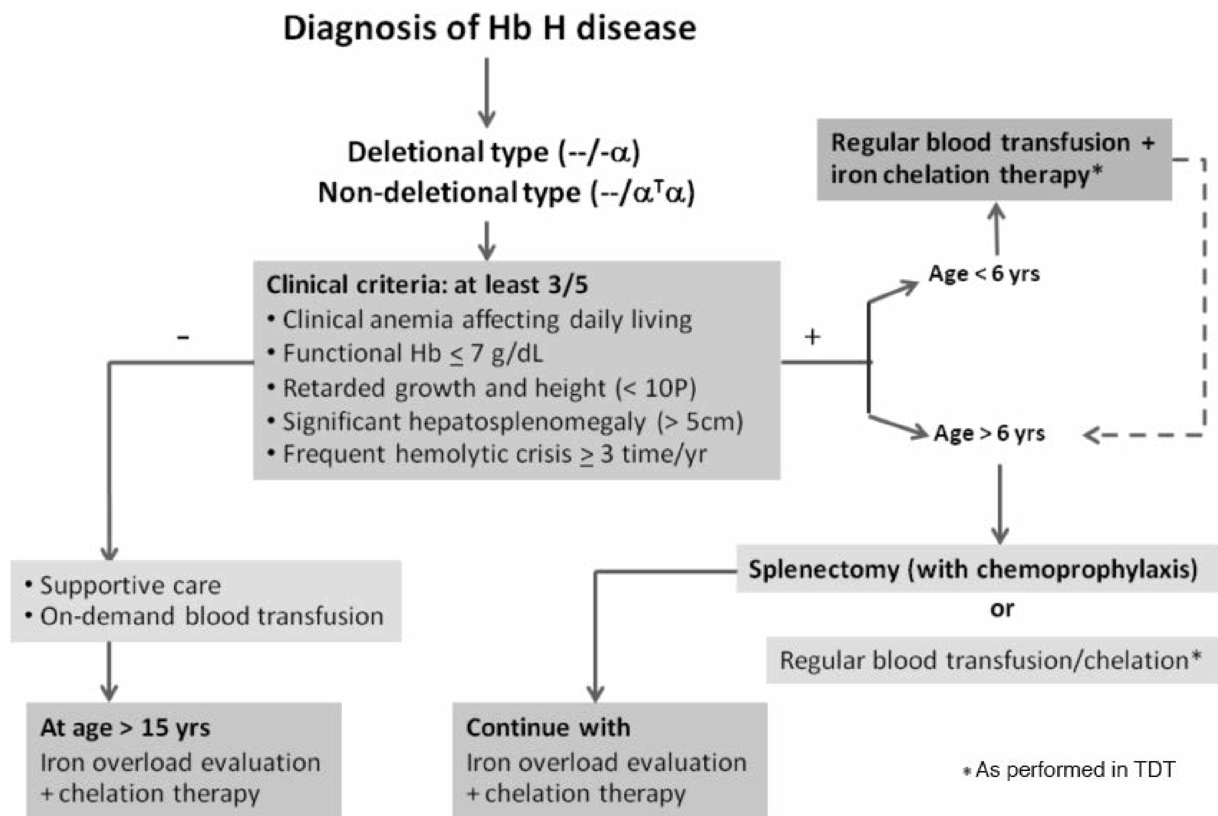


Figure 4. Management guideline in a new patient with Hb H disease. Diagnosis of Hb H disease requires a comprehensive hematology, hemoglobin and DNA analyses. [+] = with ≥3 and [-] = with <3 out of 5 criteria.

instances related to acute hemoglobin reduction during a hemolytic episode. Therefore, in a resource limited setting for a long-term safe blood transfusion support, splenectomy remains a standard of care for selected patients with Hb H disease.[77] This procedure should be performed after appropriate vaccination (pneumococcal and meningococcal vaccines) followed by antibiotic prophylaxis (penicillin V chemoprophylaxis; 250 mg/day twice a day for body weight over 20 kg for at least 3-5 years), anti-platelet drug using aspirin (80 mg/day) and regular vaccination booster every five years.[77]

During hemolytic crisis, the hemoglobin level in Hb H patients may drop significantly. Several factors, including infections and pyrexia (during or after), oxidative challenge, hypersplenism, or pregnancy may contribute to the hemolytic crisis.[79,80] Increased body temperature is the major mechanism generating Hb H inclusion bodies that can induce oxidative damage to the red blood cells and cause further extravascular hemolysis.[81,82] Moreover, if the acute hemolytic crisis is more profound with evidence of severe jaundice, hemoglobinemia and hemoglobinuria, it may result in renal damage and acute renal insufficiency.[83] This serious complication requires immediate intervention. In addition, patients with homozygous non-deletional mutations (especially Hb CS) also develop hemolytic crisis after infection as well.[52] An empirical antibiotic should be started immediately until the causative pathogens are identified. In the tropics, dengue hemorrhagic fever is probably one of the most lethal inter-current infections that cause hemolytic crisis in patients with Hb H disease.[83] Contrary to clinical dengue hemorrhagic fever or dengue shock syndrome in a normal child, Hb H patients have no evidence of hemoconcentration. They would, instead, develop hemolytic crisis that is usually misdiagnosed as gram-negative septicemia.[83] In addition, fragmented red blood cell vesicles from hemolysis can cause an erroneous count of the platelets when an automated cell counter is used, resulting in a delayed detection of thrombocytopenia.[83] More significantly, patients with evidence of poor tissue oxygenation or hypoxia must receive supportive pre-storage filtered blood transfusion at the amount of 5-12 ml/kg/dose that should be repeated if the hemolysis continues. Adequate intravenous hydration with urine alkalinization is recommended to prevent possible kidney damage from the precipitation of hemoglobin passing through the renal glomerular and tubule structures. Details of management of acute hemolysis in Hb H disease have been described previously.[18]

Iron overload may develop in Hb H disease.[25,43] However, due to milder anemia, less transfusion than in other NTDT genotypes and a lower level of ineffective erythropoiesis, iron overload in Hb H disease develops at a much slower rate.[43] Therefore, it is rare to find patients with significant iron overload before 15 years of age, except patients who have received regular or frequent blood transfusion supports (Figure 4). As in other non-transfusion-dependent thalassemias, single measurements of serum ferritin can underestimate the total body iron store in Hb H disease. Therefore, direct monitoring using magnetic resonance imaging (MRI)-evaluation is the approach of choice.[84] The use of liver biopsy to assess iron overload in Hb H patients is not recommended due to procedure-related complications and a possible bias of sampling error unless an open biopsy can be acquired during splenectomy. Once iron overload is detected, it should be treated and monitored using the same recommendation as for other types of NTDT patients.[85]

Prevention and control for severe α-thalassemia syndromes

Due to fetal lethality at mid-gestation and predisposition of the mothers to several obstetric complications including hypertension and antenatal hemorrhage,[86] a prevention and control program for Hb Bart's hydrops fetalis is now operative in Asian countries such as China and Thailand.[87-90] Through the program, carriers for α^0-thalassemia are detected at antenatal care level using a screening by osmotic fragility (OF) or MCV and MCH values.[64] A correct genotype of α^0-thalassemia will be further confirmed by DNA analysis. However, identification of α^0-thalassemia can be complicated by co-inheritance of β-thalassemia traits. Therefore, it is highly recommended to perform a combination of hemoglobin analysis and a common set of α-thalassemia genotype by DNA study as confirmation tests in individuals who come from a region with high prevalence of both α and β thalassemia. This approach can prevent a possible error by missing correct α and β globin genotypes in these individuals and successfully identify couples at risk for producing infants affected with Hb Bart's hydrops.[64] Prenatal diagnosis of Hb Bart's hydrops can be achieved by DNA analysis of chorionic villous samples or cord blood hemoglobin analysis by cordocenthesis.[88,91] For couples who would like to avoid prenatal diagnosis and a termination of pregnancy with affected fetus, an assisted *in vitro* reproduction with embryo selection after pre-implantation genetic diagnosis (PGD) for Hb Bart's hydrops is now available with modest success rates (< 30%) due to allelic drop-out and low pregnancy rate.[92] However, this technology still has to be improved and confirmation by prenatal diagnosis of this assisted pregnancy is still recommended.

As Hb H disease is generally mild and does not require life-long blood transfusion, a prenatal diagnosis for both common deletional and non-deletional types might not be ethical and is not recommended. However, concerning the rare non-deletional α-thalassemias mentioned above, a prenatal diagnosis for couples at risk of Hb H hydrops or transfusion dependent Hb H should be offered, in particular to those families with previously affected offspring. Nevertheless, it remains a challenge to provide such a service to a new couple since the molecular characterization of these rare non-deletional mutations is not routinely performed nor is it widely available. In addition, heterozygotes for these non-deletional mutations have normal or borderline MCV and MCH and simply might not be diagnosed without DNA studies.[18]

Summary

A definitive diagnosis of the disease-causing mutations in α-thalassemia syndromes is important for disease management and genetic counseling. Patients with severe α-thalassemia syndromes such as Hb H hydrops should be treated with regular blood transfusion with appropriate iron chelation therapy. Stem cell transplantation as cura-

tive therapy should be offered if a matched donor is available. The majority of patients with Hb H disease can do well using only supportive care with 'on demand' blood transfusion. Splenectomy should be reserved to more severely affected patients; in particular, to those with nondeletional Hb H disease. Couples at risk of having an affected child with severe form of α-thalassemia syndromes, such as Hb Bart's hydrops fetalis and Hb H hydrops/transfusion dependent Hb H, should be offered genetic counseling and an informed choice on reproductive options, including prenatal diagnosis, which involves fetal sampling to determine the fetal genotype. In addition, assisted reproductive technology that combines preimplantation genetic diagnosis (PGD) with *in vitro* fertilization (IVF) may help parents who have thalassemia or who are carriers of a severe defective α globin gene to give birth to healthy babies by embryo selection.

References

1. Weatherall DJ, Clegg JB. (eds.) The Thalassaemia Syndromes (4th ed.). Oxford, United Kingdom: Blackwell Science, 2001.
2. Higgs DR, Engel JD, Stamatoyannopoulos G. Thalassaemia. Lancet. 2012;379:373-83.
3. Higgs DR, Garrick D, Anguita E, et al. Understanding alpha-globin gene regulation: Aiming to improve the management of thalassemia. Ann N Y Acad Sci. 2005;1054:92-102.
4. Higgs DR. The molecular basis of alpha-thalassemia. Cold Spring Harb Perspect Med. 2013;3:a011718.
5. Engel JD, Tanimoto K. Looping, linking, and chromatin activity: new insights into beta-globin locus regulation. Cell. 2000;100:499-502.
6. Cohen AR, Galanello R, Pennell DJ, Cunningham MJ, Vichinsky E. Thalassemia. Hematology Am Soc Hematol Educ Program. 2004:14-34.
7. Higgs DR, Sharpe JA, Wood WG. Understanding alpha globin gene expression: a step towards effective gene therapy. Semin Hematol. 1998;35:93-104.
8. Higgs D. Clinical and laboratory features of the a thalassemia syndrome. In: Bowden D, Steinberg M, Forget B, Higgs D, Nagel R, (eds.) Disorders of hemoglobin. Cambridge; 2001:431-69.
9. Harteveld CL, Higgs DR. Alpha-thalassaemia. Orphanet J Rare Dis. 2010;5:13.
10. Viprakasit V. Pitfalls in laboratory diagnosis of thalassemia in Thailand. In: Prayoonwiwat V ed. Annual Conference of Thailand Society of Hematology-2009. Bangkok: Thailand Society of Hematology; 2009:15-20.
11. Viprakasit V, Limwongse C, Sukpanichnant S, et al. Problems in determining thalassemia carrier status in a program for prevention and control of severe thalassemia syndromes: A lesson from Thailand. Clinical Chem Lab Med. 2013 Mar 23. [Epub ahead of print].
12. Liebhaber SA, Griese EU, Weiss I, et al. Inactivation of human alpha-globin gene expression by a de novo deletion located upstream of the alpha-globin gene cluster. Proc Natl Acad Sci USA. 1990;87:9431-5.
13. Viprakasit V, Kidd AM, Ayyub H, Horsley S, Hughes J, Higgs DR. De novo deletion within the telomeric region flanking the human alpha globin locus as a cause of alpha thalassaemia. Br J Haematol. 2003;120:867-75.
14. De Gobbi M, Viprakasit V, Hughes JR, et al. A regulatory SNP causes a human genetic disease by creating a new transcriptional promoter. Science. 2006;312:1215-7.
15. Barbour VM, Tufarelli C, Sharpe JA, et al. alpha-thalassemia resulting from a negative chromosomal position effect. Blood. 2000;96:800-7.
16. Horsley SW, Daniels RJ, Anguita E, et al. Monosomy for the most telomeric, gene-rich region of the short arm of human chromosome 16 causes minimal phenotypic effects. Eur J Hum Genet. 2001;9:217-25.
17. Bishop AJ, Schiestl RH. Homologous recombination as a mechanism for genome rearrangements: environmental and genetic effects. Hum Mol Genet. 2000;9:2427-334.
18. Fucharoen S, Viprakasit V. Hb H disease: clinical course and disease modifiers. Hematology Am Soc Hematol Educ Program. 2009:26-34.
19. Chui DH, Waye JS. Hydrops fetalis caused by alpha-thalassemia: an emerging health care problem. Blood. 1998;91:2213-22.
20. Chui DH. Alpha-thalassemia: Hb H disease and Hb Barts hydrops fetalis. Ann N Y Acad Sci. 2005;1054:25-32.
21. Chitayat D, Silver MM, O'Brien K, et al. Limb defects in homozygous alpha-thalassemia: report of three cases. Am J Med Genet. 1997;68:162-7.
22. Sohan K, Billington M, Pamphilon D, Goulden N, Kyle P. Normal growth and development following in utero diagnosis and treatment of homozygous alpha-thalassaemia. Bjog. 2002;109:1308-10.
23. Chui DH, Fucharoen S, Chan V. Hemoglobin H disease: not necessarily a benign disorder. Blood. 2003;101:791-800.
24. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ. 2008;86:480-7.
25. Chen FE, Ooi C, Ha SY, et al. Genetic and clinical features of hemoglobin H disease in Chinese patients. N Engl J Med. 2000;343:544-50.
26. Laosombat V, Viprakasit V, Chotsampancharoen T, et al. Clinical features and molecular analysis in Thai patients with HbH disease. Ann Hematol. 2009;88:1185-92.
27. Galanello R. Recent advances in the molecular understanding of non-transfusion-dependent thalassemia. Blood Rev. 2012;26 Suppl 1:S7-S11.
28. Wenning MR, Harteveld CL, Giordano PC, et al. Hemoglobin H disease resulting from the association of the - alpha 3.7 rightward deletion and the (alpha alpha)MM deletion in a Brazilian patient. Eur J Haematol. 2002;69:179-81.
29. Clegg JB, Weatherall DJ, Milner PF. Haemoglobin Constant Spring—a chain termination mutant? Nature. 1971;234:337-40.
30. Viprakasit V, Tanphaichitr VS, Pung-Amritt P, et al. Clinical phenotypes and molecular characterization of Hb H-Pakse disease. Haematologica. 2002;87:117-25.
31. Waye JS, Eng B, Patterson M, Chui DH, Nisbet-Brown E, Olivieri NF. Novel mutation of the alpha 2-globin gene initiation codon (ATG—>A-G) in a Vietnamese girl with Hb H disease. Hemoglobin. 1997;21:469-72.
32. Viprakasit V, Chinchang W, Glomglao W, Tanphaichitr VS. A rare association of alphaO-thalassemia (—SEA) and an initiation codon mutation (ATG—>A-G) of the alpha2 gene causes Hb H disease in Thailand. Hemoglobin. 2005;29:235-40.
33. Sura T, Trachoo O, Viprakasit V, et al. Hemoglobin H disease induced by the common SEA deletion and the rare hemoglobin Quong Sze in a Thai female: longitudinal clinical course, molecular characterization, and development of a PCR/RFLP-based detection method. Ann Hematol. 2007;86:659-63.
34. Viprakasit V, Green S, Height S, Ayyub H, Higgs DR. Hb H hydrops fetalis syndrome associated with the interaction of two common determinants of alpha thalassaemia (—MED/(alpha)TSaudi(alpha)). Br J Haematol. 2002;117:759-62.
35. Fucharoen S, Winichagoon P, Pootrakul P, Piankijagum A, Wasi P. Differences between two types of Hb H disease, alpha-thalassemia 1/alpha-thalassemia 2 and alpha-thalassemia 1/Hb constant spring. Birth Defects Orig Artic Ser. 1987;23:309-15.
36. Galanello R, Aru B, Dessi C, et al. HbH disease in Sardinia: molecular, hematological and clinical aspects. Acta Haematol. 1992;88:1-6.
37. Liang R, Liang S, Jiang NH, et al. Alpha and beta thalassaemia among Chinese children in Guangxi Province, P.R. China: molecular and haematological characterization. Br J Haematol. 1994;86:351-4.
38. Oner C, Gurgey A, Oner R, et al. The molecular basis of Hb H disease in Turkey. Hemoglobin. 1997;21:41-51.
39. Kanavakis E, Papassotiriou I, Karagiorga M, et al. Phenotypic and molecular diversity of haemoglobin H disease: a Greek experience. Br J Haematol. 2000;111:915-23.
40. Mirabile E, Samperi P, Di Cataldo A, Poli A, La Spina M, Schiliro G. Phenotype-genotype correlation in Sicilian patients with Hb H. Eur J Haematol. 2000;65:306-9.
41. Waye JS, Eng B, Patterson M, et al. Hemoglobin H (Hb H) disease in Canada: molecular diagnosis and review of 116 cases. Am J Hematol. 2001;68:11-5.
42. Khan SN, Hasan F, Sollaino C, Perseu L, Riazuddin S. Molecular characterization of alpha-thalassemia in Pakistan. Hemoglobin. 2003;27:161-66.
43. Lal A, Goldrich ML, Haines DA, Azimi M, Singer ST, Vichinsky EP. Heterogeneity of hemoglobin H disease in childhood. N Engl J Med. 2011;364:710-8.
44. Bunyaratvej A, Fucharoen S, Greenbaum A, Mohandas N. Hydration of red cells in alpha and beta thalassemias differs. A useful approach to distinguish between these red cell phenotypes. Am J Clin Pathol. 1994;102:217-22.

45. Schrier SL, Bunyaratvej A, Khuhapinant A, et al. The unusual pathobiology of hemoglobin constant spring red blood cells. Blood. 1997;89:1762-9.
46. Viprakasit V, Ekwattanakit S, Chalaow N, et al. Clinical presentation and molecular identification of four uncommon a-globin variants in Thailand: initiation codon mutation of α2-globin gene (HBA2:c.1delA), donor splice site mutation of α1-globin gene (IVSI-1, HBA1:c.95+1G>A), Hemoglobin Queens Park/Chao Pra Ya (HBA1:c.98T>A) and Hemoglobin Westmead (HBA2:c.369C>G). Acta Haematol. 2013. In press.
47. Singer ST, Kim HY, Olivieri NF, et al. Hemoglobin H-constant spring in North America: an alpha thalassemia with frequent complications. Am J Hematol. 2009;84:759-61.
48. Viprakasit V. Variable clinical phenotypes and management outcomes in patients with KLF-1 mutations. In: Engle D, Higgs D, Stamatoyannopoulos G. (eds.) 18th Annual Conference on Hemoglobin Switching. Monterey: University of Michigan; 2012:21.
49. Derry S, Wood WG, Pippard M, et al. Hematologic and biosynthetic studies in homozygous hemoglobin Constant Spring. J Clin Invest. 1984;73:1673-82.
50. Pootrakul P, Winichagoon P, Fucharoen S, Pravatmuang P, Piankijagum A, Wasi P. Homozygous haemoglobin Constant Spring: a need for revision of concept. Hum Genet. 1981;59:250-5.
51. Krishnamurti L, Little JA. Homozygous hemoglobin constant spring with normal electrophoresis. A possible cause for underdiagnosis. Ann N Y Acad Sci. 1998;850:415-9.
52. Viprakasit V, Veerakul G, Sanpakit K, Pongtanakul B, Chinchang W, Tanphaichitr VS. Acute haemolytic crisis in a Thai patient with homozygous haemoglobin Constant Spring (Hb CS/CS): a case report. Ann Trop Paediatr. 2004;24:323-8.
53. Thein SL, Wallace RB, Pressley L, Clegg JB, Weatherall DJ, Higgs DR. The polyadenylation site mutation in the alpha-globin gene cluster. Blood. 1988;71:313-9.
54. Curuk MA, Kilinc Y, Evruke C, Ozgunen FT, Aksoy K, Yuregir GT. Prenatal diagnosis of Hb H disease caused by a homozygosity for the alpha2 poly A (AATAAA—>AATAAG) mutation. Hemoglobin. 2001;25:255-8.
55. Charoenkwan P, Sirichotiyakul S, Chanprapaph P, et al. Anemia and hydrops in a fetus with homozygous hemoglobin constant spring. J Pediatr Hematol Oncol. 2006;28:827-30.
56. Chan V, Chan VW, Tang M, Lau K, Todd D, Chan TK. Molecular defects in Hb H hydrops fetalis. Br J Haematol. 1997;96:224-8.
57. Lorey F, Charoenkwan P, Witkowska HE, et al. Hb H hydrops foetalis syndrome: a case report and review of literature. Br J Haematol. 2001;115:72-8.
58. Ma SK, Chan AY, Chiu EK, Chan LC. Haemoglobin H disease due to (—SEA) alpha-globin gene deletion and alpha2-codon 30 (DeltaGAG) mutation: a family study. Clin Lab Haematol. 2001;23:325-7.
59. Nainggolan IM, Harahap A, Setianingsih I. Hydrops fetalis associated with homozygosity for Hb Adana [alpha59(E8)Gly—>Asp (alpha2)]. Hemoglobin. 2010;34:394-401.
60. Viprakasit V, Tanphaichitr VS, Veerakul G, et al. Co-inheritance of Hb Pak Num Po, a novel alpha1 gene mutation, and alpha0 thalassemia associated with transfusion-dependent Hb H disease. Am J Hematol. 2004;75:157-63.
61. Sanpakit K, Viprakasit V. Management for transfusion dependent Hb H disease; from regular transfusion to a successful hematopoietic stem cell transplantation. J Led Hematol Oncol. 2013. In press.
62. Traeger-Synodinos J, Papassotiriou I, Vrettou C, Skarmoutsou C, Stamoulakatou A, Kanavakis E. Erythroid marrow activity and functional anemia in patients with the rare interaction of a single functional a-globin and beta-globin gene. Haematologica. 2001;86:363-7.
63. Ma ES, Chan AY, Au WY, Yeung YM, Chan LC. Diagnosis of concurrent hemoglobin H disease and heterozygous beta-thalassemia. Haematologica. 2001;86:432-3.
64. Viprakasit V, Limwongse C, Sukpanichnant S, et al. Problems in determining thalassemia carrier status in a program for prevention and control of severe thalassemia syndromes: A lesson from Thailand. Clinical Chem Lab Med. 2013;23:1-10.
65. Thonglairuam V, Winichagoon P, Fucharoen S, Wasi P. The molecular basis of AE-Bart's disease. Hemoglobin. 1989;13:117-24.
66. Viprakasit V, Tanphaichitr VS. Unusual phenotype of Hemoglobin EE with Hemoglobin H disease: A pitfall in clinical diagnosis and genetic counseling. J Pediatr. 2004;144:391-3.
67. Fucharoen S, Winichagoon P, Thonglairuam V, Wasi P. EF Bart's disease: interaction of the abnormal alpha- and beta-globin genes. Eur J Haematol. 1988;40:75-8.
68. Winichagoon P, Svasti S, Munkongdee T, et al. Rapid diagnosis of thalassemias and other hemoglobinopathies by capillary electrophoresis system. Transl Res. 2008;152:178-84.
69. Tan AS, Quah TC, Low PS, Chong SS. A rapid and reliable 7-deletion multiplex polymerase chain reaction assay for alpha-thalassemia. Blood. 2001;98:250-1.
70. Lee SY, Li CK, Ling SC, Shiu YK. Survival of homozygous alpha-thalassemia with aplasia/hypoplasia of phalanges and jejunal atresia. J Matern Fetal Neonatal Med. 2009;22:711-3.
71. Chik KW, Shing MM, Li CK, et al. Treatment of hemoglobin Bart's hydrops with bone marrow transplantation. J Pediatr. 1998;132:1039-42.
72. Zhou X, Ha SY, Chan GC, et al. Successful mismatched sibling cord blood transplant in Hb Bart's disease. Bone Marrow Transplant. 2001;28:105-7.
73. Yi JS, Moertel CL, Baker KS. Homozygous alpha-thalassemia treated with intrauterine transfusions and unrelated donor hematopoietic cell transplantation. J Pediatr. 2009;154:766-8.
74. Pongtanakul B, Sanpakit K, Chongkolwatana V, Viprakasit V. Normal cognitive functioning in a patient with Hb Bart's hydrops successfully cured by hematopoietic stem cell transplantation. Bone Marrow Transplant. 2013. In press.
75. Vichinsky E. Advances in the treatment of alpha-thalassemia. Blood Rev. 2012;26(1 Suppl):S31-34.
76. Vichinsky E. Emerging 'A' therapies in hemoglobinopathies: agonists, antagonists, antioxidants, and arginine. Hematology Am Soc Hematol Educ Program. 2012;2012:271-5.
77. Taher A, Musallam KM, Viprakasit V, Cappellini MD. Clinical Practice Guideline for Non-Transfusion Dependent Thalassemia (NTDT). Vol. 1. Nicosia: Thalassemia International Federation (TIF); 2013.
78. Al-Salem AH, Nasserulla Z. Splenectomy for children with thalassemia. Int Surg. 2002;87:269-73.
79. Chinprasertsuk S, Wanachiwanawin W, Pattanapanyasat K, Tatsumi N, Piankijagum A. Relation of haemolytic anaemia and erythrocyte-bound IgG in alpha- and beta-thalassaemic syndromes. Eur J Haematol. 1997;58:86-91.
80. Jetsrisuparb A, Sanchaisuriya K, Fucharoen G, et al. Development of severe anemia during fever episodes in patients with hemoglobin E trait and hemoglobin H disease combinations. J Pediatr Hematol Oncol. 2006;28:249-53.
81. Chinprasertsuk S, Piankijagum A, Wasi P. In vivo induction of intraerythrocytic inclusion bodies in hemoglobin H disease: an electron microscopic study. Birth Defects Orig Artic Ser. 1987;23:317-26.
82. Chinprasertsuk S, Wanachiwanawin W, Piankijagum A. Effect of pyrexia in the formation of intraerythrocytic inclusion bodies and vacuoles in haemolytic crisis of haemoglobin H disease. Eur J Haematol. 1994;52:87-91.
83. Pongtanakul B, Narkbunnam N, Veerakul G, et al. Dengue hemorrhagic fever in patients with thalassemia. J Med Assoc Thai. 2005;88(8 Suppl):S80-85.
84. Saiviroonporn P, Viprakasit V, Sanpakit K, Wood JC, Krittayaphong R. Intersite validations of the pixel-wise method for liver R2* analysis in transfusion-dependent thalassemia patients: a more accessible and affordable diagnostic technology. Hematol Oncol Stem Cell Ther. 2012;5:91-5.
85. Taher AT, Viprakasit V, Musallam KM, Cappellini MD. Treating iron overload in patients with non-transfusion-dependent thalassemia. Am J Hematol. 2013 Feb 6. [Epub ahead of print].
86. Traisrisilp K, Luewan S, Tongsong T. Pregnancy outcomes in women complicated by thalassemia syndrome at Maharaj Nakorn Chiang Mai Hospital. Arch Gynecol Obstet. 2009;279:685-9.
87. Chan TK, Chan V, Todd D, Ghosh A, Wong LC, Ma HK. Prenatal diagnosis of alpha- and beta-thalassemias: experience in Hong Kong. Hemoglobin. 1988;12:787-94.
88. Fucharoen S, Winichagoon P, Thonglairoam V, et al. Prenatal diagnosis of thalassemia and hemoglobinopathies in Thailand: experience from 100 pregnancies. Southeast Asian J Trop Med Public Health. 1991;22:16-29.
89. Tongsong T, Wanapirak C, Sirivatanapa P, et al. Prenatal control of severe thalassaemia: Chiang Mai strategy. Prenat Diagn. 2000;20:229-34.
90. Tongsong T, Wanapirak C, Sirivatanapa P, et al. Prenatal eradication of Hb Bart's hydrops fetalis. J Reprod Med. 2001;46:18-22.
91. Lam YH, Tang MH. Prenatal diagnosis of haemoglobin Bart's disease by cordocentesis at 12-14 weeks—experience with the first 59 cases. Prenat Diagn. 2000;20:900-4.
92. Prechapanich J, Choavaratana R, Makemaharn W, Kanjanakorn C, Thongnoppakhun W, Limwongse C. Preimplantation Genetic Diagnosis for alpha thalassemia: experience in Siriraj Hospital. Siriraj Med J. 2008;60:333-6.

How to optimize the treatment of sickle cell disease in children?

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A B S T R A C T

Important progress in the understanding of the pathophysiology and in the management of sickle cell disease in children has dramatically improved the prognosis in well resourced countries, so that mortality in children in most series in now below 2%. However, we still need to organize a better network of care so that patients can receive adequate management outside well-staffed centers of excellence. Most important clinical targets are prevention of infections, tailored management of pain, prevention of brain disease, appropriate use of transfusion, and use of hydroxyurea. Education on therapeutic options is a key factor. Mortality in young adults remains a challenge. Improving care during the transition from the pediatric to the adult period is mandatory. Screening early for complications in children will also contribute to reducing the extremely high morbidity in adults. Application of hematopoietic stem cell transplantation remains a challenge scientifically, ethically, and economically. Finally, participation in interactive international networks will surely contribute to decrease the morbidity and mortality of sickle cell disease.

Learning goals

At the conclusion of this activity, participants should know that:
- in addition to systematic preventive measures such as daily penicillin and immunization, management of fever in a child with sickle cell disease must consider the risk of overwhelming infection with *Streptococcus pneumoniae* and the use of tailored antibiotic therapy;
- children with sickle cell disease can develop acute anemia requiring immunologically matched transfusion within 2 h;
- prevention of brain disease should include annual systematic transcranial Doppler screening in homozygous SS and in S/β_0 children aged 2-16 years;
- patients with sickle cell disease must be enrolled in health care networks allowing them to find appropriate care at units with trained personnel close to their home that work in close collaboration with expert centers. All network centers must share information, protocols, and educational tools.

Introduction

Approximately 300,000 children with sickle cell disease (SCD; homozygous SS, compound heterozygous SC and S/β-thalassemic patients) are born in the world every year.[1] Although most live in Sub-Saharan Africa, India, and the Middle East, SCD is now prevalent in the United States and Western Europe as a result of migration of people from areas in which the mutation has a high prevalence.[2] More and more hospital-based pediatricians in the United States and in Western Europe have become familiar with SCD, and pediatric mortality is now below 2% in most series.[3,4] However, adults are still affected by a high morbidity and mortality.[5] The challenge in well-resourced countries is to minimize SCD-related morbidities in adulthood and to develop international collaborations to make such progress available in other countries. Here, we will develop the issues which we consider are key factors for optimization of the treatment of SCD.

Improving general knowledge about the disease

All physicians, even in Europe, may be called on to manage a patient with SCD. The exact prevalence of SCD in Europe is not known. Such data require population census screening or calculations based on accurate birth and mortality rates. Mortality rates are not well known, and prevalence is estimated using reliable birth data from countries that have implemented systematic neonatal screening of the disease. Neonatal screening is generalized and universal in England[6] and the Netherlands,[7] and generalized but targeted to populations at risk in France.[8] In Belgium, it is universal but limited to the cities of Brussels and Liege.[9] In Spain, it is universal but limited to Extremadura and Madrid, while other Spanish regions have pilot programs ongoing.[10] It is experimental in some cities in Germany[11] and Italy.[12] The numbers of babies affected with abnormal hemoglobin in these different screening programs is indicated in Table 1. Except for neonatal data, we have only approximations of the size of the popula-

tions of patients affected with SCD. An estimated 12,500 patients are affected with SCD in the UK, and 10,000 in France. The patients are concentrated in big cities to which migrants have moved to seek employment.

Most of Europe's biggest cities have developed hospital-based comprehensive care centers of expertise for patients with SCD. However, these centers are not accessible to all patients. Analysis of the causes of death in SCD patients in the UK indicated that the care of over one-third of the patients could had been improved.[13] Sixty-five percent of 110 healthcare professionals in emergency departments thought they had insufficient training to provide optimal care for patients with SCD.[14] These observations demonstrate that we must improve access to adequate care for all patients, being mindful that a majority of patients have low incomes and are frequently without independent transport. In my own series of patients, one third of mothers are isolated at home with 1-5 children and obviously will not be able to go to a center of expertise in case of emergency. All health care providers must receive SCD-specific training. They must know the main risks faced by SCD patients, which were until recently responsible for a high mortality: pneumococcal sepsis, acute splenic sequestration, strokes in young children,[15] and acute chest syndromes in older ones.[16] All centers must be able to provide simple blood transfusion with matched blood for acute stroke or acute chest syndrome, antibiotics for infection, and tailored analgesia for painful crisis. Designated centers of expertise must be able to perform exchange red cell transfusion and provide access to specialist care in sickle-related complications, in neurology, orthopedics, cardiology, ophthalmology, ENT, respiratory problems, surgery, and anesthesia. Networks including community general practitioners, proximity centers, and centers of expertise must share information, educative tools, and protocols.

Early implementation of preventive measures

Neonatal screening or early diagnosis

Observational studies have established that neonatal screening leads to major decreases in pediatric morbidity and mortality,[17,18] mainly as the result of early intervention in penicillin prophylaxis and parent education. British studies on the cost-effectiveness of neonatal screening suggest that in populations with greater than 16 sickle traits/1000 and 0.5 SCA/1000, universal screening is more effective than targeted screening.[19] In regions with lower immigration rates from northern and sub-Saharan Africa, the cost-effectiveness of universal neonatal screening of SCD may be higher. In these populations, screening could be targeted to women in early pregnancy or, when possible, adolescents, as suggested by a study showing that results were retained by young people for a mean period of 15 years between screening and pregnancy.[20]

Prevention of infections

Fulminant infections by encapsulated bacteria enabled by functional asplenia were, until recent years, the major cause of death in SCD children under five years of age.[21] A randomized, placebo-controlled study published in 1986 showed that prophylaxis with penicillin twice a day in SCD children under the age of three years at study inclusion was associated with an 84% reduction in the incidence of infection.[22] Penicillin is, therefore, recommended twice daily starting at two months of age; further research is needed to determine the age at which penicillin prophylaxis can be stopped safely.[23]

Given the risk of poor adherence to daily prophylaxis and the development of penicillin resistant *Streptococcus pneumoniae* strains, the combination of pneumococcal immunization and prophylactic penicillin is recommended.[24] Penicillin prophylaxis and 23-valent pneumococcal polysaccharide vaccine have dramatically reduced those risks in the 1990s, but strains resistant to penicillin have emerged. Furthermore, 23-valent pneumococcal polysaccharide vaccine is less effective in children under the age of two years, and the immune response declines within three years after administration. The introduction in 2000 of a 7-valent conjugate vaccine (7vPnC) induced a further reduction in invasive pneumococcal diseases in SCD reported as 90% and 68%, repectively,[25,26] but was accompanied by emergence of non-7vPnC serotypes-related infections.[27] A recently licensed 13-valent pneumococcal conjugate vaccine added serotypes 1, 3, 5, 6A, 7F and 19A to those in 7vPnC. It was shown to be immunogenic and safe in children previously immunized with the 23-valent pneumococcal polysaccharide vaccine.[28] Despite all these preventive measures, the risk of overwhelming infection related to *S. pneumoniae* must always be con-

Table 1. The numbers of babies affected with abnormal hemoglobin in different screening programs in Europe.

City, country	N. of babies affected with SCD/ screened babies	N. of SS/ screened babies	N. of SC/ screened babies	N. of Sthal/ screened babies	N. of with S trait; with C trait/ screened babies	Ref.
Brussels, Belgium		1/1954	1/11,986	1/29,965	1/65:1/508	9
Liege, Belgium		1/1714	1/5998	1/11,995	1/65:1/387	9
Madrid, Spain	1/6914					10
England	1/2000 (hemoglobin E disease included)				6	
London, England	1/549 (hemoglobin E disease included)				6	
France	1/2065					8
Ferrara, Italy	0				1/135:1/541	12

sidered in a febrile SCD infant and must lead to obtaining blood cultures and initiating targeted antibiotic therapy.

Prevention of strokes

Until recent years, 11% of patients with SCD were observed to have a clinical stroke by the age of twenty years.[29] Silent infarcts were evidenced in 37.1% of patients with SCD (95%CI: 26.3-50.7%) by the age of 14 years,[4] and these silent infarcts were associated with possible impairment of cognitive function. Adams and his associates demonstrated in 1992 that it was possible to identify children at risk of developing an overt stroke using transcranial Doppler ultrasonography screening; 40% of the children with increased blood flow velocity in the internal carotid or middle cerebral artery will have an overt stroke within three years.[30] Six years later, Adams *et al.* demonstrated that a first stroke could be prevented by monthly transfusions to children with abnormal TCD findings, as evidenced in a randomized study by a 92% difference in the risk of stroke between the transfused and non-transfused arms.[31] These well designed studies provide the basis for the recommendations that transcranial Doppler ultrasonography be performed annually in SCD children aged 2-16 years and that regular blood transfusions should be strongly considered in those with abnormal transcranial Doppler ultrasonography findings.[32] The possibility of preventing a first stroke in children with SCD using hydroxyurea is undergoing current investigation through the TWitCH trial, which compares outcomes of children with pathological TCD allocated to hydroxyurea or to chronic transfusions. The optimal management of silent cerebral infarcts is also currently being explored. The ongoing SIT trial has allocated transfusion or observation in children with silent infarcts; the results are not expected until after June 2013.[33]

Screening of sickle-related complications starting during childhood

SCD patients have a dramatically increased number of complications as they get older. A 4-decade observational study of 1056 patients, initiated in 1956, showed that 232 patients died and that by the fifth decade nearly one-half of the survivors had documented irreversible organ damage.[34] A recent series in Europe showed prevalences in adult SCD patients of pulmonary hypertension, renal failure, retinopathy, avascular necrosis, and iron overload of 32%, 8%, 24%, 16% and 17%, respectively.[35] Median age at death was 42 years for male and 48 years for female SS patients in the United States in 1994,[36] and remained low (45 years) in 2012.[37] The range for the mean age at death is notably wide in this last series: 24 to 86 years, which may be explained by a combination of access to care, by genetically determined variations of the phenotypes, and environmental factors. Main causes for death are pulmonary (pulmonary hypertension and acute chest syndromes), cerebrovascular events, and renal failure. It is likely that many adult complications could have been prevented or minimized in childhood. Early screening of renal impairment (microalbuminuria), regular pulmonary function testing, liver and gallbladder ultrasound, hip X-ray, electrocardiogram (ECG) and echocardiography, and ophthalmologic evaluation are recommended.[38] Although there has been no evidence that angiotensin-converting enzyme inhibitors reduce hyperfiltration in SCD patients, these agents have been shown to reduce proteinuria.[39] Based on parallel responses in proteinuria and hyperfiltration in other diseases, early and systematic screening may be justified. Concerning pulmonary protection, prevention and prompt treatment of bacterial pulmonary infections, and screening for and management of asthma are likely to prevent further worsening of pulmonary function. Careful surveillance of asthma status must be conducted if patients are to be treated with β-blocking drugs. Consequences of finding elevated tricuspid regurgitation velocity (TRV) in young children are debatable; both the determination of accurate TRV diagnostic criteria and clinical trials to evaluate strategies aiming to prevent or delay the development of pulmonary hypertension are warranted.[40] Lastly, protection of cerebral function remains a challenge (see above). Chronic transfusion protects from most secondary and primary strokes but not from worsening of neuroimaging findings.[41,42] It appears that it is mandatory to intervene therapeutically before a threshold of vascular damage ensues beyond which vascular function can no longer be improved.

Improving education and transition programs from child-centered care to adult-oriented care

Education may disrupt the vicious cycle of pain/fear of having pain. Adolescence is a crucial period during which disease control should be optimized since new complications may occur. However, adolescent patients may feel overwhelmed by the burden of daily care and want to escape from what is perceived as unfair limits imposed by their family and doctors. Furthermore, SCD adolescents are vulnerable to pubertal delay and/or severe jaundice. Adolescents with SCD may experience academic difficulties related in part to frequent absences from school and, in some patients, to cerebral vasculopathy of variable severity. Many adolescents have been forbidden by their doctors to engage in sports. The adolescent may also be concerned about becoming infertile from hydroxyurea therapy. Parental overprotection may cause additional distress. These factors in combination all contribute to depression, low self-esteem, and even post-traumatic stress disorder.[43] Furthermore, there are major differences between pediatric and adult wards, with most pediatric units offering games, schooling, other activities, and even holiday camps; activities that are lacking from adult facilities. Importantly, urgent treatment of pain and accelerated admission pathways are more often available in pediatric than in adult units receiving SCD patients. Transition must be a gradual process, starting early (12-13 years). There should be a hospital transition policy in place. At age 15-16 years a detailed review of the patient should be conducted to include knowledge and understanding about management of SCD, concerns about the adult healthcare setting, and readiness to transfer. The adolescent and his or her parents should also meet the adult sickle cell team.[44]

Intensifying treatment in some patients

A decisional tree to intensify treatment is proposed in Figure 1.

Hydroxyurea

Hydroxyurea has been used in children with SCD affected by severe forms of the disease for more than 20 years. A Belgian controlled trial in children with severe SCD showed that hydroxyurea decreased the number of hospital admissions and the number of days spent in hospital.[45] There are now many reports about the use of hydroxyurea in SCD children afflicted with severe forms of the disease.[46] Globally, hydroxyurea is now recommended for the treatment of children with SCD to prevent recurrence of acute pain crises and acute chest syndrome. Many physicians also use chronic severe anemia as an indication for hydroxyurea therapy in children with SCD. In the United States, the Food and Drug Administration has approved hydroxyurea for use only in adult SCD patients, while children must be enrolled in hydroxyurea study protocols. European regulatory authorities have approved a coated, breakable 1000 mg tablet for adults and children and 100 mg tablets for children. Starting doses are generally approximately 15 gm/kg/day and may be escalated by 5 mg/kg/day until there is either evidence of clinical benefit or the maximum tolerated dose is reached. Clinical effects required to assess the efficacy of the drug may not be observed in some patients until after a delay of 6-9 months. Short- and mid-term tolerance of hydroxyurea in children is good, the main side effect being transient myelosuppression that usually resolves after decreasing the dosage or temporarily interrupting the drug therapy.[47]

Many issues remain on the use of hydroxyurea therapy in SCD children. The most challenging is whether hydroxyurea therapy is not only indicated in severe patients to prevent progression of organ damage, but also in asymptomatic children to prevent the onset of such complications. A major challenge will be to establish the risk/benefit ratio of the use of the drug in infants, which is not yet known. The BABY HUG study was a multicenter, randomized controlled trial designed to assess whether hydroxyurea given in infants could prevent organ dysfunction, choosing as primary end points splenic and kidney function.[48] Infants aged 9-18 months were allocated to receive hydroxyurea 20 mg/kg/d, or placebo for two years. One hundred and sixty-seven of the 193 children completed the study. Children receiving hydroxyurea experienced a marked reduction in painful events, toxicity was limited to mild to moderate neutropenia, but no significant differences were seen between the groups for the primary splenic and renal end points. The short 2-year duration of the study or a poor choice of end points may explain the lack of protection against progressive splenic and renal

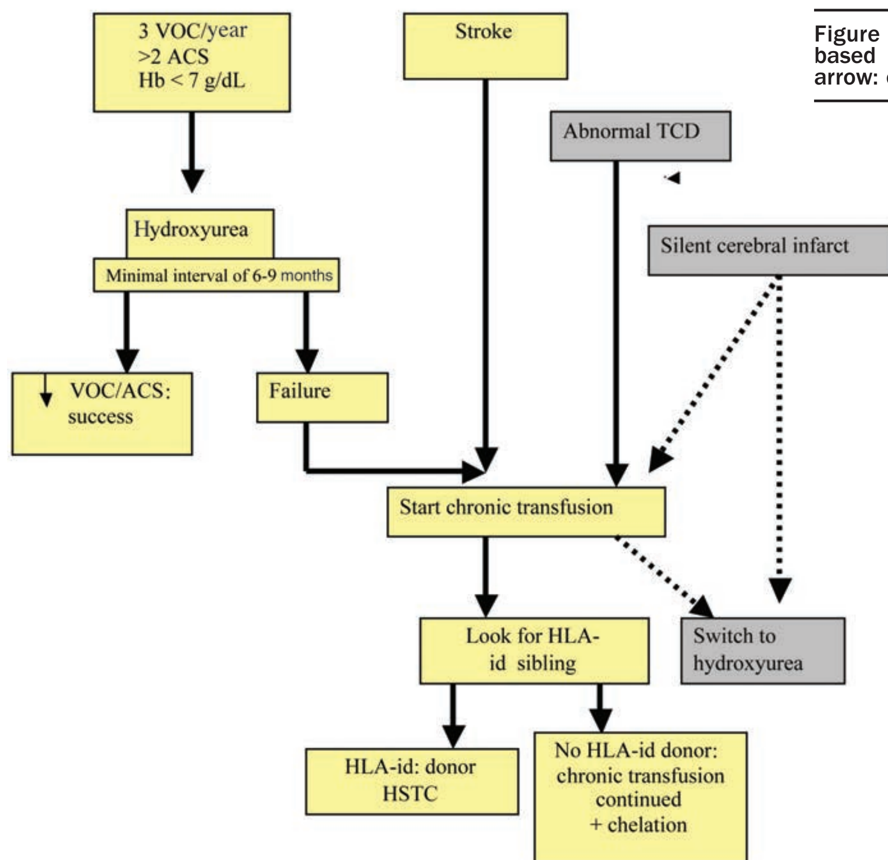

Figure 1. Unbroken arrow: evidence-based or consensus treatment. Broken arrow: currently under investigation.

impairments observed. Differently designed studies are needed. All hydroxyurea studies must keep in mind a possible toxic effect of the drug on spermatic function, which has been shown after six months of treatment in SCD adults.[49] Uncertainty regarding long-term consequences on fertility are especially important for boys treated with hydroxyurea early and for several years. Storage of frozen sperm can be offered to mature boys and adults, though it is rarely accepted.

Chronic transfusion

Most frequent indications for chronic transfusion in children are cerebral vasculopathy and failure of hydroxyurea treatment for preventing recurrent painful crises and/or acute chest syndromes, the latter possibility becoming more frequent as children get older. Chronic transfusion may also be used in children under the age of five years with recurrent splenic sequestrations to delay the time until splenectomy. Chronic transfusion may be performed through simple or exchange transfusion. The advantages of the latter include the avoidance of an excessive increase in hematocrit and to reduce the amount of transfused iron. Exchange transfusion can be performed manually or using a cell separator (erythrocytapheresis).

Considering brain protection, transfusion programs have been found to decrease by 90% the risk of a first stroke[31] and from 70% to 13% the risk of overt recurrent strokes, although transient neurological events can still occurr.[50] However, these programs raise several questions. First, there is a risk that some countries may have difficulty collecting enough immunologically matched blood, given the blood group disparities between usual blood donors and Afro-Caribbean recipients.[51,52] Second, chronic transfusion does not always protect against worsening of macro-and micro-vessel abnormalities,[41,42] even when HbS percentage is permanently kept below 30%. We need to better study the longitudinal outcomes of specific cerebral vessels lesions observed in childhood. In addition, chronic transfusion induces significant toxicities, the most important of which is iron overload.[53] Iron toxicity seems to spare the heart, compared with what is observed in thalassaemia, but induces significant hepatic toxicity.[54] Recently, a once-daily oral chelator, deferasirox, was demonstrated to have acceptable tolerability and similar efficacy to deferoxamine in reducing iron burden in children with SCD.[55] Paucity of venous access leads in many cases to the use of subcutaneous central venous access devices. Transfusion-transmitted infections, although rare in industrialized countries with established blood transfusion services, still require awareness and surveillance.

The risk of shortage of blood supplies, the toxicities of transfusion, and the burdensome nature of chronic transfusion define a need for alternatives to blood transfusions. In particular, attempts have been made to switch from chronic transfusion to hydroxyurea in children who have had a stroke. The SWiTCH study was a non-inferiority trial randomizing an alternative treatment (hydroxyurea/phlebotomy) to standard treatment (transfusion/chelation) in 134 children who have had a stroke and been transfused for 18 months or more, with transfusional iron overload.[56] The study was closed by the National Heart, Lung and Blood Institute (NHLBI)-appointed Data and Safety Monitoring Board because the interim analysis indicated that the reduction in liver iron content was not superior on the hydroxyurea/phlebotomy arm and because seven strokes had been observed in the 67 subjects in this arm compared to none in the 66 subjects on transfusion/chelation. Without superiority in iron elimination, the increased stroke risk in the hydroxyurea arm (10% *vs.* 0% in the transfusion arm) was no longer justifiable. Transfusion remains, therefore, the best option to prevent recurrences in SCD children who have had a stroke, unless the patient can benefit from hematopoietic stem cell transplantation.

Hematopoietic stem cell transplantation

Transplantation of hematopoietic stem cells (HSCT) from HLA-identical siblings is the only curative therapy for SCD. Many issues still have to be considered. First, there is a debate about the indications of HSCT. Currently, HSTC, using as stem cell sources either bone marrow or cord blood originating from an HLA identical sibling, can be curative for children and adolescents affected with a severe form of the disease. In a series of 87 patients transplanted between 1988 and 2004, the overall and event-free survival rates were 93.1% and 86.1%, respectively.[57] Cord blood transplantation is associated with less acute graft-*versus*-host disease but delayed neutrophil engraftment compared to bone marrow transplantation. Sibling cord blood banking should be encouraged to avoid discomfort and risks of bone marrow harvest.[58] Some specialists propose to widen the indications to almost all SCD patients having a donor, given the current burden of morbidity and mortality in adults. Many parents, however, would opt for HSCT in their child independent of the objective severity of their child's disease, because HSCT offers a cure.[59] However, both the immediate risk of death and the long-term uncertainties about fertility lead other specialists to restrict the indications to patients with severe disease.[60] Concerning fertility in females, ovarian tissue can be cryopreserved, but it is currently unclear how useful this approach is for allowing future pregnancies.[61] Cryopreservation of sperm is, of course, possible only for mature males. Non-myeloablative peripheral blood stem cell transplants have been used in adults to reduce the toxicity of the procedure.[62] In a series published by Hsieh *et al.*, 9 patients out of 10 engrafted, but with a high incidence of mixed chimerism, and no successful discontinuation of immunosuppression with sirolimus. A major issue relates to not all patients having an HLA-identical sibling, and the despair experienced when parents and patients learn the procedure is not possible. Important difficulties in finding suitable unrelated donors in the registers relate to the ethnic variability and shortage of donations from minority groups. A recent publication on non-myeloablative bone marrow transplants from haploidentical donors in 14 adult patients reported a graft failure in 43%.[63] Reports of unrelated stem cell transplants in sickle cell disease have not provided encouraging results.[64,65] Prospective protocols including well selected patients, homogeneous conditioning regimen, and high cell doses are needed to better define the role of unrelated cord blood transplant.[66] Gene therapy will perhaps be an answer in selected patients in the near future.[67] HSCT and gene therapy are expensive and not affordable by patients living in countries with low resources. Strong co-operation between more and less developed countries has to be promoted.

Understanding the pathophysiology of SCD allows new therapeutic targets to be found

SCD pathophysiology cannot be explained solely by polymerization of desoxyhemoglobin S. Endothelial dysfunction related to inflammation, nitric oxide (NO) dysregulation, cell adhesion, oxidative injury, and disordered coagulation have emerged as key determinants of the onset of complications.[68,69] Many novel therapeutic agents are undergoing investigations, one of the major problems in assessing their efficacy being the choice of appropriate end points.

Developing international networks

From its independent origins, at least three times in Africa and once in India, the sickle cell gene has spread across continents and skin colors through forced slave trading and modern economic migrations. International organizations such as UNESCO, WHO, and the UN have recognized SCD as a global public health issue. Still, to date, we have no sound epidemiological data to estimate the actual health burden of SCD, not only in Europe or the United Sates, but also in the most affected poorer countries. Here, improved nutrition and better control of infectious diseases have lowered childhood mortality rates, only to unmask the prevalence of genetic diseases including SCD. We know almost nothing about the natural history of SCD in its natural environment and comparative cohort studies are badly needed. We have no idea on how safe hydroxyurea treatment is in places where infectious diseases, including malaria, are prevalent. To answer these questions, and to share expertise, international collaboration is crucial. This can only be accomplished through coordinated efforts to establish not only North-South but also South-South and triangular equitable and sustainable collaborations. The recent advent of several regional and international networks is a remarkable and promising step toward this goal. For example, the Central African SCD network (REDAC) and the Caribbean network of researchers on sickle cell disease and thalassemias (CAREST) co-ordinate efforts to improve SCD management and develop collaborative research projects in eleven countries of central Africa and eleven countries of the Caribbean, respectively. The mission of the global sickle cell disease network (GSCDN) is to further research and clinical care globally by facilitating joint research and clinical programs, training, and education. Initiatives of this kind should contribute to a better understanding of the different factors that modify disease phenotypes, and constitute a driving force to not only improve management of patients, both in the developing countries and well-resourced countries.

References

1. Weatherall D. Genomics and global health: time for a reappraisal. Science. 2003; 302(5645):597-9.
2. Roberts I, de Montalembert M. Sickle cell disease as a paradigm of immigration hematology: new challenges for hematologists in Europe. Haematologica. 2007;92(7):865-71.
3. Telfer P, Coen P, Chakravorty S, Wilkey O, Evans J, Newell H, et al. Clinical outcomes in children with sickle cell disease living in England: a neonatal cohort in East London. Haematologica. 2007;92(7):905-12.
4. Bernaudin F, Verlhac S, Arnaud C, Kamdem A, Chevret S, Hau I, et al. Impact of early transcranial Doppler screening and intensive therapy on cerebral vasculopathy outcome in a newborn sickle cell anemia cohort. Blood. 2011;117(4):1130-40.
5. Lanzkron S, Haywood C. Trends in mortality rates and age of death in sickle cell disease (SCD): 1979-2005. Blood. 2010;116:325.
6. Streetly A, Latinovic R, Hall K, Henthrorn J. Implementation of universal newborn bloodspot screening and other clinically significant heamoglobinopathies in England: screening results for 2005-7. J Clin Pathol. 2009;62:26-30.
7. Giordano GP. Starting neonatal screening for haemoglobinpathies in the Netherlands. J Clin Pathol. 2009;62:49-52.
8. Bardakdjian-Michau J, Bahuau M, Hurtrel D, Godart C, Riou J, Mathis M, et al. Neonatal screening for sickle cell disease in France. J Clin Pathol. 2009;62:31-3.
9. Gulbis B, Cotton F, Ferster A, Ketelsegers O, Dresse MF, Rongé-Collard E, et al. Neonatal haemoglobinpathy screening in Belgium. J Clin Pathol 2009;62:49-52
10. Manu Perreira M, Vives Corrons JL. Neonatal haemoglobinopathy screening in Spain. J Clin Pathol. 2009;62:22-5.
11. Dickerhoff R, Genzel-Boroviczeny O, Kohne E. Haemoglobinopathies and newborn haemoglobinopathies in Germany. J Clin Pathol. 2009;62:34.
12. Ballardini E, Tarocco A, Marsella M, Bernardoni R, Carandina G, Melandri C, et al. Universal neonatal screening for sickle cell disease and other haemoglobinopahties in Ferrara, Italy. Blood Transfusion. 2012 Sep 12:1-6. doi: 10.2450/2012.0030-12. [Epub ahead of print].
13. Mayor S. UK enquiry shows lack of knowledge about complications in patients with sickle cell disease. BMJ. 2008; 336:112.
14. Mayor S. Group publishes standards for adult sickle cell disease to reduce the number of unexplained deaths. BMJ. 2008;337;132.
15. Gill FM, Sleeper A, Weiner SJ, Brown AK, Bellevue R, et al. Clinical events in the first decade in a cohort of infants with sickle cell disease. Blood. 1995;86(2):776-83.
16. Quinn CT, Rogers ZR, McCavit TL, Buchanan GR. Improved survival of children and adolescents with sickle cell disease. Blood. 2010;115(17):3447-52.
17. Lees CM, Davies S, Dezateux C. Neonatal screening for sickle cell disease. Cochrane Database of Systematic Reviews 2000; Issue1. Art. N. CD001913. doi:10.1002/1465 1858.CD001913
18. Vichinsky E, Hurst D, Earles A, Kleman K, Lubin B. Newborn screening for sickle cell disease: effect on mortality. Pediatrics. 1988(6);81:749-55.
19. Davies SC, Cronin E, Gill M, Greengross P, Hickman M, Normand C. Screening for sickle cell disease and thalassaemia: a systematic review with supplementary research. Health Technol Assess. 2004;4(3):1-99.
20. Lena-Russo D, Badens C, Aubinaud M, Merono F, Paolasso C, et al. Outcome of a school screening programme for carriers of haemoglobin disease. J Med Screen. 2002;9(2):67-9.
21. Leikin SL, Gallagher D, Kinney TR, Sloane D, Klug P, Rida W. Mortality in children and adolescents with sickle cell disease. Cooperative study of Sickle Cell Disease. Pediatrics. 1989;84(3):500-8.
22. Gaston MH, Verter JI, Woods G, Pegelow C, Kelleher J, Presbury G, et al. Prophylaxis with oral penicillin in children with sickle cell anemia. N Engl J Med. 1986; 314(25):1593-9.
23. Hirst C, Owusu-Ofori S. Prophylactic antibiotics for preventing pneumococcal infection in children with sickle cell disease. Cochrane Database Syst Rev 2002;3:CD003427.
24. Davies EG, Riddington C, Lottenberg R, Dower N. Pneumococcal vaccines for sickle cell disease. Cochrane Database Syst Rev. 2004;(1):CD003885.pub2
25. Halasa NB, Shankar SM, Talbot TR, Arbogast PG, Mitchel EF, Wang WC, et al. Incidence of invasive pneumococcal disease among individuals with sickle cell disease before and after the introduction of the pneumococcal conjugate vaccine. Clin Infect Dis. 2007;44:1428-33.
26. Adamkiewicz TV, Silk BJ, Howgate J, Baughman W, Strayhorn G, Sullivan K, et al. Effectiveness of the 7-valent pneumococcal conjugate vaccine in children with sickle cell disease in the first decade of life. Pediatrics. 2008;121(3):562-9.
27. McCavit TL, Quinn CT, Techasaensiri C, Rogers ZT. Increase in invasive Streptococcus pneumoniae infections in children with sickle cell disease since pneumococcal conjugate vaccine licensure. J Pediatr. 2011;158(3):505-7.
28. De Montalembert M, Abboud MR, Fiquet A, Inati AI,

Lebenburg JD, Kaddah N, et al. A 2-dose schedule of 13-valent pneumococcal conjugate vaccine (PCV13.
given to children with sickle cell disease previously immunized with 23-valent pneumococcal polysaccharide vaccine (PPSV23). 54th ASH meeting, Atlanta, USA, 2012.
29. Ohene-Frempong K, Weiner SJ, Sleeper LA, Miller ST, Embury S, Moohr JW, et al. Cerebrovascular accidents in sickle cell disease: rates and risk factors. Blood. 1998;91(1):288-94.
30. Adams R, McKie V, Nichols F, Carl E, Zhang DL, McKie K, et al. The use of transcranial ultrasonography to predict stroke in sickle cell disease. N Engl J Med. 1992;326(9):605-10.
31. Adams RJ, McKie V, Hsu L, Files B, Vichinsky E, Pegelow C, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial doppler ultrasonography. N Engl J Med. 1998;339(1):5-11.
32. National Institutes of Health. The management of sickle cell disease. 4th ed. 2002. (NIH publication No 02-2117. www.nhlbi.nih.gov/health/prof/blood/sickle/index.htm
33. DeBaun MR, Amstrong FD, McKinstry RC, Ware RE, Vichinsky E, Kirkham FJ. Silent cerebral infarcts: a review on a prevalent and progressive cause of neurological injury in sickle cell anemia. Blood. 2012;119:4587-96.
34. Powars DR, Chan LS, Hiti A, Ramicone E, Johnson C. Outcome of sickle cell anemia: a 4-decade observational study of 1056 patients. Medicine (Baltimore). 2005;84(6):363-76.
35. van Beers EJ, van Tuijn CF, MacGillavry MR, van der Giessen A, Schnog JJ, Biemond BJ; CURAMA study group. Sickle cell disease-related organ damage occurs irrespective of pain rate: implications for clinical practice. Haematologica. 2008;93(5):757-60.
36. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J Med. 1994;330(23):1639-44.
37. Elmariah H, Garrett ME, Ataga KI, Ashely-Koch AE, Telen M. Clinical characteristics associated with survival in adult sickle cell disease. 54th ASH meeting, Atlanta, USA, 2012.
38. De Montalembert M, Ferster A, Colombatti R, Rees DC, Gulbis B; European Network for Rare and Congenital Anaemias. ENERCA clinical recommendations for disease management and prevention of complications of sickle cell disease in children. Am J Hematol. 2011;86(1):72-5.
39. Sharpe CC, Thein SL. Sickle cell nephropathy- a practical approach. Br J Haematol. 2011;155(3):287-97.
40. Gordeuk VR, Minniti CP, Nouraie M, Campbell AD, Rana SR, Luchtman-Jones L, et al. Elevated tricuspid regurgitation velocity and decline in exercise capacity over 22 months of follow up in children and adolescents with sickle cell anemia. Haematologica. 2011;96(8):33-40.
41. Hulbert ML, McKinstry RC, Lacey JL, Moran CJ, Panepinto JA, Thompson AA, et al. Silent cerebral infarcts occur despite regular blood transfusion therapy after first strokes in children with sickle cell disease. Blood. 2011;117(3):772-9.
42. Brousse V, Hertz-Pannier L, Consigny Y, Bresson JL, Girot R, Mirre E, et al. Does regular blood transfusion prevent progression of cerebrovascular lesions in children with sickle cell disease? Ann Hematol. 2009;88(8):785-8.
43. Hofman M, de Montalembert M, Beauquier-Maccotta B, de Villartay P, Golse B. Posttraumatic stress disorder in children affected by sickle cell disease and their parents. Am J Hematol. 2007;82(2):171-2.
44. DeBaun MR, Telfer J. Transition and sickle cell disease. Pediatrics. 2012; doi: 10.1542/peds.2011-3049
45. Ferster A, Vermylen C, Cornu G, Buyse M, Corazza F, Devalck C, et al. Hydroxyurea for treatment of severe sickle cell anemia: a pediatric clinical trial. Blood. 1996;88(8):1960-4.
46. Stouse JJ, Lanzkron S, Beach MC, Haywood C, Park H, Witkop C, et al. Hydroxyurea for sickle cell disease : a systematic review for efficacy and toxicity in children. Pediatrics. 2008;122(6):1332-42.
47. De Montalembert M, Brousse V, Elie C, Bernaudin F, Shi J, Landais P. Long-term hydroxyurea treatment in children with sickle cell disease: tolerance and clinical outcomes. Haematologica. 2006;91(1):125-8.
48. Wang WC, Ware RE, Miller ST, Iyer RI, Casella JF, Minniti CP, et al. Hydroxycarbamide in very young children with sickle-cell anaemia: a multicentre, randomized, controlled trial (BABY HUG). Lancet. 2011;377(9778):1663-72.
49. Bachir D, Lionnet F, Gellen Dautremer J, Stankovic K, Habibi A, Bartolucci P, et al. Prospective comparison of sperm parameters in SCD adult patients before and after a 6 months treatment with hydroxyurea (HU). 54th ASH meeting Atlanta, USA, 2012.
50. Pegelow CH, Adams RJ, McKie V, Abboud M, Berman B, Miller ST, et al. Risk of recurrent stroke in patients with sickle cell disease treated with erythrocyte transfusions. J Pediatr. 1995;126(6):896-9.
51. Drasar E, Igbineweka N, Vasavda N, Free M, Awogbade M, Allman M, et al. Blood transfusion usage among adults with sickle cell disease - a single institution experience over ten years. Br J Haematol. 2011;152(6):766-70.
52. Yazdanbakhsh K, Ware RE, Noizat-Pirenne F. Red blood cell alloimmunization in sickle cell disease: pathophysiology, risk factors, and transfusion management. Blood. 2012;120(3):528-37.
53. Vichinsky EP, Ohene-Frempong K; Transfusion Committee. Approaches to transfusion therapy and iron overload in patients with sickle cell disease: results of an international survey. Pediatr Hematol Oncol. 2011;28(1):37-42.
54. Vichinsky E, Butensky E, Fung E, Hudes M, Theil E, Ferrell L, et al. Comparison of organ dysfunction in transfused patients with SCD or beta thalassemia. Am J Hematol. 2005;80(1):70-4.
55. Vichinsky E, Bernaudin F, Forni GL, Gardner R, Hassell K, Heeney MM, et al. Long-term safety and efficacy of Deferasirox (Exjade. for up to 5 years in transfusional iron-overloaded patients with sickle cell disease. Br J Haematol. 2011;154(3):387-97.
56. Ware RE, Helms RW. Stroke with transfusions changing to Hydroxyurea (SWiTCH). Blood. 2012;26(17):3925-32.
57. Bernaudin F, Socie G, Kuentz M, Chevret S, Duval M, Bertrand Y, et al. Long-term results of related myeloablative stem-cell transplantation to cure sickle cell disease. Blood. 2007;110(7):2749-56.
58. Rocha V, Kabbara N, Ionescu I, Ruggeri A, Purtill D, Gluckman E. Diseases. Bone Marrow Transplant. 2009;44(10):653-9.
59. Kodish E, Lantos J, Stocking C, Singer PA, Siegler M, Johnson FL. Bone marrow transplantation for sickle cell disease. A study of parents' decisions. N Engl J Med. 1991;325(19):1349-53.
60. De Montalembert, M, Roberts I. Sickle cell disease: Primum non nocere (first do no harm). Haematologica. 2010;95(1):4-5.
61. Poirot C, Abirached F, Prades M, Coussieu C, Bernaudin F, Piver P. Induction of puberty by autograft of cryopreserved ovarian tissue. Lancet. 2012;379(9815):588.
62. Hsieh MH, Kang EM, Fitzhugh CD, Link B, Bolan CD, Kurlander R. Allogeneic hematopoietic stem-cell transplantation for sickle cell disease. N Engl J Med. 2009;361(24):2309-17.
63. Balanos-Meade J, Fuchs EJ, Luznik L, Lanzkron SR, Gamper CJ. HLA haploidentical bone marrow transplantation with post-transplant cyclophosphamide expands the donor pool for patients with sickle cell disease. Blood. 2012;102(22):4285-91.
64. Adamkiewicz TV, Szabolcs P, Haight A, Baker KS, Staba S, Kedar A, et al. Unrelated cord blood transplantation in children with sickle cell disease: review of four-center experience. Pediatr Transplant. 2007;11(6):641-4.
65. Kamani NR, Walters MC, Carter S, Aquino V, Brochstein JA, Chaudhury S. Unrelated donor cord blood transplantation for children with severe sickle cell disease: results of one cohort from the phase II study from the Blood and Marrow transplant Clinical Trial Network (BMT CTN). Biol Blood Marrow Transplant. 2012;18(8):1265-72.
66. Ruggeri A, Eapen M, Scaravadou A, Cairo MS, Bhatia M, Kurtzberg J, et al. Umbilical cord blood transplantation for children with thalassemia and sickle cell disease. Biol Blood Marrow Transplant. 2011;17(9):1375-82.
67. Payen E, Leboulch P. Advances in stem cell transplantation and gene therapy in the beta-hemoglobinopathies. ASH Education Program. 54th ASH meeting Atlanta, USA, 2012, pp 276-83.
68. Embury SH. The not-so-simple process of sickle cell vasoocclusion. Microcirculation. 2004;11:101-13.
69. Gladwin MT, Vichinsky E. Pulmonary complications of sickle cell disease. N Engl J Med. 2008;359(21):2254-65.

Gastrointestinal graft-versus-host disease: from biomarkers to pathophysiology

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A B S T R A C T

Acute graft-*versus*-host disease (GVHD) of the gastrointestinal tract (GI) affects up to 60% of patients receiving allogeneic hematopoietic cell transplantation (HCT). It causes nausea, vomiting, anorexia, secretory diarrhea and, in severe cases, abdominal pain and/or hemorrhage. Acute GVHD is often clinically indistinguishable from other causes of GI dysfunction such as conditioning regimen toxicity, infection or medications. Endoscopic biopsy is often used to confirm the diagnosis, but histological severity on biopsy does not consistently correlate with clinical outcome. Clinical stage II or greater (more than 1 L of diarrhea per day) is associated with reduced survival, but daily stool volume can vary considerably. Lower GI GVHD responds poorly to treatment compared to other target organs, and treatment with high-dose systemic steroid therapy carries significant risks, especially infectious complications in profoundly immunosuppressed patients.

Learning goals

At the conclusion of this activity, participants should:
- know the three parameters that define risk for death at the onset of gastrointestinal graft-*versus*-host disease;
- know the functional importance of Paneth cells in the gastrointestinal tract;
- know the relationship between Reg3α and the gastrointestinal microbiome.

Acute graft-*versus*-host disease (GVHD) of the gastrointestinal tract (GI) affects up to 60% of patients receiving allogeneic hematopoietic cell transplantation (HCT).[1,2] It causes nausea, vomiting, anorexia, secretory diarrhea and, in severe cases, abdominal pain and/or hemorrhage.[3] Acute GVHD is often clinically indistinguishable from other causes of GI dysfunction such as conditioning regimen toxicity, infection or medications. Endoscopic biopsy is often used to confirm the diagnosis,[4] but histological severity on biopsy does not consistently correlate with clinical outcome.[4-6] Clinical stage II or greater (more than 1 L of diarrhea per day) is associated with reduced survival,[1,2] but daily stool volume can vary considerably. Lower GI GVHD responds poorly to treatment compared to other target organs, [2] and treatment with high-dose systemic steroid therapy carries significant risks, especially infectious complications in profoundly immunosuppressed patients.[7,8]

The standard treatment of acute GVHD is higher dose systemic steroids, which has not changed in 40 years. One reason for this lack of progress is the lack of validated biomarkers for acute GVHD. We have recently identified and validated laboratory regenerating islet-derived 3-alpha (REG3α), a C-type lectin secreted by Paneth cells,[9,10] as a non-invasive, reliable blood biomarker specific for GVHD of the GI tract with diagnostic and prognostic utility.[11]

The research effort that identified REG3α used a proteomics approach that analyzed pooled plasma samples from 10 patients with biopsy-proven GI GVHD and 10 patients without GVHD taken at similar times after HCT.[12,13] We identified and quantified 562 proteins, of which 74 were increased at least 2-fold in patients with GVHD. Of the 5 proteins preferentially expressed in the GI tract, only one had commercially available antibodies suitable for quantification of plasma concentrations by ELISA. This process identified REG3α as the lead candidate for a biomarker of GI GVHD.

We validated REG3α as a biomarker by measuring plasma concentration in samples from 850 allogeneic HCT recipients from the University of Michigan, USA. Plasma REG3α concentrations were 3 times higher in patients at the onset of GI GVHD than in all other patients, including those with non-GVHD enteritis. Serum REG3α concentrations were also higher in GI GVHD in an independent validation set of 143 HCT patients from Regensburg, Germany, and Kyushu, Japan, although the absolute values were lower. This difference may be due to a center effect that depends on several factors, including variations in transplant conditioning regimens and supportive care. For example, all patients in Regensburg and Kyushu received oral antibiotics as GVHD prophylaxis, whereas Michigan patients did not, and thus increased

GI flora might account for greater REG3α secretion.

We next analyzed REG3α concentrations according to diagnosis and type of GI symptom in all 1000 patients. In patients with diarrhea caused by GVHD, REG3α concentrations at the onset of GVHD were 5 times higher than in patients with diarrhea from other causes. REG3α concentrations at the onset of symptoms continued to distinguish between GVHD and non-GVHD etiologies in patients with both small and large volumes of diarrhea.

The clinical utility of any biomarker increases if it provides prognostic information regarding the future status of a disease and/or patient, e.g. the likelihood of response to treatment. We, therefore, evaluated the prognostic significance of REG3α plasma levels in more than 160 patients taken at the time of diagnosis of lower GI GVHD. Four weeks is a common time point at which to evaluate response.[14] REG3α concentrations were 3-fold lower at the time of GVHD diagnosis in patients who did not respond *versus* those in patients who experienced a complete or partial response to therapy (635±132 ng/mL *vs.* 240±61 ng/mL; $P<0.001$). Patients responding to therapy still exhibited REG3α concentrations more than 3 times that of non-GVHD controls (77±22 ng/mL; $P<0.001$). Because the response to treatment at four weeks strongly correlates with non-relapse mortality (NRM), we hypothesized that the REG3α concentration at GVHD diagnosis would also correlate with NRM. We, therefore, divided the patients into two equal groups based upon the median REG3α concentration. NRM was twice as high in patients with high REG3α concentrations, and this difference remained significant after adjusting for known risk factors of donor type, degree of HLA match, conditioning intensity, age, and baseline disease severity: 59% (95%CI: 4%-69%) *vs.* 34% (95%CI: 2%-46%); $P<0.001$).

Data regarding the clinical stage, histological grade, REG3α concentration, and level of GVHD at onset were available in 140 patients. The plasma concentration of REG3α (above *vs*. below the median), the clinical severity of GVHD (stage 1 *vs*. stage 2-4), and the histological severity (stage 1-3 *vs*. stage 4) at the time of GVHD diagnosis independently predicted both lack of response to GVHD therapy and 1-year NRM after adjustment for the aforementioned risk factors. When lack of response to therapy and NRM were modeled simultaneously on all four parameters, all the three parameters remained statistically significant. The inclusion of all three characteristics that remained statistically significant on simultaneous modeling demonstrated that patients with increasing numbers of risk factors present at onset had increasing risk for NRM.

Paneth cells are major producers of REG3α. Given the importance of these biomarkers, we evaluated 118 patients who had duodenal biopsies obtained at the time of GVHD-related diarrhea, and 15 patients who had minimal or no GI symptoms but had small duodenal biopsies available. We quantitated the number of Paneth cells per high powered field (HPF) and found a very strong statistical correlation between the number of Paneth cells per HPF and the clinical severity of GI GVHD at the time of biopsy ($P<0.0001$) (Figure 1). Contrary to our hypothesis, however, the number of Paneth cells *decreased* as the severity of GVHD increased. Likewise, small intestine pathological grade also correlated with clinical GVHD severity (*data not shown*). We next determined whether Paneth cell number or pathological grade correlated better with clinical GVHD severity. Proportional odds logistical regression analysis demonstrated that Paneth cell numbers retained a strong correlation with GVHD severity after accounting for pathological grade ($P=0.008$), but pathological grade did not correlate with GVHD severity after Paneth cell numbers were accounted for ($P=0.27$). Thus Paneth cell number in the duodenum correlates better with clinical GI GVHD severity than histological grade.

Patients with a complete response (CR) to therapy also had significantly more Paneth cells than patients with a partial response (PR); patients with no response (NR) had the fewest Paneth cells corresponding to a highly significant difference among the three treatment responses ($P<0.0001$) (Figure 2). The number of Paneth cells also strongly correlated with maximum GI GVHD severity (*data not shown*).

We found that patients whose duodenal biopsies contained a mean of 4 or fewer Paneth cells at the onset of GVHD were more than twice as likely to die from GVHD-related causes as compared to patients with 5 or more Paneth cells (55% *vs.* 23%; $P<0.0001$). This large difference in GVHD-related mortality translated into a significant difference in overall survival at six months from the onset of GI GVHD (16% *vs.* 42%, $P=0.01$).

The current GI GVHD pathological grading system has major limitations. First, the correlation between severity of colonic changes and clinical GVHD severity is poor and so many pathologists do not report pathological grade in order to avoid confusion by clinicians. Second, the grading system has not been standardized. Third, there is no definition as to how to resolve the variable severity within biopsy sections from the same patient. These findings demonstrate that a straightforward quantification of Paneth cell number can help establish the diagnosis of GI GVHD and has prognostic importance.

Recent research supports the correlation between Paneth cells and GI GVHD, and our observations provide insights into physiological mechanisms and suggest Paneth cells secrete α-defensins and REG3α, which are antimicrobial peptides. These peptides function to regulate the microbiota of the intestine through selective activity against non-commensal bacteria, while generally sparing commensal

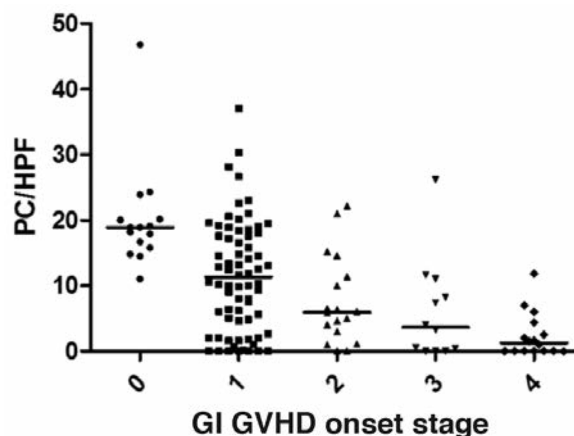

Figure 1. Paneth cell count by clinical GI GVHD onset stage.

bacteria. A loss of intestinal microbiota diversity, characterized by expansion of Lactobacillus species and contracture of the commensal bacteria flora, has been observed in both mice and patients who developed GVHD.[15] In a separate study, mice with GVHD exhibited decreased numbers of Paneth cells, similar to our observations in clinical GVHD.[16] Other immune-mediated inflammatory conditions, such as inflammatory bowel disease and reduced Paneth cell numbers characterize and correlate with changes in the intestinal microbiota.[17,18] Interestingly, plasma concentration of REG3α and Paneth cell numbers do not correlate with each other (*data not shown*), suggesting that these two parameters measure different biological processes, and that Paneth cells may not be the primary drivers of the increased appearance of REG3α in the plasma. Paneth cells are not normally present in the distant colon, although they are found in the proximal colon close to the ileal-cecal junction. This focus on changes in the small intestine has precedent in other diseases, where small intestinal pathology can account for the diarrhea observed in viral gastroenteritis,[19] celiac disease,[20] and some cases of bacterial overgrowth.[21] Nonetheless, although Paneth cell quantification eliminates much of the subjectivity and variability within the current GI GVHD pathological grading system, incorporation of this measure into GI GVHD management will require acquisition of duodenal biopsies. The additional expense and risk associated with either upper GI endoscopy or colonoscopy that includes the terminal ileum may be offset by improved risk stratification early in the GI GVHD course, a time when interventions are most likely to be effective.

The surprising inverse relationship between Paneth cell number and REG3α concentrations led to a further examination of the role of Paneth cells and REG3α in a well-defined animal model of acute GVHD. B6D2F1 mice received 12 Gy total body irradiation on Day 1 and on Day 0 were injected intravenously (iv) with 5 million BM cells and 2.5 million B6 spleen cells. Syngeneic B6 BMT recipients served as non-GVHD controls. Mice were killed on Day +14 and samples of the ileum were scored for GVHD, as for human samples. GVHD scores were 2.3±0.4 in the allogeneic BMT group and 1.0±0.1 in the syngeneic group (P<0.001). As shown in Figure 2, Paneth cells were readily identified by immuno-histochemical (IHC) staining of lysozyme. On Day +14, GVHD caused an 80% decrease in Paneth cell numbers. Similarly, GVHD caused more than an over 95% decrease in REG3α protein. Thus, the number of Paneth cells decreased dramatically in both clinical and experimental GVHD. Yet, despite this decrease, the levels in the plasma increased.

One possible explanation for this paradox could be that the correlation of mucosal denudation with high REG3α concentrations suggests that microscopic breaches in the mucosal epithelial barrier caused by severe GVHD permit REG3α to traverse into the systemic circulation. The tight proximity of Paneth cells with ISCs concentrates their secretory contents in that vicinity, so that mucosal barrier disruption caused by stem cell dropout may preferentially allow Paneth cell secretions, including REG3α, to enter into the bloodstream. REG3α is a large molecule that is concentrated in the mucus covering the apical surface of the cells so that when the cell dies, highly concentrated REG3α translocates into the circulation system (Figure 3; REG3a is represented by the red circle). Plasma levels of REG3α may, therefore, serve as a surrogate marker for the cumulative area of these breaches to GI mucosal barrier integrity, a parameter impossible to measure by individual tissue biopsies and current endoscopic technology. Such an estimate of total damage to the mucosal barrier may also help explain the prognostic value of REG3α with respect to therapy responsiveness and NRM.

The importance of REG3α as an anti-microbial peptide refocuses our attention on the potential role of the microbiota in the pathogenesis of GI GVHD. The importance of the GI microbial flora to systematic GVHD was noted almost forty years ago when germ-free mice developed significantly less GVHD.[22,23] Clinical trials confirmed these findings and led to the use of oral non-absorbable antibiotics as part of GVHD prophylaxis.[24,25] As mentioned above, the crypts are the primary sites of GVHD damage in the GI tract, and intestinal stem cells (ISCs) appear to be the principal cellular targets because their damage amplifies systemic GVHD, and their protection through wnt signals reduces alloreactive cascade of

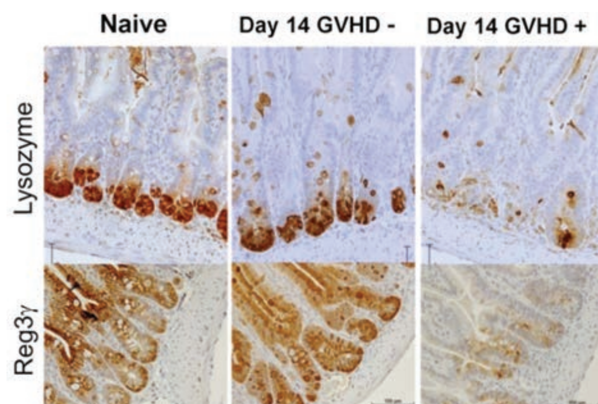

Figure 2. GVHD damage to GI mucosal immunity.

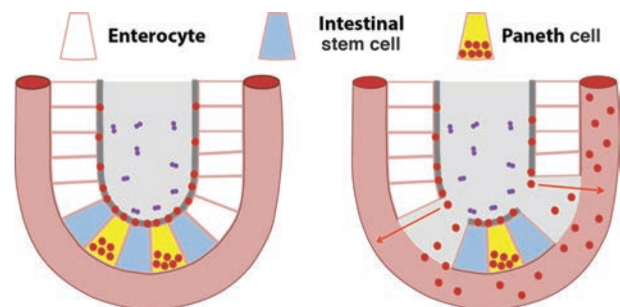

Figure 3. REG3α translocation into the bloodstream during GVHD.

GVHD.[26] ISCs reside in the crypts juxtaposed to Paneth cells, and Paneth cells are key to ISC function *in vitro*,[27,28] giving rise to their description as "guardians of ISCs".[29] Paneth cells secrete antimicrobial peptides such as alpha defensins and regenerative3 alpha (REG3α) that sterilize the mucus covering the luminal surface of enterocytes, creating a protective barrier.[30]

References

1. Martin PJ, McDonald GB, Sanders JE, Anasetti C, Appelbaum FR, Deeg HJ, et al. Increasingly frequent diagnosis of acute gastrointestinal graft-versus-host disease after allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transpl. 2004;10(5):320-7.
2. MacMillan ML, Weisdorf DJ, Wagner JE, DeFor TE, Burns LJ, Ramsay NK, et al. Response of 443 patients to steroids as primary therapy for acute graft-versus-host disease: comparison of grading systems. Biol Blood Marrow Transpl. 2002;8(7):387-94.
3. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transpl. 1995;15(6):825-8.
4. Shulman HM, Kleiner D, Lee SJ, Morton T, Pavletic SZ, Farmer E, et al. Histopathologic diagnosis of chronic graft-versus-host disease: National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: II. Pathology Working Group Report. Biol Blood Marrow Transpl. 2006;12(1):31-47.
5. Mowat A, Socie G. Intestinal Graft-vs.-Host Disease. In: Ferrara JLM, Cooke KR, Deeg HJ (eds.) Graft-vs-Host Disease. 3rd ed. New York: Marcel Dekker; 2004. p. 279-327.
6. Ertault-Daneshpouy M, Leboeuf C, Lemann M, Bouhidel F, Ades L, Gluckman E, et al. Pericapillary hemorrhage as criterion of severe human digestive graft-versus-host disease. Blood. 2004;103(12):4681-4.
7. Weisdorf D, Haake R, Blazar B, Miller W, McGlave P, Ramsay N, et al. Treatment of moderate/severe acute graft-versus-host disease after allogeneic bone marrow transplantation: an analysis of clinical risk features and outcome. Blood. 1990;75(4):1024-30.
8. Deeg HJ. How I treat refractory acute GVHD. Blood. 2007;109(10):4119-26.
9. Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science. 2006;313(5790):1126-30.
10. Gironella M, Iovanna JL, Sans M, Gil F, Penalva M, Closa D, et al. Anti-inflammatory effects of pancreatitis associated protein in inflammatory bowel disease. Gut. 2005;54(9):1244-53.
11. Ferrara JL, Harris AC, Greenson JK, Braun TM, Holler E, Teshima T, et al. Regenerating islet-derived 3 alpha is a biomarker of gastrointestinal graft-versus-host disease. Blood. 2011;118(25):6702-8.
12. Faca V, Pitteri SJ, Newcomb L, Glukhova V, Phanstiel D, Krasnoselsky A, et al. Contribution of protein fractionation to depth of analysis of the serum and plasma proteomes. J Proteome Res. 2007;6(9):3558-65.
13. Paczesny S, Braun TM, Levine JE, Hogan J, Crawford J, Coffing B, et al. Elafin is a biomarker of graft-versus-host disease of the skin. Science Translational Medicine. 2010;2(13):13ra2.
14. Levine JE, Logan B, Wu J, Alousi AM, Ho V, Bolanos-Meade J, et al. Graft-versus-host disease treatment: predictors of survival. Biol Blood Marrow Transplant. 2010;16(12):1693-9.
15. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. J Exp Med. 2012;209(5):903-11.
16. Eriguchi Y, Takashima S, Oka H, Shimoji S, Nakamura K, Uryu H, et al. Graft-versus-host disease disrupts intestinal microbial ecology by inhibiting Paneth cell production of α-defensins. Blood. 2012;120(1):223-31.
17. Gersemann M Wehkamp J, Stange EF. Innate immune dysfunction in inflammatory bowel disease. J Intern Med. 2012;271(5):421-8.
18. Bevins CL Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol. 2011;9(5):356-68.
19. Baldi F Bianco MA, Nardone G, Pilotto A, Zamparo E. Focus on acute diarrhoeal disease. World J Gastroenterol 2009;15(27):3341-8.
20. Bao F Green PH, Bhagat G. An update on celiac disease histopathology and the road ahead. Arch Pathol Lab Med 2012;136(7):735-45.
21. Pyleris E Giamarellos-Bourboulis EJ, Tzivras D, Koussoulas V, Barbatzas C, Pimentel M. The prevalence of overgrowth by aerobic bacteria in the small intestine by small bowel culture: relationship with irritable bowel syndrome. Dig Dis Sci. 2012;57(5):1321-9.
22. Jones JM, Wison R, Bealmear PM. Mortality and gross pathology of secondary disease in germfree mouse radiation chimeras. RadiatRes. 1971;45:577.
23. van Bekkum DW, Roodenburg J, Heidt PJ, van der Waaij D. Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. J Natl Cancer Inst. 1974;52(2):401-4.
24. Storb R, Prentice RL, Buckner CD, Clift RA, Appelbaum F, Deeg J, et al. Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. N Engl J Med. 1983;308(6):302-7.
25. Beelen DW, Haralambie E, Brandt H, Linzenmeier G, Muller KD, Quabeck K, et al. Evidence that sustained growth suppression of intestinal anaerobic bacteria reduces the risk of acute graft-versus-host disease after sibling marrow transplantation. Blood. 1992;80(10):2668-76.
26. Takashima S, Kadowaki M, Aoyama K, Koyama M, Oshima T, Tomizuka K, et al. The Wnt agonist R-spondin1 regulates systemic graft-versus-host disease by protecting intestinal stem cells. J Exp Med. 2011;208(2):285-94.
27. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature. 2009;459(7244):262-5.
28. Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature. 2011;469(7330):415-8.
29. Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol. 2003;3(9):710-20.
30. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science. 2011;334(6053):255-8.

Markers to predict relapse of acute myeloid leukemia

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A B S T R A C T

With the introduction of novel genomic technologies, a large number of genetic alterations have been identified in acute myeloid leukemia (AML) in the last decade. These genetic lesions, that in part define clinicopathological entities, have been shown to be one of the most informative prognostic factors and may be used for risk-adapted therapeutic approaches. Beyond the diagnosis of genetic risk factors at diagnosis, monitoring of minimal residual disease using quantitative polymerase chain reaction (RQ-PCR) has become a powerful tool to identify patients who are at high risk of relapse. Molecular markers that predict higher rates of relapse include *FLT3* internal tandem duplications, mutations of *RUNX1*, *ASXL1*, and *TP53* genes, or deregulated expression of the *EVI1* gene. Some of these molecular markers may have predictive value in that they identify patients for whom allogeneic hematopoietic stem cell transplantation (HSCT) may be a good treatment option in first-line therapy. In addition, novel therapies are now being evaluated that target some of these mutations or their deregulated downstream signaling pathway. One prominent example is the development of FLT3 inhibitors for patients with *FLT3*-mutated AML. This review will discuss the role of selected genetic markers in refining the decision making process for risk-adapted treatment approaches.

Learning goals

At the conclusion of this activity, participants should:
- have an overview on genetic markers that are known to predict relapse in patients with acute myeloid leukemia;
- appreciate the role of minimal residual disease assessment by quantitative RQ-PCR for predicting relapse;
- discuss allogeneic hematopoietic stem cell transplantation as a treatment option for patients with poor prognostic genetic markers.

Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous clonal disorder characterized by the accumulation of somatically acquired genetic changes in hematopoietic progenitor cells that disturb normal mechanisms of self-renewal, proliferation, and differentiation. In recent years, gene mutations and deregulated expression of genes have been identified that gave new insights into the molecular pathogenesis and the enormous clinical heterogeneity of the disease.[1] With progress in genomics technology, in particular the next-generation sequencing (NGS) techniques, a systematic characterization of AML genomes has become possible, and we can expect that virtually all acquired gene mutations will be known in the near future. Looking at the function of genes, it has become clear that involved genes not only play a role in proliferation and differentiation, but also in a variety of other cellular processes such as epigenetic regulation, DNA repair, RNA splicing, or the splicesome complex.[2-9] The novel NGS techniques also allow us to unravel the complex clonal architecture that is found in many, if not most cases, of AML. Beyond the disease-founding mutation, various subclonal mutations may be present at the time of diagnosis that are either eradicated by therapy, or undergo selection and clonal expansion. Moreover, new mutations may be acquired leading to clonal evolution of the disease.[10-12] Both clonal expansion and evolution may eventually contribute to chemotherapy resistance at the time of relapse.

From a clinical point of view, genetic markers have acquired great value. First, the current WHO classification of AML reflects the fact that an increasing proportion of AML can be classified on the basis of their underlying genetic defects that define clinicopathological entities.[13] Second, both cytogenetic and molecular genetic lesions have been shown to be one of the most informative prognostic factors and may be used for risk-adapted therapeutic approaches.[1,14,15] Table 1 shows the standardized reporting system for risk classification that was established by an international group of experts on behalf of the European LeukemiaNet.[14] Finally, novel therapies are being developed that target some of these gene mutations or their deregulated downstream signaling pathway. One prominent example is the clinical development of FLT3 inhibitors for AML with activating

FLT3 mutations.[16] Nonetheless, there is a time gap between the rapid development in unraveling the molecular pathogenesis and the successful clinical development of such molecularly targeted therapies.

The backbone of conventional chemotherapy in AML has more or less remained unchanged over the last 20 years.[14] Risk-adapted therapeutic approaches have been limited to the question as to whether a patient should be recommended to proceed to allogeneic hematopoietic stem cell transplantation (HSCT) or not. Allogeneic HSCT is associated with the lowest rates of relapse but benefits of allogeneic HSCT may be outweighed by high treatment-related mortality (TRM). Thus, in the decision-making process it is generally recommended to consider both AML-related prognostic factors, best assessed by the genetic profile, and factors predicting HSCT-related non-relapse mortality.[17]

Beyond the identification of genetic risk factors at the time of diagnosis, monitoring of minimal residual disease (MRD) using sensitive quantitative polymerase chain reaction (RQ-PCR) assays has become an important tool to identify patients at very high risk of relapse.[18]

This review will focus on selected genetic markers in AML, assessed at the time of diagnosis or in a time-dependent manner using MRD monitoring, that are associated with higher rates of relapse and that have contributed to refining the decision-making process for risk-adapted and pre-emptive therapy.

Molecular markers to predict relapse in AML

With respect to the molecular markers, so far only screening of *NPM1, CEBPA*, and *FLT3* mutations has entered clinical practice affecting diagnosis, prognosis, and guidance of therapy.[14] Therefore, these markers are currently recommended to be analyzed in clinical trials and routine practice, at least in patients who will receive treatment other than low-dose chemotherapy and/or best supportive care. Nevertheless, additional molecular markers have been identified that are associated with an increased risk of relapse, for example, mutations of *RUNX1*,[19-22] *ASXL1*,[23-27] *TP53*,[28-30] or deregulated expression of the EVI1 gene.[31,32] Some of these markers have been shown to have predictive value, in that they identify patients who may benefit from allogeneic HSCT. However, it should be taken into account that most of these data stem from retrospective analyses, often involving small cohorts of patients. Therefore, validation of these findings by further retrospective or prospective studies will be needed before these markers can be used in clinical routine for guidance of therapy.

FLT3 internal tandem duplications

Activating mutations of the tyrosine kinase receptor FLT3 are diagnosed in approximately 30% of adult patients with AML.[1] Mutations are found in two functional domains of the receptor, the juxtamembrane domain (JM) and the tyrosine kinase domain (TKD). The most frequent (approx. 25%) type of mutations are internal tandem duplications (ITD) that cluster in the JM domain; more recently, it was found that approximately 30% of the ITDs insert within the TK1 domain of the receptor.[33,34] The activation loop in the carboxy-terminal lobe of the TKD is affected by point mutations, small insertions, or deletions, mainly at codons 835 and 836, in 5-10% of AML.

When treated with conventional chemotherapy, prognosis of AML with *FLT3*-ITD is significantly worse compared with AML without the mutation. The evidence is most compelling in cytogenetically normal (CN)-AML. Besides the mere presence of the ITD, the ratio of the mutated versus wild-type allele is related to outcome in that a high burden of the mutated allele predicts for particularly poor outcome;[35-40] in few cases, there is homozygosity for the mutated allele, arising from somatic recombination leading to uniparental disomy (UPD).[41] Furthermore, AML with *FLT3*-ITD that inserts within the TK1 domain appear to be significantly worse compared to AML with *FLT3*-ITD located in the JM-domain.[34] The prognostic significance of *FLT3*-TKD mutations has remained controversial.

There are two important aspects to be considered in guiding therapy in AML with *FLT3*-ITD. First, whenever possible patients should be entered on a trial evaluating FLT3 inhibitors. The compound most advanced in clinical development is midostaurin (PKC412). Based on encouraging results of a Phase Ib study with midostaurin,[42] a large international randomized trial was conducted that reached its accrual goal of 719 patients with *FLT3*-mutated AML in September 2011 (*clinicaltrials.gov identifier: NCT00651261*). If positive, these results will impact the treatment of patients with AML and *FLT3* mutations. Quizartinib (AC220) is a novel 2nd generation compound expressly developed as an FLT3 inhibitor for the treat-

Table 1. Standardized reporting for correlation of cytogenetic and molecular genetic data in AML with clinical data.*

Genetic group	Subsets
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Mutated *CEBPA* (normal karyotype)
Intermediate-I†	Mutated *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); *MLLT3*-MLL Cytogenetic abnormalities not classified as favorable or adverse‡
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* t(6;9)(p23;q34); *DEK-NUP214* t(v;11)(v;q23); *MLL* rearranged -5 or del(5q); -7; abnl(17p); complex karyotype§

**Frequencies, response rates and outcome measures should be reported by genetic group and, if sufficient numbers are available, by specific subsets indicated; excluding cases of acute promyelocytic leukemia. †Includes all AMLs with normal karyotype except for those included in the favorable subgroup. Most of these cases are associated with poor prognosis, but they should be reported separately because of the potential different response to treatment. ‡For most abnormalities, adequate numbers have not been studied to allow firm conclusions to be drawn regarding their prognostic significance. §Three or more chromosome abnormalities in the absence of one of the WHO-designated recurring translocations or inversions, i.e. t(15;17), t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23), t(6;9), inv(3)/t(3;3); indicate how many complex karyotype cases have involvement of chromosome arms 5q, 7q, and 17p. Adopted from Döhner et al.[14]*

ment of AML.[43] In phase II studies, quizartinib given as a single agent has proven to be highly efficacious, leading to high response rates in relapsed and refractory AML exhibiting *FLT3*-ITD.[44,45] Second, there is increasing evidence that patients with *FLT3*-ITD positive AML may benefit from allogeneic HSCT in first complete remission (CR), although the mutation remains an important prognostic factor even in the context of allogeneic transplantation. In a donor *versus* no-donor analysis performed by the German-Austrian AML Study Group (AMLSG), a beneficial effect of allogeneic HSCT was found for younger patients with CN-AML with unfavorable molecular genotypes that included AML with *FLT3*-ITD.[46] These data are supported by other studies and by a recent retrospective analysis of the European Group for Blood and Marrow Transplantation (EBMT).[47,48]

Thus, although evidence from prospective trials is not available, allogeneic HSCT should be considered in patients whose leukemic cells have *FLT3*-ITD. An attractive future approach will be to combine the concepts of allogeneic HSCT and pharmacological FLT3 inhibition, before and after transplantation. Such a trial was recently initiated by the AMLSG (*clinicaltrials.gov identifier: NCT01477606;* Figure 1).

RUNX1 mutations

The gene encoding *runt-related transcription factor 1* (*RUNX1*) is targeted by chromosomal rearrangements such as t(8;21)(q22;q22) and intragenic mutations. There are only a few studies evaluating the frequency and clinical impact of intragenic *RUNX1* mutations in AML. Mutations are relatively infrequent, varying between 5.6% and 13.2%, and in all studies *RUNX1* mutations have been associated with inferior outcome.[19-22] In the Taiwanese study, *RUNX1* mutations predicted for lower CR rate and shorter disease-free (DFS) and overall survival (OS); in multivariate analysis, *RUNX1* mutations were an independent prognostic factor for OS.[19] A negative impact on DFS, OS and event-free-survival (EFS) for both younger (age < 60 years, n=175) and older (age ≥ 60 years, n=225) CN-AML patients was also described by the Cancer and Leukemia Group B (CALBG).[22] *RUNX1* mutations were associated with *ASXL1* mutations and inversely correlated with *NPM1* and *CEBPA* mutations, and its negative impact persisted also in *ASXL1*-wild-type patients. In a study by the AMLSG of 945 unselected younger adult AML patients, *RUNX1* mutations were found in only 5.6%.[21] *RUNX1* mutations clustered in the intermediate-risk cytogenetic group (CN-AML 6.3%) and were significantly associated with *MLL* partial tandem duplications and with *IDH1/IDH2* mutations. Mutations predicted for resistance to chemotherapy as well as inferior EFS, relapse-free survival (RFS), and OS. Explorative analysis revealed that allogeneic HSCT had a favorable impact on RFS in patients with *RUNX1* mutations (Figure 2A), demonstrating that allogeneic transplantation may have a role in the management of these patients.[21]

Deregulated *EVI1* expression

In addition to structural genetic lesions, changes in expression of specific genes impact prognosis. Deregulated expression of *EVI1* (ecotropic viral integration site 1) is found in all AML cases with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) leading to rearrangement of the *RPN1* and *EVI1* genes.[31,32] *EVI1* overexpression is not restricted to this entity, but is also found in approximately 10% of unselected AML as

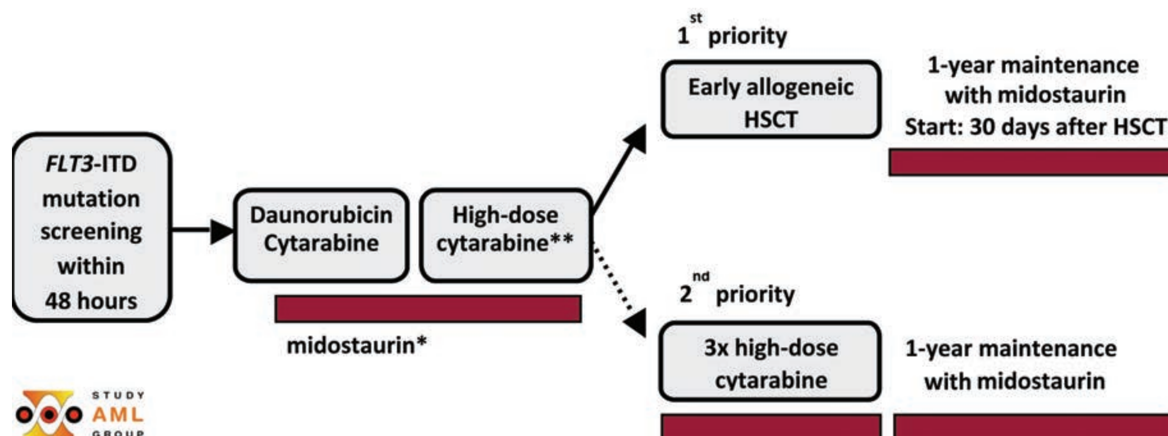

Figure 1. Design of the AMLSG 16-10 clinical trial in AML with *FLT3*-ITD combining the concepts of pharmacological FLT3 inhibition using midostaurin and allogeneic hematopoietic stem cell transplantation (HSCT) as first priority for postremission therapy (*clinicaltrials.gov identifier: NCT01477606*). *Midostaurin: start on Day 8, thereafter continuous dosing, only interrupted on days of chemotherapy. **Optional consolidation before allogeneic HSCT.

assessed by RQ-PCR.[31,32] *EVI1* expression is associated with specific cytogenetic subsets, in particular monosomy 7 and translocations involving the *MLL* locus at chromosomal band 11q23 [t(11q23)], while expression is virtually absent in favorable subsets, such as core-binding factor (CBF)-AML and AML with *NPM1* mutation.[32] In a large study of 1,382 younger adult AML patients performed by the Dutch-Belgian Hemato-Oncology Cooperative Group and the AMLSG, *EVI1* expression was associated with inferior outcome, in particularly in cytogenetic intermediate-risk AML. Interestingly, patients with deregulated *EVI1* expression who received allogeneic HSCT in first CR had significantly better 5-year RFS (Figure 2B).[32]

As already mentioned, *EVI1* overexpression is frequently found in the subgroup of AML with t(11q23). A recent study specifically analyzed the frequency and clinical impact of *EVI1* expression in a cohort of 286 AML with t(11q23).[49] *EVI1* overexpression was found in 45.8% of all patients with t(11q23), with t(6;11) showing the highest frequency (83.9%), followed by t(9;11) at 40.0%, and t(v;11q23) at 34.8%. *EVI1* overexpression was the sole prognostic factor for all survival endpoints, that is, EFS, RFS, and OS, in all AML with t(11q23) as well as in the subset of AML with t(9;11). *EVI1* overexpressing AML with t(11q23) in first CR had a significantly better RFS after allogeneic HSCT compared with other consolidation therapies (Figure 2C) resulting in a significantly better long-term outcome (5-year OS, 54.7% vs. 0%).[49]

These two studies, although retrospective in nature, provide evidence that patients with this molecular marker significantly benefit from allogeneic HSCT in first-line treatment.

Complex karyotype, monosomal karyotype, and *TP53* mutations

AML with complex karyotype (CK) and AML with monosomal karyotype (MK) are descriptive in nature and do not define distinct disease entities.[50,51] Complex karyotype is defined by the presence of 3 or more chromosome abnormalities in the absence of aberrations defined in the WHO category "AML with recurrent genetic abnormalities". A new descriptive cytogenetic category was proposed that identifies AML of particularly unfavorable risk, that is, the monosomal karyotype.[51] In this study, the MK was defined by the presence of one single monosomy (excluding isolated loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding CBF-AML). The majority of AML with MK also falls into the category of AML with CK. However, in the initial study by Breems *et al.*, AML with MK outperformed AML with CK as poor prognostic markers. This effect was confirmed in subsequent studies.[52-55]

The non-random pattern of chromosome abnormalities in AML with CK includes a predominance of unbalanced rearrangements.[30,50] One of the prominent features is the frequent loss of 17p and/or *TP53* gene mutation occurring in approximately 70% of the cases.[28-30] Loss of 17p/*TP53* mutation in turn is highly correlated with the presence of the MK.[30] In a recent study of a large cohort of CK-AML, the MK lost its prognostic power when adjusted for the variable loss of 17p/*TP53* mutation.[30] Thus, loss of TP53

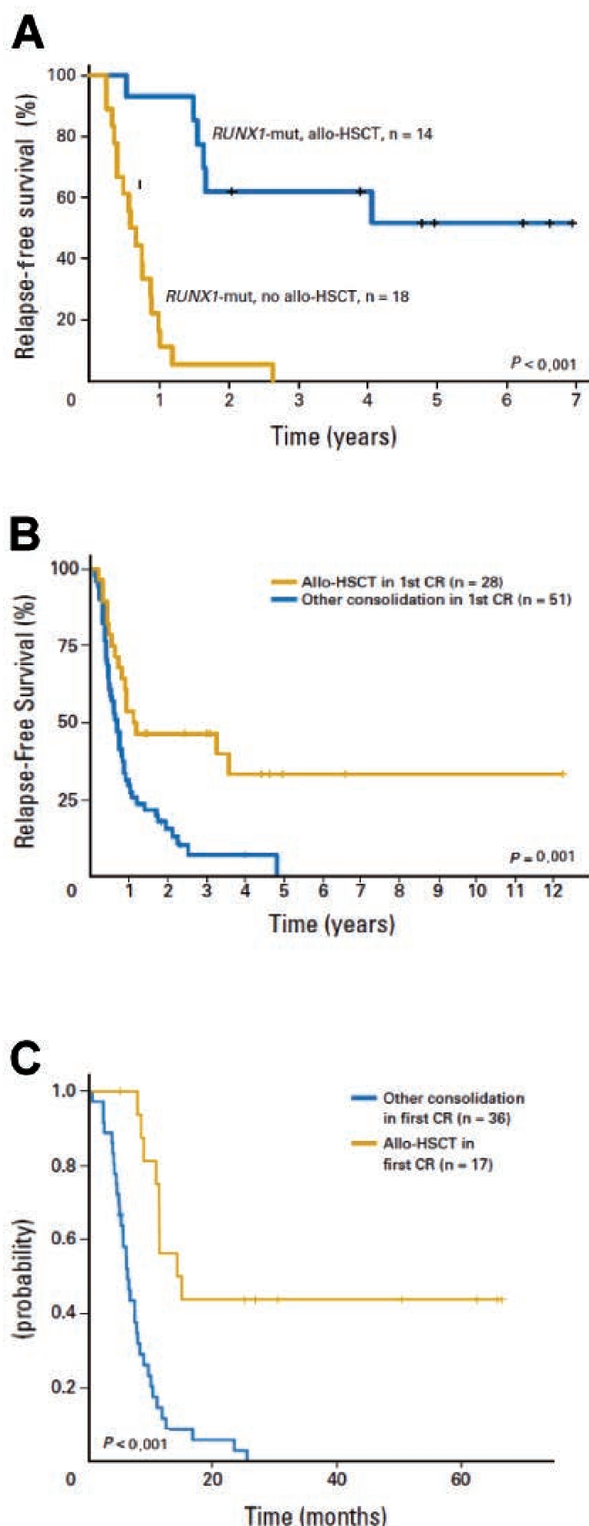

Figure 2. Role of allogeneic hematopoietic stem cell transplantation (HSCT) in molecular subsets of AML. Relapse-free survival after allogeneic HSCT in first complete remission: (A) according to *RUNX1* mutation status;[21] (B) in *EVI1*+ AML as compared to other consolidation;[32] and (C) in the *EVI1*+ AML subset with t(11q23) as compared to other consolidation.[49]

function by *TP53* loss and/or mutation is an important predictor of very poor prognosis.

In general, allogeneic HSCT is advocated as the treatment of choice for younger adult patients with intermediate-I/II and high-risk genetics (according to the European LeukemiaNet classification) who achieve CR and provided an appropriate donor is available.[17,56-59] This also applies for patients whose leukemic cells exhibit a CK. More recent studies specifically looked at the effect of allogeneic HSCT in patients with MK-AML.[54,60-64]

In the study by HOVON-SAKK of patients under 61 years of age, allogeneic HSCT, applied as consolidation in CR1, was associated with a significant reduction in relapse and improvement in survival, with the same relative reduction in relapse and death as in other cytogenetic risk categories.[62] The 5-year OS was 19% for patients with MK-AML after allogeneic HSCT compared with 9% for those who received chemotherapy or autologous HSCT. Positive effects of allogeneic HSCT in patients with MK-AML were also found in retrospective analyses performed by the AMLSG and investigators at the Fred Hutchinson Cancer Center.[54,61] In a study of a small cohort of patients with loss of 17p/*TP53* mutation, allogeneic HSCT did not improve outcome.[30]

In summary, although in these studies looking at the effect of allogeneic HSCT in patients with MK-AML there was some improvement in survival end points, the beneficial effects appear to be marginal, stressing the need for novel therapeutic agents for these poor prognosis AML subsets.

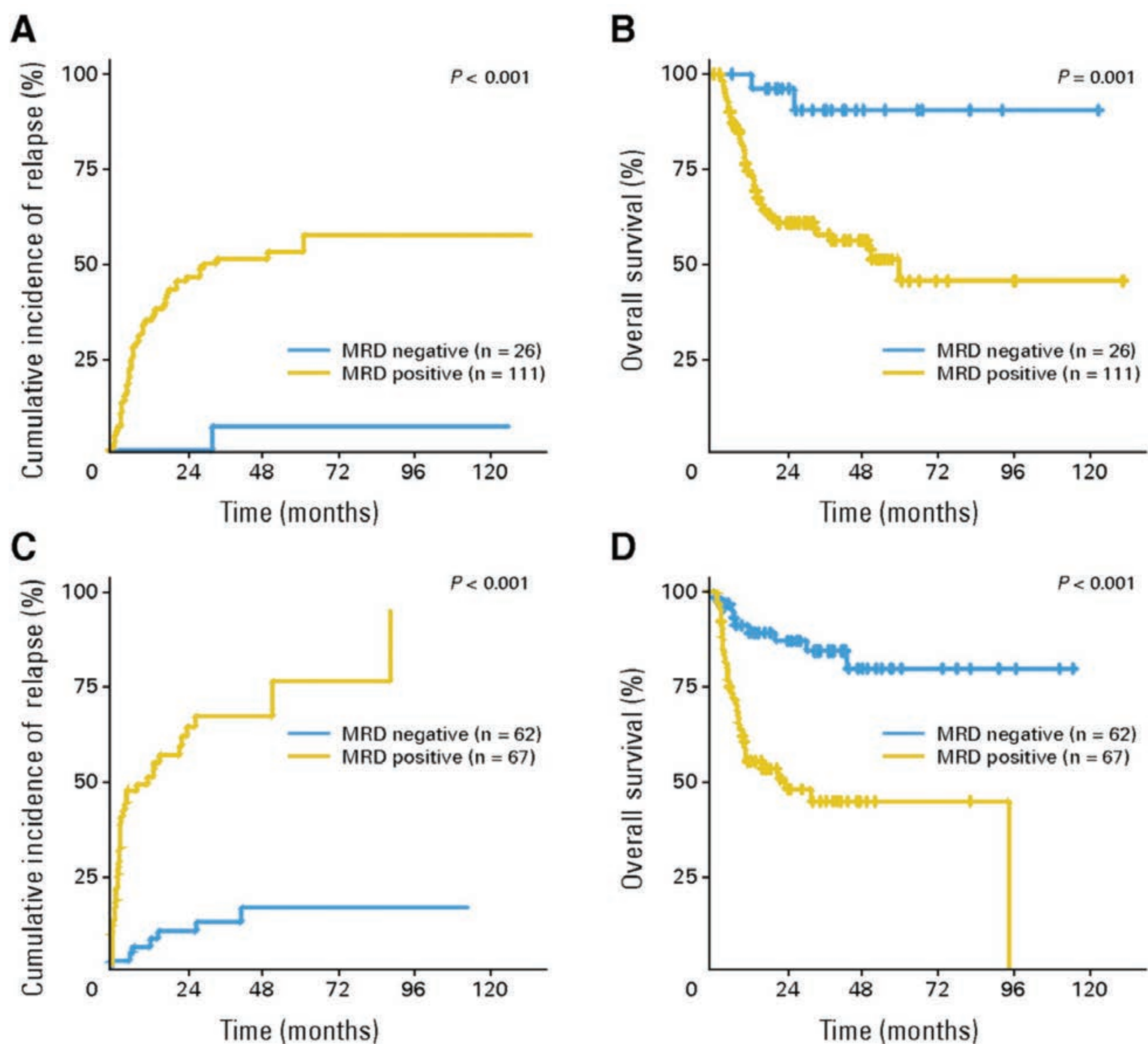

Figure 3. Prognostic relevance of minimal residual disease (MRD) by using mutated *NPM1* as target gene.[70] (A) Cumulative incidence of relapse (CIR) and (B) overall survival (OS) for patients in complete remission according to MRD status after induction therapy in bone marrow (negative *vs.* any positive NPM1 mutation transcript level value). (C) CIR and (D) OS according to MRD status after completion of therapy in bone marrow.

Role of minimal residual disease assessment for relapse prediction

Monitoring of minimal residual disease (MRD) in AML is emerging as a very powerful tool to identify patients who are at high risk of relapse. Monitoring of MRD can be determined either by RQ-PCR detecting leukemia-specific targets, such as gene fusions, gene mutations, and deregulated expression of genes, or by multiparametric flow cytometry identifying leukemia-associated aberrant phenotypes.[18] In the past, in AML the clinical value of MRD monitoring was mainly restricted to acute promyelocytic leukemia. However, more recently, the predictive power has also been shown for other subsets, such as AML with inv(16)(p13.3q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*,[65-67] AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*,[66-68] and AML with *NPM1* mutation.[69,70]

There are various useful applications of MRD monitoring. One of the most informative time points to guide further post-remission therapy is after induction or early consolidation therapy. This also relates to the question as to whether in case of molecular disease persistence the patient should be referred to allogeneic HSCT. Beyond guidance of post-remission therapy, MRD monitoring early on may become a powerful instrument to assess the efficacy of novel, investigational compounds, as a surrogate marker for survival end points. In addition, MRD monitoring can be used post-treatment to diagnose impending relapse and guide pre-emptive therapy.

As a paradigm, *NPM1* mutant alleles have become one of the most interesting molecular targets for MRD, because of their high frequency and the availability of sensitive RQ-PCR assays. In a study by AMSLG,[70] *NPM1* mutant transcript levels were highly predictive for treatment outcome, and clinically relevant time points could be identified (Figure 3). After induction therapy, achievement of RQ-PCR negativity identified patients with a low cumulative incidence of relapse and, therefore, allowed a refined risk assessment. Second, after completion of therapy, *NPM1* mutant transcript levels again were an independent factor for survival. Finally, in the follow-up period, serial MRD measurement allowed early prediction of relapse. Not unexpectedly, the reduction in *NPM1* mutant transcript levels correlated with the *FLT3*-ITD status, with *FLT3*-ITD negative patients achieving a significantly better molecular response.

Given the high power of predicting relapse, monitoring of MRD by RQ-PCR is now being integrated in prospective trials for guidance of therapy, in case of molecular persistence or molecular relapse of the disease.

References

1. Marcucci G, Haferlach T, Döhner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. J Clin Oncol. 2011;29(5):475-86.
2. Ley TJ, Mardis ER, Ding L, Fulton B, McLellan MD, Chen K, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. Nature. 2008;456(7218):66-72.
3. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med. 2009;361 (11):1058-66.
4. Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, et al. Array-based genomic resequencing of human leukemia. Oncogene. 2010;29(25):3723-31.
5. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010;363(25):2424-33.
6. Yan XJ, Xu J, Gu ZH, Pan CM, Lu G, Shen Y, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet. 2011;43(4):309-15.
7. Papaemannuil E, Cazzola M, Boultwood J, Malcovati L, Vyas P, Bowen D, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. N Engl J Med. 2011; 365(15):1384-95.
8. Dolnik A, Engelmann JC, Scharfenberger-Schmeer M, Mauch J, Kelkenberg-Schade S, Haldemann B, et al. Commonly altered genomic regions in acute myeloid leukemia are enriched for somatic mutations involved in chromatin-remodeling and splicing. Blood. 2012;120(18):e83-92.
9. Link DC. Molecular genetics of AML. Best Pract Res Clin. Haematol. 2012;25(4):409-14.
10. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, et al. The origin and evolution of mutations in acute myeloid leukemia. Cell. 2012;150(2):264-78.
11. Walter MJ, Shen D, Ding L, Shao J, Koboldt DC, Chen K, et al. Clonal architecture of secondary acute myeloid leukemia. N Engl J Med. 2012;366(12):1090-8.
12. Ding L, Ley TJ, Larson DE, Miller CA, Koboldt DC, Welch JS, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012;481(7382):506-10.
13. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. eds. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2008.
14. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115(3):453-74.
15. Patel JP, Gönen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, et al. Prognostic relevance of genomic profiling in acute myeloid leukemia. N Engl J Med. 2012;366(12):1079-89.
16. Faith H, Levis M. FLT3 inhibitors: a story of the old and the new. Curr Opin Hematol. 2011;18(2):71-6.
17. Cornelissen JJ, Gratwohl A, Schlenk RF, Sierra J, Bornhäuser M, Juliusson G, et al. The European LeukemiaNet AML Working Party consensus statement on allogeneic HSCT for patients with AML in remission: an integrated-risk adapted approach. Nat Rev Clin Oncol. 2012;9(10):579-90.
18. Grimwade D, Vyas P, Freeman S. Assessment of minimal residual disease in acute myeloid leukemia. Curr Opin Oncol. 2010;22(6):656-63.
19. Tang JL, Hou HA, Chen CY, Liu CY, Chou WC, Tseng MH, et al. AML1/RUNX1 mutations in 470 adult patients with de novo acute myeloid leukemia: prognostic implication and interaction with other gene alterations. Blood. 2009;114(26):5352-61.
20. Schnittger S, Dicker F, Kern W, Wendland N, Sundermann J, Alpermann T, et al. RUNX1 mutations are frequent in de novo AML with noncomplex karyotype and confer an unfavorable prognosis. Blood. 2011;117(8):2348-57.
21. Gaidzik VI, Bullinger L, Schlenk RF, Zimmermann AS, Röck J, Paschka P, et al. RUNX1 mutations in acute myeloid leukemia: results from a comprehensive genetic and clinical analysis from the AML Study Group. J Clin Oncol. 2011;29(10):1364-72.
22. Mendler JH, Maharry K, Radmacher MD, Mrózek K, Becker H, Metzeler KH, et al. RUNX1 mutations are associated with poor outcome in younger and older patients with cytogenetically normal acute myeloid leukemia and with distinct gene and MicroRNA expression signatures. J Clin Oncol. 2012;30(25):3109-18.
23. Gelsi-Boyer V, Trouplin V, Adélaïde J, Bonansea J, Cervera N, Carbuccia N, et al. Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. Br J Haematol. 2009;145(6):788-800.
24. Carbuccia N, Trouplin V, Gelsi-Boyer V, Murati A, Rocquain J, Adélaïde J, et al. Mutual exclusion of ASXL1 and NPM1 mutations in a series of acute myeloid leukemias. Leukemia. 2010;24(2):469-73.
25. Chou WC, Huang HH, Hou HA, Chen CY, Tang JL, Yao M, et al. Distinct clinical and biological features of de novo acute myeloid leukemia with additional sex comb-like 1 (ASXL1)

mutations. Blood. 2010;116(20):4086-94.
26. Metzeler KH, Becker H, Maharry K, Radmacher MD, Kohlschmidt J, Mrózek K, et al. ASXL1 mutations identify a high-risk subgroup of older patients with primary cytogenetically normal AML within the ELN favorable genetic category. Blood. 2011;118(26):6920-9.
27. Schnittger S, Eder C, Jeromin S, Alpermann T, Fasan A, Grossmann V, et al. ASXL1 exon 12 mutations are frequent in AML with intermediate risk karyotype and are independently associated with an adverse outcome. Leukemia. 2013;27(1):82-91.
28. Haferlach C, Dicker F, Herholz H, Schnittger S, Kern W, Haferlach T. Mutations of the TP53 gene in acute myeloid leukemia are strongly associated with a complex aberrant karyotype. Leukemia. 2008;22(8):1539-41.
29. Bowen D, Groves MJ, Burnett AK, Patel Y, Allen C, Green C, et al. TP53 gene mutation is frequent in patients with acute myeloid leukemia and complex karyotype, and is associated with very poor prognosis. Leukemia. 2009;23(1):203-6.
30. Rücker FG, Schlenk RF, Bullinger L, Kayser S, Teleanu V, Kett H, et al. TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. Blood. 2012;119(9):2114-21.
31. Lugthart S, van Drunen E, van Norden Y, van Hoven A, Erpelinck CA, Valk PJ, et al. High EVI1 levels predict adverse outcome in acute myeloid leukemia: prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. Blood. 2008;111(8):4329-37.
32. Gröschel S, Lugthart S, Schlenk RF, Valk PJ, Eiwen K, Goudswaard C, et al. High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. J Clin Oncol. 2010;28(12):2101-17.
33. Breitenbuecher F, Schnittger S, Grundler R, Markova B, Carius B, Brecht A, et al. Identification of a novel type of ITD mutations located in nonjuxtamembrane domains of the FLT3 tyrosine kinase receptor. Blood. 2009;113(17):4074-7.
34. Kayser S, Schlenk RF, Londono MC, Breitenbuecher F, Wittke K, Du J, et al; for the German-Austrian AML Study Group (AMLSG). Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. Blood. 2009;114(12):2386-92.
35. Whitman SP, Archer KJ, Feng L, Baldus C, Becknell B, Carlson BD, et al. Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a Cancer and Leukemia Group B study. Cancer Res. 2001;61(19):7233-9.
36. Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98(6):1752-9.
37. Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99(12):4326-35.
38. Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, et al. The impact of FLT3 internal tandem duplication mutant level, number, size and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood. 2008;111(5):2776-84.
39. Pratcorona M, Brunet S, Nomdedéu J, Ribera JM, Tormo M, Duarte R, et al. Favorable outcome of patients with acute myeloid leukemia harboring a low-allelic burden FLT3-ITD mutation and concomitant NPM1 mutation: relevance to postremission therapy. Blood. 2013 Feb 1. [Epub ahead of print]
40. Kayser S, Schlenk RF, Krauter J, Köhne CH, Germing U, Held G, et al. Prognostic impact of mutant to wild-type ratio and insertion site in acute myeloid leukemia with FLT3 internal tandem duplication. Blood. 2012;120:785(Abstract).
41. Raghavan M, Lillington DM, Skoulakis S, Debernardi S, Chaplin T, Foot NJ, et al. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. Cancer Res. 2005;65(2):375-8.
42. Stone RM, Fischer T, Paquette R, Schiller G, Schiffer CA, Ehninger G, et al. Phase 1B study of the FLT3 kinase inhibitior midostaurin with chemotherapy in younger newly diagnosed adult patients with acute myeloid leukemia. Leukemia. 2012;26(9):2061-8.
43. Zarrinkar PP, Gunawardane RN, Cramer MD, Gardner MF, Brigham D, Belli B, et al. AC220 is a uniquely potent and selective inhibitor of FLT3 for the treatment of acute myeloid leukemia (AML). Blood. 2009;114(14):2984-92.
44. Levis MJ, Perl AE, Dombret H, Döhner H, Steffen B, Rousselot P, et al. Final results of a phase 2 open-label, monotherapy efficacy and safety study of quizartinib (AC220) in patients with FLT3-ITD positive or negative relapsed/refractory acute myeloid leukemia after second-line chemotherapy or hematopoietic stem cell transplantation. Blood. 2012;120:673(Abstract).
45. Cortes JE, Perl AE, Dombret H, Kayser S, Steffen B, Rousselot P, et al. Final results of a phase 2 open-label, monotherapy efficacy and safety of quizartinib (AC220) in patients ≥ 60 years of age with FLT3 ITD positive or negative relapsed/refractory acute myeloid leukemia. Blood. 2012;120:48(Abstract).
46. Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358(18):1909-18.
47. DeZern AE, Sung A, Kim S, Smith BD, Karp JE, Gore SD, et al. Role of allogeneic transplantation for FLT3/ITD acute myeloid leukemia: outcomes from 133 consecutive newly diagnosed patients from a single institution. Biol Blood Marrow Transplant. 2011;17(9):1404-9.
48. Brunet S, Labopin M, Esteve J, Cornelissen J, Socié G, Iori AP, et al. Impact of FLT3 internal tandem duplication on the outcome of related and unrelated hematopoietic transplantation for adult acute myeloid leukemia in first remission: a retrospective analysis. J Clin Oncol. 2012;30(7):735-41.
49. Gröschel S, Schlenk RF, Engelmann J, Rockova V, Teleanu V, Kühn MW, et al. Deregulated expression of EVI1 defines a poor prognostic subset of MLL-rearranged acute myeloid leukemias: a study of the German-Austrian Acute Myeloid Leukemia Study Group and the Dutch-Belgian-Swiss HOVON/SAKK Cooperative Group. J Clin Oncol. 2013;31(1):95-103.
50. Mrózek K. Cytogenetic, molecular genetic, and clinical characteristics of acute myeloid leukemia with a complex karyotype. Semin Oncol. 2008;358(4):365-77.
51. Breems DA, van Putten WLJ, De Greef GE, Van Zelderen-Bhola SL, Gerssen-Schoorl KB, Mellink CH, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. J Clin Oncol. 2008;26(29):4791-7.
52. Medeiros BC, Othus M, Fang M, Roulston D, Appelbaum FR. Prognostic impact of monosomal karyotype in young adult and elderly acute myeloid leukemia: the Southwest Oncology Group (SWOG) experience. Blood. 2010;116(13):2224-8.
53. Perrot A, Luquet I, Pigneux A, Mugneret F, Delaunay J, Harousseau JL, et al. Dismal prognostic value of monosomal karyotype in elderly patients with acute myeloid leukemia: a GOELAMS study of 186 patients with unfavorable cytogenetic abnormalities. Blood. 2011;118(3):679-85.
54. Kayser S, Zucknick M, Döhner K, Krauter J, Köhne CH, Horst HA, et al. Monosomal karyotype in adult acute myeloid leukemia: prognostic impact and outcome after different treatment strategies. Blood. 2012;119(2):551-8.
55. Haferlach C, Alpermann T, Schnittger S, Kern W, Chromik J, Schmid C, et al. Prognostic value of monosomal karyotype in comparison to complex aberrant karyotype in acute myeloid leukemia: a study on 824 cases with aberrant karyotype. Blood. 2012;119(9):2122-5.
56. Cornelissen JJ, van Putten WLJ, Verdonck LF, Theobald M, Jacky E, Daenen SM, et al. Results of a HOVON/SAKK donor versus no-donor analysis of myeloablative HLA-identical sibling stem cell transplantation in first remission acute myeloid leukemia in young and middle-aged adults: benefits for whom? Blood. 2007;109(9):3658-66.
57. Yanada M, Matsuo K, Emi N, Naoe T. Efficacy of allogeneic hematopoietic stem cell transplantation depends on cytogenetic risk for acute myeloid leukemia in first disease remission: a metaanalysis. Cancer. 2005;103(8):1652-8.
58. Koreth J, Schlenk R, Kopecky KJ, Honda S, Sierra J, Djulbegovic BJ, et al. Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. JAMA. 2009;30 1(22):2349-61.
59. Schlenk RF, Döhner K, Mack S, Stoppel M, Király F, Götze K, et al. Prospective evaluation of allogeneic hematopoietic

stem cell transplantation from matched related and matched unrelated donors in younger adults with high-risk acute myeloid leukemia: Results of German-Austrian AMLSG treatment trial AMLHD98A. J Clin Oncol. 2010;28:4642-46488;35(4):449-57.
60. Oran B, Dolan M, Cao Q, Brunstein C, Warlick E, Weisdorf D. Monosomal karyotype provides better prognostic prediction after allogeneic stem cell transplantation in patients with acute myelogenous leukemia. Biol Blood Marrow Transplant. 2011;17(3):356-64.
61. Fang M, Storer B, Estey E, Othus M, Zhang L, Sandmaier BM, et al. Outcome of patients with acute myeloid leukemia with monosomal karyotype who undergo hematopoietic cell transplantation. Blood. 2011;118(6):1490-4.
62. Cornelissen JJ, Breems D, van Putten WL, Gratwohl AA, Passweg JR, Pabst T, et al. Comparative analysis of the value of allogeneic hematopoietic stem-cell transplantation in acute myeloid leukemia with monosomal karyotype versus other cytogenetic risk categories. J Clin Oncol. 2012;30(17):2140-6.
63. Yanada M, Kurosawa S, Yamaguchi T, Yamashita T, Moriuchi Y, Ago H, et al. Prognosis of acute myeloid leukemia harboring monosomal karyotype in patients treated with or without allogeneic hematopoietic cell transplantation after achieving complete remission. Haematologica. 2012;97(6):915-8.
64. Middeke JM, Beelen D, Stadler M, Göhring G, Schlegelberger B, Baurmann H, et al. Outcome of high-risk acute myeloid leukemia after allogeneic hematopoietic cell transplantation: negative impact of abnl(17p) and -5/5q-. Blood. 2012;120(12):2521-8.
65. Corbacioglu A, Scholl C, Schlenk RF, Eiwen K, Du J, Bullinger L, et al. Prognostic impact of minimal residual disease in CBFB-MYH11-positive acute myeloid leukemia. J Clin Oncol. 2010;28(23):3724-9.
66. Yin JA, O'Brien MA, Hills RK, Daly SB, Wheatley K, Burnett AK. Minimal residual disease monitoring by quantitative RT-PCR in core binding factor AML allows risk stratification and predicts relapse: results of the United Kingdom MRC AML-15 trial. Blood. 2012;120(14):2826-35.
67. Jourdan E, Boissel N, Chevret S, Delabesse E, Renneville A, Cornillet P, et al. Prospective evaluation of gene mutations and minimal residual disease (MRD) in patients with core binding factor acute myeloid leukemia (CBF-AML). Blood. 2013 Jan 15. [Epub ahead of print]
68. Döhner K, Corbacioglu A, Eiwen K, Späth D, Germing U, Lübbert M, et al. Prognostic impact of minimal residual disease in RUNX1-RUNX1T1 acute myeloid leukemia: a study of the German-Austrian AML Study Group (AMLSG). Haematologica. 2012. 97(s1):538(Abstract).
69. Schnittger S, Kern W, Tschulik C, Weiss T, Dicker F, Falini B, et al. Minimal residual disease levels assessed by NPM1 mutation-specific RQ-PCR provide important prognostic information in AML. Blood. 2009;114(11):2220-31.
70. Krönke J, Schlenk RF, Jensen KO, Tschürtz F, Corbacioglu A, Gaidzik VI, et al. Monitoring of minimal residual disease in NPM1 mutated acute myeloid leukemia: A study of the German-Austrian AML Study Group (AMLSG). J Clin Oncol. 2011;29(19):2709-16.

Immune impairment and infection predisposition in graft-versus-host disease

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A B S T R A C T

Infections and graft-*versus*-host disease (GVHD) have from the early days been major obstacles to allogeneic hematopoietic stem cell transplantation (HSCT). It is likely that the relationship between both acute and chronic GVHD and infectious complications is bidirectional with pathogens resulting in an inflammatory cascade activating the immune system resulting in acute GVHD, and the immunosuppressive effect of GVHD and its therapy increasing the risk for severe infections. An example of this complex relationship is cytomegalovirus (CMV), chronic GVHD, and the graft-versus-leukemia effect resulting in lower risks for leukemia relapse although the mechanism need still to be clarified. Other examples are acute GVHD and candida colonization and varizella-zoster virus and chronic GVHD. This review discusses this interplay between pathogens and the reactions of the host.

Learning goals

At the conclusion of this activity, participants should be able to:
- clarify the complex interactions between pathogens and graft-*versus*-host disease;
- identify patients at increased risk for infectious complications.

Introduction

Graft-*versus*-host disease (GVHD) and infections have been the most important complications since the early days of hematopoietic stem cell transplantation. There are several links between the two entities such as the immunosuppressive effect of GVHD prophylaxis and treatment, the immunosuppressive effect by GVHD itself, and the increased risk for GVHD thought to be caused by infections. Furthermore, genetic polymorphisms in the innate immune system have been associated both with acute GVHD and the risk for severe viral and fungal infections.[1] Recently the polymorphisms of the genes in the Th17/IL23 pathway has been suggested to influence both the risk for candida infections and acute GVHD.[2,3] Furthermore, polymorphisms in the gene for CCR6 were associated with both chronic GVHD and invasive aspergillosis.[4]

Acute and chronic GVHD profoundly impact the immune system and its regeneration after allogeneic HSCT. T-cell dysfunction and poor immune reconstitution is common in patients with GVHD. Early after the HSCT, the T-cell reconstitution is mostly dependent on expansion of mature T cells from the graft. In contrast, naïve T cells are needed to obtain a long-term immune reconstitution with broad responses to different pathogens and this requires a functional thymus. Older patients have poorer reconstitution of the thymic function and a narrow T-cell repertoire, and have an increased risk for late opportunistic infections.[5] The late immune reconstitution is negatively influenced by GVHD.[6] Chronic GVHD is associated both with T-cell and B-cell dysfunction.[7] Inadequate reconstitution of naive B cells and the persistence of high levels of B-cell activating factor have been found in patients with chronic GVHD.[8]

Infection as a risk factor for graft-*versus*-host disease

An early hypothesis for the pathogenesis of acute GVHD was the influence of gut bacterial flora. This was supported by studies in germ-free animals and also data from human studies of the protective effects of transplants in laminar air flow rooms.[9-11] It has been shown in both murine and human recipients of allogeneic bone marrow grafts that intestinal inflammation secondary to GVHD is associated with major shifts in the composition of the intestinal microbiota. The microbiota, in turn, can modulate the severity of intestinal inflammation.[12] The risk for acute GVHD can be affected by reduction of the intestinal bacterial flora, The mechanism might be upregulation of minor histocompatibility antigens on the T-cell repertoire.[13] Damage to the gastrointestinal tract allowing activation of antigen presenting cells by microbial products such as lipopolysaccharide has also been suggested to be a pathogenetic factor in the development of acute GVHD, especially of the gastrointestinal tract.[14] Also secretion of inflammatory cytokines such as TNFα can be stimulated by

microbial products[14] contributing to the inflammatory cascade that increase the risk for development of severe acute GVHD. It has also recently been shown that colonization with candida of the gastrointestinal tract increases the risk for acute GVHD.[15]

Acute graft-*versus*-host disease and bacterial infections

Acute GVHD grades II-IV has been reported to be an independent risk factor for blood stream infections[16] and *S. Aureus* bacteremias.[17] Organ damage induced by acute GVHD, such as that in the gut, predisposes to bacterial infections.[16] *Clostridium difficile* infections have at some centers become a major clinical problem in patients undergoing HSCT. A recent study showed that acute GVHD of the gastrointestinal tract was associated with *C. difficile* infections.[18] Although pneumococcal infections are usually associated with late infections, also early severe infections do occur.[19]

Acute graft-*versus*-host disease and viral infections

CMV

Cytomegalovirus (CMV) has been one of the major obstacles to successful allogeneic HSCT. Several studies have suggested that the interrelationship between CMV and acute and chronic GVHD is complex. Although some early studies suggested that CMV seropositivity was associated with a higher risk for acute GVHD, this has not been supported. But more recent reports have instead observed that CMV seronegative patients with seronegative donors had an increased risk for acute GVHD grade II-IV.[20] It has been shown that the allogeneic reaction can reactivate CMV from latency,[21] and acute GVHD has been shown to be a risk factor for CMV infection.[22] Furthermore, several studies have shown that acute GVHD, especially grades II-IV, is a risk factors for CMV disease.[23-26]

Adenovirus

Acute GVHD is a risk factor for disseminated adenovirus disease.[27-29] However, adenovirus is controlled mainly by specific T cells and so there is a conflict in management. It is logical to try to decrease immunosuppression to control adenovirus but this might increase the risk for acute GVHD. Therefore, the use of adeno-specific T-cells is being evaluated to improve specific immunity.[30,31]

Human herpesvirus 6

Recently, the two subtypes of human herpesvirus 6 (HHV-6) A and B have been classified as two different viruses. HHV-6 B infects most children early in life and is then associated with skin rash (exanthem subitum).[32] HHV-6 is frequently detected early after HSCT and is then temporarily associated with the development of acute GVHD. However, given the characteristic of HHV-6 to integrate into the germline, data must be interpreted with caution.[33] Data have been presented supporting the observation that acute GVHD grade II-IV is more common in patients with early HHV-6 reactivation.[34-37] However, a recent study suggests a bidirectional relationship through which HHV-6 predisposed for acute GVHD and also the opposite was found.[38] Furthermore, HHV-6 DNA has been detected in the skin of patients with a skin rash resembling acute GVHD.[39] HHV-6 also has immunosuppressive properties, and has been suggested to predispose for CMV infection[37] and to inhibit development of CMV-specific T-cell response.[40]

Respiratory viruses

Progression to lower respiratory tract disease in community acquired respiratory viral infections, such as RSV and influenza, has been associated with lymphopenia[41-43] and poor outcome.[44,45]

Acute graft-*versus*-host disease and invasive fungal infections

Invasive fungal infections have been one of the major infectious complications after allogeneic HSCT contributing to transplant-related mortality. Acute GVHD has been identified in several studies as one of the major risk factors, especially for mold infections.[46,47] In a classic paper, Wald *et al.* showed that invasive aspergillosis occurs with two peaks after allogeneic HSCT; the first peak occurring during neutropenia, and the second and stronger peak occurring after engraftment during the time where acute GVHD is occurring.[47] It is difficult to differentiate between the relative contributions of acute GVHD and the immunosuppression given as treatment. High-dose corticosteroids used as therapy for GVHD have been found to increase the risk for invasive aspergillosis,[47,48] outcome of aspergillosis,[49-51] and development of disseminated candida infections. High doses of corticosteroids have several effects on the immune system possibly contributing to this increased risk. These include effects on lymphocytes (induction of lymphopenia, especially $CD4^+$ cells, decreased proliferation capacity of lymphocytes, decreased lymphokine production, and a shift from Th1 to Th2 cells), and effects on granulocytes, monocytes, and macrophages (reduced phagocytosis, chemotaxis, and killing by granulocytes and monocytes, reduced production of NO, IL-1, IL-6, and TNFα, and reduced maturation to macrophages and dendritic cells). Furthermore, corticosteroids have effects on the fungal pathogens such as increased growth rate of aspergillus and increased capacity for candida to pass from the gut wall to the blood stream.

Chronic graft-*versus*-host disease as a risk factor for bacterial and fungal infections

Patients are at risk for severe infections many years after HSCT if they have had chronic GVHD. It has been shown that chronic GVHD is an independent risk factor for late infectious disease mortality.[52] Furthermore, extensive chronic GVHD was shown to be a risk factor for late severe bacterial and fungal infections and non-hepatitis C virus infections.[53] Chronic GVHD has also been shown to be a risk factor for invasive aspergillosis.[48,54]

Chronic GVHD is a risk factor for pneumonia.[55,56] An important infectious complication in patients with chronic GVHD is pneumococcal disease. It was shown that chron-

Table 1. Examples of proposed relationships between pathogens and acute and chronic graft-*versus*-host disease.

Pathogen	Infection increases the risk for acute GVHD	Acute GVHD increases the risk for infection/disease	Infection increases the risk for chronic GVHD	Chronic GVHD increases the risk for infection/disease
Intestinal bacteria	Likely	Yes	No	No
Pneumococci	No	Likely	No	Yes
Candida	Likely	Yes	No	Unlikely
Aspergillus	No	Yes	No	Yes
CMV	Unlikely	Yes	Yes	Yes
HHV-6B	Likely	Likely	No	No
Adenovirus	No	Yes	No	No

ic GVHD was a risk factor for pneumococcal infection.[19,57] Early after transplantation, and in patients with chronic GVHD, the antibody response to pneumococcal polysaccaride is immature, similar to that in young children, with mainly IgG1 response. In contrast, later after transplant in patients without chronic GVHD, the patients respond with IgG2.[58] This effect can be circumvented by the use of conjugate vaccines that are able to induce good immune responses also early after HSCT.[59] However, the likelihood of achieving protective antibody titers was lower in patients with chronic GVHD also when using a pneumococcal conjugate vaccine.[59]

Chronic graft-*versus*-host disease and viral infections

The relationship between CMV infection, chronic GVHD, and late immune reconstitution is complex. CMV infection has been associated with the occurrence of late lethal infections.[52] Chronic GVHD has also been correlated to development of late CMV pneumonia.[60] Several studies using different techniques have suggested that CMV drives the development of extensive chronic GVHD. Early on, a significant correlation was found between CMV serological status in the recipient and/or the donor and the development of chronic GVHD.[61-63] A possible mechanism has been proposed through CD13 since patients with chronic GVHD had CD13 specific antibodies in skin biopsies.[64] Recently, it was found that antibodies against a late CMV protein UL94 were correlated to scleroderma-like skin lesions in chronic GVHD.[65] Finally, the use of early pre-emptive therapy reduced the risk for extensive chronic GVHD.[66] A correlation was also found with CMV serological status or CMV infection and the graft-*versus*-leukemia effect.[67,68] A recent study showed that early CMV infection was strongly associated with a decreased relapse rate in patients with AML.[69] The underlying mechanism of this effect needs still to be clarified. A recent large study from the Infectious Diseases Working Party failed to show any relationship between CMV serological status of either patient or donor with the risk for relapse in patients transplanted for AML (P Ljungman *et al*., unpublished data, 2012).

Other viral infections and chronic graft-*versus*-host disease

Extensive chronic GVHD is a risk factor for herpes zoster after allogeneic HSCT.[53,70] It has also been suggested that herpes zoster can stimulate the development of chronic GVHD.[71] Chronic GVHD is also a risk factor for becoming seronegative to measles and thereby having increased risk for infection.[72] Late deaths from influenza have been documented in patients with chronic GVHD

Conclusion

Pathogens and graft-*versus*-host disease strongly influence each other. The relationship can be uni- or bidirectional and is different for acute and chronic GVHD. Table 1 shows a summary of information regarding some of the relationships between pathogens and GVHD discussed in this review. More biological and clinical studies are needed.

References

1. Loeffler J, Ok M, Morton OC, Mezger M, Einsele H. Genetic polymorphisms in the cytokine and chemokine system: their possible importance in allogeneic stem cell transplantation. Curr Top Microbiol Immunol. 2010;341:83-96.
2. Zelante T, De Luca A, D'Angelo C, Moretti S, Romani L. IL-17/Th17 in anti-fungal immunity: what's new? Eur J Immunol. 2009;39(3):645-8.
3. van der Velden WJ, Plantinga TS, Donnelly JP, Kullberg BJ, Blijlevens NM, Netea MG. Host-microbe interactions in stem cell transplantation: recognizing Candida in infection and inflammation. Virulence. 2010;1(3):180-4.
4. Broen K, van der Waart AB, Greupink-Draaisma A, Metzig J, Feuth T, Schaap NP, et al. Polymorphisms in CCR6 are associated with chronic graft-versus-host disease and invasive fungal disease in matched-related hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2011;17(10):1443-9.
5. Toubert A, Glauzy S, Douay C, Clave E. Thymus and immune reconstitution after allogeneic hematopoietic stem cell transplantation in humans: never say never again. Tissue Antigens. 2012;79(2):83-9.
6. Clave E, Busson M, Douay C, Peffault de Latour R, Berrou J, Rabian C, et al. Acute graft-versus-host disease transiently impairs thymic output in young patients after allogeneic hematopoietic stem cell transplantation. Blood. 2009;113(25):6477-84.
7. Toubai T, Sun Y, Reddy P. GVHD pathophysiology: is acute different from chronic? Best Practice and Research Clinical Haematology. 2008;21(2):101-17.

8. Sarantopoulos S, Stevenson KE, Kim HT, Bhuiya NS, Cutler CS, Soiffer RJ, et al. High levels of B-cell activating factor in patients with active chronic graft-versus-host disease. Clin Cancer Res. 2007;13(20):6107-14.
9. Navari R, Buckner C, Clift R, Storb R, Sanders J, Stewart P, et al. Prophylaxis of infection in patients with aplastic anemia receiving marrow transplants. Am J Med. 1986;76:564-72.
10. Storb R, Prentice R, Buckner C, Clift R, Appelbaum F, Deeg J, et al. Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. N Engl J Med. 1983;308:302-7.
11. Mahmoud HK, Schaefer UW, Schuning F, Schmidt CG, Bamberg M, Haralambie E, et al. Laminar air flow versus barrier nursing in marrow transplant recipients. Blut. 1984;49(5):375-81.
12. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. J Exp Med. 2012;209(5):903-11.
13. Sawamura SA, Tanaka K, Noda S, Koga Y. The role of intestinal bacterial flora in the tuning of the T cell repertoire. Immunobiology. 1999;201(1):120-32.
14. Hill GR, Ferrara JL. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. Blood. 2000;95(9):2754-9.
15. van der Velden WJ, Netea MG, de Haan AF, Huls GA, Donnelly JP, Blijlevens NM. Role of the Mycobiome in Human Acute Graft-versus-Host Disease. Biol Blood Marrow Transplant. 2013;19(2):329-32.
16. Poutsiaka DD, Munson D, Price LL, Chan GW, Snydman DR. Blood stream infection (BSI) and acute GVHD after hematopoietic SCT (HSCT) are associated. Bone Marrow Transplant. 2011;46(2):300-7.
17. Mihu CN, Schaub J, Kesh S, Jakubowski A, Sepkowitz K, Pamer EG, et al. Risk factors for late Staphylococcus aureus bacteremia after allogeneic hematopoietic stem cell transplantation: a single-institution, nested case-controlled study. Biol Blood Marrow Transplant. 2008;14(12):1429-33.
18. Alonso CD, Treadway SB, Hanna DB, Huff CA, Neofytos D, Carroll KC, et al. Epidemiology and outcomes of Clostridium difficile infections in hematopoietic stem cell transplant recipients. Clin Infect Dis. 2012;54(8):1053-63.
19. Engelhard D, Cordonnier C, Shaw PJ, Parkalli T, Guenther C, Martino R, et al. Early and late invasive pneumococcal infection following stem cell transplantation: a European Bone Marrow Transplantation survey. Br J Haematol. 2002;117(2):444-50.
20. Hahn T, McCarthy PL Jr, Zhang MJ, Wang D, Arora M, Frangoul H, et al. Risk factors for acute graft-versus-host disease after human leukocyte antigen-identical sibling transplants for adults with leukemia. J Clin Oncol. 2008;26(35):5728-34.
21. Söderberg-Naucler C, Fish K, Nelson J. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. Cell. 1997;91:119-26.
22. Miller W, Flynn P, McCullough J, Balfour HH Jr, Goldman A, Haake R, et al. Cytomegalovirus infection after bone marrow transplantation: an association with acute graft-v-host disease. Blood. 1986;67(4):1162-7.
23. Meyers JD, Ljungman P, Fisher LD. Cytomegalovirus excretion as a predictor of cytomegalovirus disease after marrow transplantation: importance of cytomegalovirus viremia. J Infect Dis. 1990;162(2):373-80.
24. Ljungman P, Perez-Bercoff L, Jonsson J, Avetisyan G, Sparrelid E, Aschan J, et al. Risk factors for the development of cytomegalovirus disease after allogeneic stem cell transplantation. Haematologica. 2006;91(1):78-83.
25. Boeckh M, Leisenring W, Riddell SR, Bowden RA, Huang ML, Myerson D, et al. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. Blood. 2003;101(2):407-14.
26. Ljungman P, Aschan J, Azinge JN, Brandt L, Ehrnst A, Hammarstrom V, et al. Cytomegalovirus viraemia and specific T-helper cell responses as predictors of disease after allogeneic marrow transplantation. Br J Haematol. 1993;83(1):118-24.
27. Flomenberg P, Babbitt J, Drobyski WR, Ash RC, Carrigan DR, Sedmak GV, et al. Increasing incidence of adenovirus disease in bone marrow transplant recipients. J Infect Dis. 1994;169(4):775-81.
28. Robin M, Marque-Juillet S, Scieux C, Peffault de Latour R, Ferry C, Rocha V, et al. Disseminated adenovirus infections after allogeneic hematopoietic stem cell transplantation: incidence, risk factors and outcome. Haematologica. 2007;92(9):1254-7.
29. La Rosa AM, Champlin RE, Mirza N, Gajewski J, Giralt S, Rolston KV, et al. Adenovirus infections in adult recipients of blood and marrow transplants. Clin Infect Dis. 2001;32(6):871-6.
30. Feuchtinger T, Richard C, Joachim S, Scheible MH, Schumm M, Hamprecht K, et al. Clinical grade generation of hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. J Immunother. 2008;31(2):199-206.
31. Leen AM, Christin A, Myers GD, Liu H, Cruz CR, Hanley PJ, et al. Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation. Blood. 2009;114(19):4283-92.
32. Yamanishi K, Okuno T, Shiraki K, Takahashi M, Kondo T, Asano Y, et al. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet. 1988;1:1065-67.
33. Hubacek P, Virgili A, Ward KN, Pohlreich D, Keslova P, Goldova B, et al. HHV-6 DNA throughout the tissues of two stem cell transplant patients with chromosomally integrated HHV-6 and fatal CMV pneumonitis. Br J Haematol. 2009;145(3):394-8.
34. Dulery R, Salleron J, Dewilde A, Rossignol J, Boyle EM, Gay J, et al. Early human herpesvirus type 6 reactivation after allogeneic stem cell transplantation: a large-scale clinical study. Biol Blood Marrow Transplant. 2012;18(7):1080-9.
35. Wang LR, Dong LJ, Zhang MJ, Lu DP. Correlations of human herpesvirus 6B and CMV infection with acute GVHD in recipients of allogeneic haematopoietic stem cell transplantation. Bone Marrow Transplant. 2008;42(10):673-7.
36. Zerr DM, Corey L, Kim HW, Huang ML, Nguy L, Boeckh M. Clinical outcomes of human herpesvirus 6 reactivation after hematopoietic stem cell transplantation. Clin Infect Dis. 2005;40(7):932-40.
37. Zerr DM, Boeckh M, Delaney C, Martin PJ, Xie H, Adler AL, et al. HHV-6 Reactivation and Associated Sequelae after Hematopoietic Cell Transplantation. Biol Blood Marrow Transplant. 2012;18(11):1700-8.
38. Pichereau C, Desseaux K, Janin A, Scieux C, Peffault de Latour R, Xhaard A, et al. The complex relationship between human herpesvirus 6 and acute graft-versus-host disease. Biol Blood Marrow Transplant. 2012;18(1):141-4.
39. Yoshikawa T, Ihira M, Ohashi M, Suga S, Asano Y, Miyazaki H, et al. Correlation between HHV-6 infection and skin rash after allogeneic bone marrow transplantation. Bone Marrow Transplant. 2001;28(1):77-81.
40. Wang FZ, Larsson K, Linde A, Ljungman P. Human herpesvirus 6 infection and cytomegalovirus-specific lymphoproliferative responses in allogeneic stem cell transplant recipients. Bone Marrow Transplant. 2002;30(8):521-6.
41. Ljungman P, Ward KN, Crooks BN, Parker A, Martino R, Shaw PJ, et al. Respiratory virus infections after stem cell transplantation: a prospective study from the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant. 2001;28(5):479-84.
42. Ljungman P, de la Camara R, Perez-Bercoff L, Abecasis M, Nieto Campuzano JB, Cannata-Ortiz MJ, et al. Outcome of pandemic H1N1 infections in hematopoietic stem cell transplant recipients. Haematologica. 2011;96(8):1231-5.
43. Nichols WG, Guthrie KA, Corey L, Boeckh M. Influenza infections after hematopoietic stem cell transplantation: risk factors, mortality, and the effect of antiviral therapy. Clin Infect Dis. 2004;39(9):1300-6.
44. Tramontana AR, George B, Hurt AC, Doyle JS, Langan K, Reid AB, et al. Oseltamivir resistance in adult oncology and hematology patients infected with pandemic (H1N1) 2009 virus, Australia. Emerg Infect Dis. 2010;16(7):1068-75.
45. Khanna N, Widmer AF, Decker M, Steffen I, Halter J, Heim D, et al. Respiratory syncytial virus infection in patients with hematological diseases: single-center study and review of the literature. Clin Infect Dis. 2008;46(3):402-12.
46. Martino R, Parody R, Fukuda T, Maertens J, Theunissen K, Ho A, et al. Impact of the intensity of the pretransplantation conditioning regimen in patients with prior invasive aspergillosis undergoing allogeneic hematopoietic stem cell transplantation: A retrospective survey of the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Blood. 2006;108(9):2928-36.
47. Wald A, Leisenring W, van Burik JA, Bowden RA. Epidemiology of Aspergillus infections in a large cohort of

patients undergoing bone marrow transplantation. J Infect Dis. 1997;175(6):1459-66.
48. Marr KA, Carter RA, Boeckh M, Martin P, Corey L. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. Blood. 2002;100(13):4358-66.
49. Upton A, Kirby KA, Carpenter P, Boeckh M, Marr KA. Invasive aspergillosis following hematopoietic cell transplantation: outcomes and prognostic factors associated with mortality. Clin Infect Dis. 2007;44(4):531-40.
50. Ribaud P, Chastang C, Latge JP, Baffroy-Lafitte L, Parquet N, Devergie A, et al. Survival and prognostic factors of invasive aspergillosis after allogeneic bone marrow transplantation. Clin Infect Dis. 1999;28(2):322-30.
51. Cordonnier C, Ribaud P, Herbrecht R, Milpied N, Valteau-Couanet D, Morgan C, et al. Prognostic factors for death due to invasive aspergillosis after hematopoietic stem cell transplantation: a 1-year retrospective study of consecutive patients at French transplantation centers. Clin Infect Dis. 2006;42(7):955-63.
52. Bjorklund A, Aschan J, Labopin M, Remberger M, Ringden O, Winiarski J, et al. Risk factors for fatal infectious complications developing late after allogeneic stem cell transplantation. Bone Marrow Transplant. 2007;40(11):1055-62.
53. Robin M, Porcher R, De Castro Araujo R, de Latour RP, Devergie A, Rocha V, et al. Risk factors for late infections after allogeneic hematopoietic stem cell transplantation from a matched related donor. Biol Blood Marrow Transplant. 2007;13(11):1304-12.
54. Mikulska M, Raiola AM, Bruno B, Furfaro E, Van Lint MT, Bregante S, et al. Risk factors for invasive aspergillosis and related mortality in recipients of allogeneic SCT from alternative donors: an analysis of 306 patients. Bone Marrow Transplant. 2009;44(6):361-70.
55. Chen CS, Boeckh M, Seidel K, Clark JG, Kansu E, Madtes DK, et al. Incidence, risk factors, and mortality from pneumonia developing late after hematopoietic stem cell transplantation. Bone Marrow Transplant. 2003;32(5):515-22.
56. Aguilar-Guisado M, Jimenez-Jambrina M, Espigado I, Rovira M, Martino R, Oriol A, et al. Pneumonia in allogeneic stem cell transplantation recipients: a multicenter prospective study. Clin Transplant. 2011;25(6):E629-38.
57. Kulkarni S, Powles R, Treleaven J, Riley U, Singhal S, Horton C, et al. Chronic graft versus host disease is associated with long-term risk for pneumococcal infections in recipients of bone marrow transplants. Blood. 2000;95(12):3683-6.
58. Hammarström V, Pauksen K, Azinge J, Öberg G, Ljungman P. The influence of graft versus host reaction on the response to pneumococcal vaccination in bone marrow transplant patients. J Support Care Cancer. 1993;1:195-9.
59. Cordonnier C, Labopin M, Chesnel V, Ribaud P, De La Camara R, Martino R, et al. Randomized study of early versus late immunization with pneumococcal conjugate vaccine after allogeneic stem cell transplantation. Clin Infect Dis. 2009;48(10):1392-401.
60. Nguyen Q, Champlin R, Giralt S, Rolston K, Raad I, Jacobson K, et al. Late cytomegalovirus pneumonia in adult allogeneic blood and marrow transplant recipients. Clin Infect Dis. 1999;28(3):618-23.
61. Lonnqvist B, Ringden O, Wahren B, Gahrton G, Lundgren G. Cytomegalovirus infection associated with and preceding chronic graft-versus-host disease. Transplantation. 1984;38(5):465-8.
62. Bostrom L, Ringden O, Jacobsen N, Zwaan F, Nilsson B. A European multicenter study of chronic graft-versus-host disease. The role of cytomegalovirus serology in recipients and donors—acute graft-versus-host disease, and splenectomy. Transplantation. 1990;49(6):1100-5.
63. Jacobsen N, Andersen HK, Skinhoj P, Ryder LP, Platz P, Jerne D, et al. Correlation between donor cytomegalovirus immunity and chronic graft-versus-host disease after allogeneic bone marrow transplantation. Scand J Haematol. 1986;36(5):499-506.
64. Soderberg C, Larsson S, Rozell BL, Sumitran-Karuppan S, Ljungman P, Moller E. Cytomegalovirus-induced CD13-specific autoimmunity—a possible cause of chronic graft-vs-host disease. Transplantation. 1996;61(4):600-9.
65. Pastano R, Dell'Agnola C, Bason C, Gigli F, Rabascio C, Puccetti A, et al. Antibodies against human cytomegalovirus late protein UL94 in the pathogenesis of scleroderma-like skin lesions in chronic graft-versus-host disease. Int Immunol. 2012;24(9):583-91.
66. Larsson K, Aschan J, Remberger M, Ringden O, Winiarski J, Ljungman P. Reduced risk for extensive chronic graft-versus-host disease in patients receiving transplants with human leukocyte antigen-identical sibling donors given polymerase chain reaction-based preemptive therapy against cytomegalovirus. Transplantation. 2004;77(4):526-31.
67. Jacobsen N, Badsberg JH, Lonnqvist B, Ringden O, Volin L, Rajantie J, et al. Graft-versus-leukaemia activity associated with CMV-seropositive donor, post-transplant CMV infection, young donor age and chronic graft-versus-host disease in bone marrow allograft recipients. The Nordic Bone Marrow Transplantation Group. Bone Marrow Transplant. 1990;5(6):413-8.
68. Lönnqvist B, Ringdén O, Ljungman P, Wahren B, Gahrton G. Reduced risk of recurrent leukaemia in bone marrow transplant recipients after cytomegalovirus infection. Br J Haematol. 1986;63(4):671-9.
69. Elmaagacli AH, Steckel NK, Koldehoff M, Hegerfeldt Y, Trenschel R, Ditschkowski M, et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. Blood. 2011;118(5):1402-12.
70. Koc Y, Miller KB, Schenkein DP, Griffith J, Akhtar M, DesJardin J, et al. Varicella zoster virus infections following allogeneic bone marrow transplantation: frequency, risk factors, and clinical outcome. Biol Blood Marrow Transplant. 2000;6(1):44-9.
71. Kawano N, Gondo H, Kamimura T, Aoki K, Iino T, Ishikawa F, et al. Chronic graft-versus-host disease following varicella-zoster virus infection in allogeneic stem cell transplant recipients. Int J Hematol. 2003;78(4):370-3.
72. Ljungman P, Aschan J, Barkholt L, Broliden PA, Gustafsson B, Lewensohn-Fuchs I, et al. Measles immunity after allogeneic stem cell transplantation; influence of donor type, graft type, intensity of conditioning, and graft-versus host disease. Bone Marrow Transplant. 2004;34(7):589-93.

Downregulation of the clotting cascade by the protein C pathway

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A B S T R A C T

The protein C pathway provides important biological activities to maintain the fluidity of the circulation, prevent thrombosis, and protect the integrity of the vasculature in response to injury. Activated protein C (APC), in concert with its co-factors and cell receptors, assembles in specific macromolecular complexes to provide efficient proteolysis of multiple substrates that result in anticoagulant and cytoprotective activities. Numerous studies on APC's structure-function relation with its co-factors, cell receptors, and substrates provide valuable insights into the molecular mechanisms and presumed assembly of the macromolecular complexes that are responsible for APC's activities. These insights allow for molecular engineering approaches specifically targeting the interaction of APC with one of its substrates or co-factors. Thus far, these approaches resulted in several anticoagulant-selective and cytoprotective-selective APC mutants, which provide unique insights into the relative contributions of APC's anticoagulant or cytoprotective activities to the beneficial effects of APC in various murine injury and disease models. Because of its multiple physiological and pharmacological activities, the anticoagulant and cytoprotective protein C pathway have important implications for the (patho)physiology of vascular disease and for translational research exploring novel therapeutic strategies to combat complex medical disorders such as thrombosis, inflammation, ischemic stroke and neurodegenerative disease.

Learning goals

At the conclusion of this activity, participants should know that:
- the protein C pathway provides multiple important functions to maintain a regulated balance between hemostasis and host defense systems;
- APC's anticoagulant activities prevent thrombosis whereas APC's cytoprotective activities protect cells;
- APC's different activities require assembly of different macromolecular complexes with different co-factors that can be targeted by mutagenesis to obtain activity-selective APC mutants;
- anticoagulant-selective and cytoprotective-selective APC mutants provide insights into the relative contributions of these APC activities to beneficial effects in various murine injury and disease models.

Introduction

The hemostatic system safeguards the patency of the vasculature. Platelet aggregation, coagulation, and fibrinolysis operate in concert with the endothelium and other vascular cells to arrest bleeding and prevent thrombosis. The coagulation pathway contributes to regulation of the hemostatic balance via multiple mechanisms and pathways that ensure a balanced and confined hemostatic response at the site of injury. Despite these feedback mechanisms to fine-tune the hemostatic response, genetic and/or acquired defects often tilt the balance sufficiently to increase the risk for thrombophilia. In the current model of coagulation,[1,2] clot formation is initiated by the extrinsic pathway with little or no role for the contact system in the initiation of physiological coagulation, although platelet-derived polyphosphates may provide an endogenous activation mechanism for FXII.[3,4] Thrombin formation occurs during two mechanistically different phases. In the first phase, referred to as primary thrombin formation, the extrinsic pathway generates the clot initiated by the tissue factor/activated factor VIIa (FVIIa) complex. However, primary thrombin formation is short-lived due to rapid inactivation of the tissue factor/FVIIa initiator complex by tissue factor pathway inhibitor (TFPI). In the second phase, when sufficient thrombin is generated to initiate FXI activation, secondary thrombin formation will continue inside the clot via thrombin-mediated FXI activation and amplification by the intrinsic pathway.[5] This secondary thrombin formation contributes to clot strength and conveys resistance to fibrinolysis via the activation of FXIII and thrombin activatable fibrinolysis inhibitor (TAFI).[6,7] Control of coagulation is generally provided by three different mechanisms. First, the γ-carboxyglutamic acid (Gla)-domain, common to most coagulation factors, requires the presence of negatively charged phosphatidylserine for calcium-dependent binding to lipid surfaces.[8] Thus, assembly of the tenase (activated factor

IXa, X, and VIII (FIXa, FX and FVIIIa)) and prothrombinase (FXa, FII, and FVa) complex is limited by the presence of negatively charged lipid surfaces (such as on activated platelets).[8,9] Second, serine protease inhibitors (SERPINs) rapidly inhibit activated coagulation factors, thereby blunting coagulation and preventing the escape of active coagulation factors in the circulation.[10] Finally, the protein C pathway actively inhibits coagulation by proteolysis of the tenase and prothrombinase complex co-factors, FVa and FVIIIa, thereby providing a dynamic regulation of coagulation.[11-15]

The focus of this review will be on the protein C pathway. Because activated protein C (APC) inactivates both FVa and FVIIIa, it has important effects on the downregulation of both primary and secondary thrombin formation that manifest as potent anti-thrombotic effects *in vivo*. Furthermore, APC's relatively new activities on cells provide physiological and pharmacological relevant protective effects on the endothelium and the vasculature. Thus APC conveys multiple activities that require assembly of macromolecular complexes with different co-factors, cell receptors and substrates. Structure-function analysis of these APC complexes, discussed in the next sections, provides a unique understanding of how a single enzyme can mediate multiple biologically and therapeutically relevant activities.

The protein C pathway

The protein C pathway provides important contributions to maintain the fluidity of the circulation, prevent thrombosis, and protect the integrity of the vasculature in response to injury. The reactions of the protein C pathway encompass protein C activation on endothelial cells, the anticoagulant protein C pathway on activated platelets, the cytoprotective protein C pathway on cell membranes, and inactivation of APC by plasma serine protease inhibitors (SERPINs) in the fluid phase (Figure 1). Each of these aspects of the protein C pathway will be discussed in the sections below.

The physiological importance of the protein C system is best illustrated by the manifestation of massive thrombotic complications in infants with protein C deficiency.[16,17] Neonatal purpura fulminans, a rapidly progressing hemorrhagic necrosis of the skin due to microvascular thrombosis, inflammation, and disseminated intravascular coagulation (DIC), is typically observed in severe protein C deficiency, whereas heterozygous protein C deficiency in adults carries a significantly increased risk for venous thrombosis.[18-20] A rare complication referred to as warfarin-induced skin necrosis with clinical symptoms similar to that of purpura fulminans, may present within days after

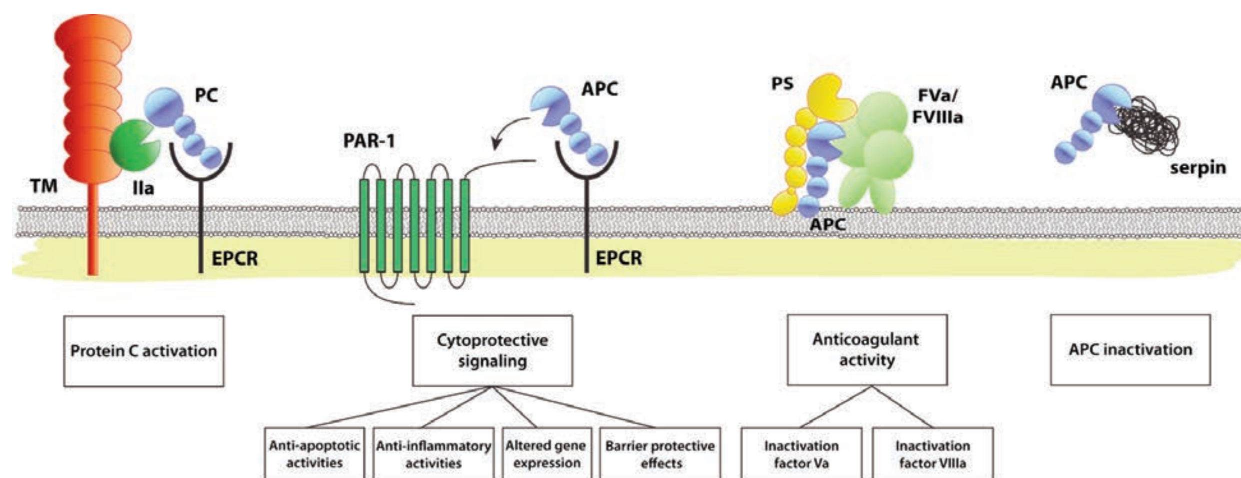

Figure 1. Reactions of the protein C pathway. Schematic representation of the protein C pathway reactions, from left to right: protein C activation, the cytoprotective protein C pathway, the anticoagulant protein C pathway and the inactivation of APC by serine protease inhibitors (SERPINs). Protein C activation: physiological activation of protein C (PC) by the thrombin (IIa)-thrombomodulin (TM) complex occurs on the surface of endothelial cell membranes when protein C is bound to the endothelial protein C receptor (EPCR). Since protein C and APC have a similar affinity for EPCR, after activation APC can remain bound to EPCR to initiate cytoprotective signaling. The cytoprotective protein C pathway: APC's direct effects on endothelial cells require the cellular receptors EPCR and PAR1. These cellular activities of APC include anti-apoptotic and anti-inflammatory activities, alteration of gene expression profiles, and protection of endothelial barrier functions and are collectively referred to as APC's cytoprotective activities. The anticoagulant protein C pathway: APC anticoagulant activities involve proteolytic cleavages of FVa and FVIIIa. Different protein co-factors, such as protein S (PS), FV, and various lipid co-factors (e.g. phosphatidylserine, phosphatidylethanolamine cardiolipin, glucosylceramide, etc.), enhance the inactivation of FVa and FVIIIa by APC. APC inactivation: inactivation of APC in plasma by serine protease inhibitors (SERPINs) is slow, which contributes to a remarkably long circulation half-life of APC (approx. 20 min). Most important inhibitors of APC in plasma are protein C inhibitor (PCI), plasminogen activator inhibitor-1 (PAI-1), and α1-antitrypsin and, to a lesser extent, α_2-macroglobulin and α_2-antiplasmin.

initiation of oral anticoagulant therapy with coumarin derivatives. This is due to a temporary functional protein C deficiency caused by the shorter circulation half-life of protein C (8 h) compared to the other procoagulant coagulation factors (24-72 h).[17,18,21] Acquired protein C deficiency is also found in patients with severe infection and sepsis, most likely due to consumption and poor synthesis in the liver, and low protein C levels correlate with poor clinical outcome and death.[22]

Protein C activation

The protein C zymogen is synthesized in the liver and circulates in plasma at 4 μg/mL, which is equivalent to approximately 70 nM based on a molecular weight of 62,000 Da. The domain topology of protein C is typical of vitamin K-dependent coagulation factors.[23] The N-terminal protein C light chain contains nine γ-carboxylated Glu residues (Gla-domain) and two epidermal growth factor (EGF)-like domains. The C-terminal heavy chain contains an N-terminal acidic protein C activation peptide that is removed upon activation and the protease domain with a typical His211 (mature protein C numbering), Asp257 and Ser360 active site triad (residues His57, Asp102 and Ser195 in chymotrypsin nomenclature. For a conversion table see Mather *et al*.[24]).

Protein C is activated by thrombin through limited proteolysis at Arg169. Physiological activation of protein C on the endothelial cell surface requires binding of thrombin to thrombomodulin (TM) and binding of protein C to the endothelial protein C receptor (EPCR) (Figure 1).[14,25-27] The binding surface for TM on protein C shows a partial overlap with the exosite for interactions with FVa, and includes residues in loop 37 (Lys191 and Lys192), loop 60 (Lys217 and Lys218), loop 70-80 (Arg229 and Arg230), and possibly loop 20 (Lys174, Arg177, and Arg178), although the direct interaction of these latter residues with TM remains controversial (loops are referred to by their chymotrypsin numbering[24]).[28-30]

Protein C activation by thrombin in the absence of TM is very inefficient and is inhibited by calcium. Presumably, this limitation ensures that APC generation is initiated only when the clot covers the intact endothelium and thrombin comes in contact with TM.[14] Several residues surrounding the Arg169 activation site in protein C (i.e. P3-P9' residues relative to Arg169 denoted as P1)[31] are responsible for the inhibitory effect of calcium on the activation of protein C by free thrombin. Mutation of these residues allows for efficient protein C activation by thrombin in the presence of calcium that is no longer dependent on the presence of TM.[32-34] *In vivo* proof-of-principal that TM-independent protein C activation by thrombin results in enhanced APC generation was provided by a transgenic mouse (named the APC^{high} mouse) expressing human protein C with mutations of the P3 and P3' residues (Asp167Phe/Asp172Lys).[33,35] Interestingly, increased blood loss after tail amputation in these mice suggest that uncoupling of protein C activation from TM disrupts the regulation of normal thrombus formation.

Inactivation of APC

Inactivation of APC in plasma is driven by serine protease inhibitors (SERPINs) of which protein C inhibitor (PCI), plasminogen activator inhibitor-1 (PAI-1), α_1-antitrypsin and, to a lesser extent, α_2-macroglobulin and α_2-antiplasmin are most relevant for APC (Figure 1).[36] Even though heparin and vitronectin accelerate the reaction of APC with PCI and PAI-1 several orders of magnitude, the reaction of APC with SERPINs is relatively slow, resulting in an approximately 20 min half-life of APC in the circulation.[36] APC exosites required for interactions of APC with the various inhibitors and heparin largely overlap with those required for interaction with FVa and include residues in loop 37 and the autolysis loop.[36,37] Interestingly, some residues that affect interactions with SERPINs are not shared with FVa and these include Leu194 in loop 37, Lys217 and Lys218 in loop 60, Thr254, and Ser336.[36,38,39]

The anticoagulant protein C pathway

The protein C pathway is best known for its anticoagulant activity that involves proteolytic inactivation of FVa and FVIIIa on negatively charged phospholipid membranes and that is enhanced by co-factors protein S and FV (Figure 1).[40-42] Because APC inactivates both FVa and FVIIIa, it has important effects on the downregulation of both primary and secondary thrombin formation. Inhibition of primary thrombin formation results in delayed clot formation, whereas inhibition of secondary thrombin formation results in diminished activation of TAFI and subsequently in an enhanced susceptibility of the clot to fibrinolysis. The latter effects of APC on secondary thrombin formation are also referred to as APC's profibrinolytic effects.[43]

APC's anticoagulant activity requires binding of the Gla-domain to negatively charged phospholipid membranes. Although membranes containing phosphatidylethanolamine in addition to phosphatidylserine generally improve APC's lipid-dependent functions, binding of APC to negatively charged phospholipids is relatively poor compared to other vitamin K-dependent coagulation factors.[44] Therefore, anticoagulant activity of APC can be enhanced by strategies aimed at improving APC's affinity for membranes, such as Gla-domain swaps or targeted mutagenesis of the Gla-domain.[45,46]

Inactivation of FVa and FVIIIa

FVa is a non-covalent heterodimeric complex composed of a heavy chain (domains A1-A2) and a light chain (domains A3-C1-C2).[47] Since FVa enhances prothrombinase approximately 10,000-fold, inactivation of FVa by APC effectively shuts down thrombin formation.[40,48] Inactivation of FVa involves APC-mediated cleavages at Arg306 and Arg506. The rapid cleavage at Arg506 is kinetically favored over cleavage at Arg306, but results only in partial inactivation of FVa, whereas the slower cleavage at Arg306 results in a complete loss of FVa function.[40,41] The importance of APC-mediated FVa inactiva-

tion is clear from the increased risk for thrombosis associated with mutations of the APC cleavage sites in FV (Arg506Gln a.k.a. FV_{Leiden}, Arg306Thr a.k.a. $FV_{Cambridge}$ and Arg306Gly a.k.a. $FV_{Hong\ Kong}$). In fact, FV_{Leiden} is the most common identifiable hereditary risk factor for venous thrombosis among Caucasians.[47,49]

Mutagenesis studies have identified several positively charged exosites on the APC protease domain surface that are required for rapid inactivation of FVa (Figure 2B).[36,37,51,52] This extended FVa exosite on APC is comprised of residues in loop 37 (Lys191, Lys192, and Lys193), loop 60 (Asp214, Glu215 and Arg222), loop 70 (Arg229 and Arg230) and the autolysis loop (Lys306, Lys311, Arg312 and Arg314). [36,37,51,52] Remarkably, these residues primarily contribute to APC cleavage of FVa at Arg506 but have little effect on APC-mediated cleavage at Arg306, suggesting that the FVa Arg306 cleavage site does not rely on APC exosite interactions or that the exosite for Arg306 has not been found yet.[51,52] Instead, protein S enhances APC-mediated cleavage at Arg306 (see below).

In the circulation, tight non-covalent binding (K_D approx. 0.5 nM) of FVIII to von Willebrand factor (vWF) prevents the incorporation of factor VIII into the tenase complex.[53] FVIIIa dissociates from vWF after activation and enhances FXa formation by the tenase complex approximately 200,000-fold.[54] Despite a domain topology similar to that of FVa, FVIIIa circulates as a heterotrimer and not a heterodimer due to different cleavage patterns that cause their respective activations. As a consequence of being a heterotrimer, FVIIIa is quite unstable with a half-life of only 2 min due to spontaneous dissociation of the A2-domain.[53] Nevertheless, several observations, including stabilization of FVIIIa by FIXa in the tenase complex, support a role for APC in the inactivation of FVIIIa.[55] Homologous to inactivation of FVa, inactivation of FVIIIa by APC occurs upon cleavage at Arg336 and Arg562; but in contrast to FVa, cleavage of FVIIIa at either site results in a complete loss of cofactor activity.[56,57] Both protein S and FV but not FVa enhance inactivation of FVIIIa by APC.[57,58]

Protein S

Protein S is best known for its function as a non-enzymatic co-factor to APC in the inactivation of FVa and FVIIIa. In addition, protein S has APC-independent anticoagulant effects and also has direct (cytoprotective) effects on cells that are independent of its anticoagulant functions but instead require interactions with receptors on cells. (The reader is referred to the references provided as a starting point for a more detailed discussion of these protein S activities).[59,60]

The important anticoagulant contributions of protein S are clear from the thrombotic complications and increased risk of venous thromboembolism associated with homozygous and heterozygous protein S deficiency.[61] Functionally, five distinct domains can be identified that include an N-terminal Gla domain, a thrombin sensitive region (TSR), a repeat of four EGF-like domains, and a sex hormone-binding globulin (SHBG) domain composed of two laminin G-type domains.[23] Protein S predominantly stimulates FVa cleavage at

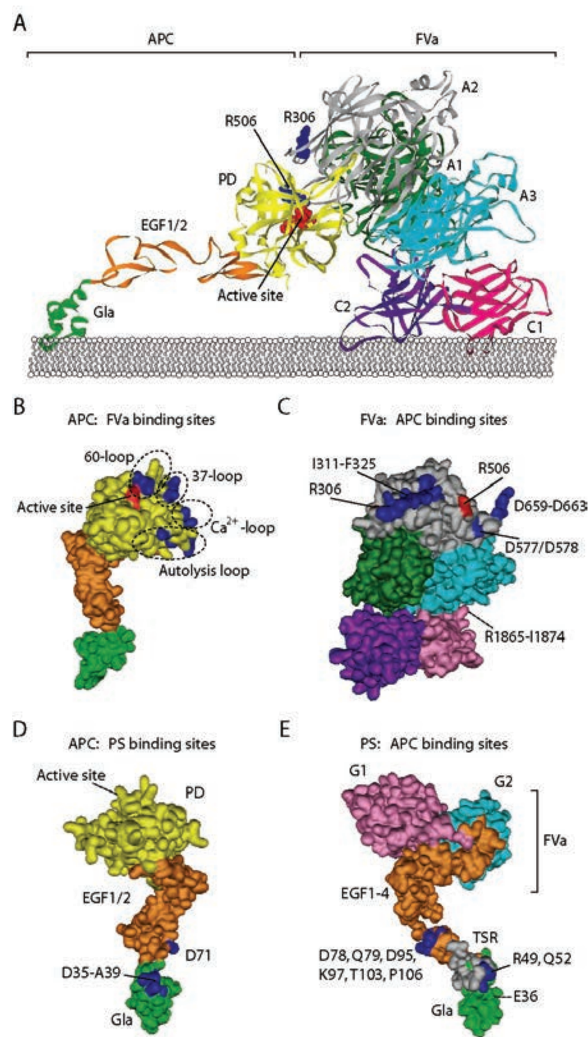

Figure 2. Schematic structural model of the anticoagulant protein C pathway. (A) Proposed model for the APC-mediated inactivation of FVa at Arg506 based on the interaction of an extended basic exosite on the protease domain (PD) of APC with negatively charged residues on the surface of FVa (adapted from Pellequer *et al.*[50]). In this model, the protease domain of APC (yellow) interacts with the A1 (green) and A2 (gray) domains of FVa. The interactions between APC and FVa position Arg506 (blue) of FVa in the active site pocket (red) of APC. The Gla-domain of APC (green) and the C1 (pink) and C2 (purple) domains of FVa interact with the phospholipid layer. Although biochemical data strongly support additional interactions between the APC Gla-domain and the FVa light chain (A3-C1-C2), especially residues within the A3 (light blue) sequence Arg1865-Ile1874, the extended projection of these domains in this model illustrate the need for a more flexible orientation of the APC protease domain to accommodate this (see text for additional details). (B) Schematic overview of the extended exosite (blue) on the surface of the APC protease domain, comprised of residues in loop 37, loop 60, the calcium-binding loop (loop70-80) and the autolysis loop that are required for interactions with FVa to accommodate cleavage at Arg506. (C) Schematic overview of the residues on the FVa surface (blue) that are involved in interactions of FVa with APC. Important residues are located on the A2 domain surrounding the Arg306 and Arg506 cleavage sites (red) and between the A1 and A3 domain at the back of the protein (not visible). (D) A schematic overview of APC residues implicated in the interaction of APC with protein S. Important residues (blue) in the Gla-domain (Asp35-Ala39) and the EGF1-domain (Asp71) are highlighted. (E) Schematic overview of the protein S residues (blue) implicated in the interaction of protein S with APC. Important residues (blue) in the Gla-domain (Glu36), TSR (Arg49 and Gln52), and EGF1-domain (Asp78, Gln79, Asp95, Lys97, Thr103 and Pro106) are highlighted.

Arg306 by APC but also neutralizes the FXa-mediated protection of FVa against APC cleavage at Arg506.[62,63] Molecular mechanisms for enhanced APC-mediated cleavage of FVa at Arg306 by protein S have been partially clarified and involve a protein S-induced change in the geometry of APC relative to the membrane. Presumably, protein S binding lowers APC's active site closer to the membrane, placing it in a better position to cleave Arg306.[64] This provides a molecular explanation for how APC can inactivate FVa at Arg306 without extensive exosite interactions between APC and FVa. APC residues that are implicated in interactions with protein S (Figure 2D) include Gla-domain residues 35 to 39 (in particular Leu38), Asp71 that contains a post-translational β-hydroxyaspartic acid modification in EGF1, and potentially the C-terminus of the light-chain.[65-68]

Molecular interaction between APC, protein S, and FVa

Since limited structural information is available, the perceived assembly of APC with protein S and the interactions with FVa remain highly speculative.[24,50,69,70] An APC-FVa model for cleavage at Arg506 (Figure 2A), based on the interaction of the extended positively charged exosite of APC (Figure 2B) with a negatively charged region on FVa that includes Asp513, Asp577, and Asp578 in the A2-domain and Asp659, Asp660, Glu661, Glu662, and Asp663 that follows the A2-domain (Figure 2C), projects the APC Gla-domain rather far away from FVa.[50,52] In complex with protein S and FVa, the APC Gla-EGF1 domains are anticipated to be orientated in closer proximity to FVa, with a flexible conformation of the APC protease domain that bends down to Arg506 (or Arg306). This hypothesis is consistent with biochemical data that indicates binding of APC to the FVa light chain.[71,72] Thus, protein S is likely to have important implications for the spatial orientation of APC in the ternary APC-protein S-FVa complex.

The APC co-factor function of protein S involves a complex set of interactions of protein S with APC and factor Va.[73] The minimal structure of protein S to support APC co-factor activity requires the Gla-domain, the TSR and EGF1 (approx. 30% compared with native protein S), although EGF2 and part of the SHBG domain provide additional interactions with APC and FVa and are required for full protein S cofactor activity.[74-76] Direct APC-binding to protein S seems contained to protein S EGF1 with important contributions of Asp78, Gln79, Asp95, and Thr103 (Figure 2E).[75,77] The protein S Gla-domain, TSR and EGF-2 are unlikely to contribute to direct interactions with APC but rather play a supporting structural role. Important residues in these supporting domains identified thus far include Glu36 in the Gla-domain and Arg49 and Gln52 in the TSR.[70,74-78]

The SHBG domain of protein S is projected to extend above the protease domain of APC since protein S contains two additional EGF-like domains compared to APC. The contributions of the SHBG-domain to protein S co-factor activity seem contained to the C-terminus of the SHBG laminin G2-domain and are likely derived from mediating interactions with FVa rather than APC.[79] Especially residues Lys630, Lys631, and Lys633 in the G2-domain seem to provide important contributions for binding of protein S to FVa, which possibly facilitates directing the APC protease domain to the FVa cleavage sites consistent with protein S decreasing the distance of the APC active site to the membrane.[64,80]

The cytoprotective protein C pathway

In addition to its anticoagulant activity, APC can convey direct effects on cells, collectively referred to as APC cytoprotective activities, that require endothelial protein C receptor (EPCR) and protease activated receptor 1 (PAR1).[11,81-84] Dependent on the cell type and injury, these cellular activities of APC include anti-apoptotic and anti-inflammatory activities, alteration of gene expression profiles, and protection of endothelial barrier function. Other receptors may also contribute to APC-initiated signaling such as sphingosine-1-phosphate receptor 1 (S1P1), apolipoprotein E receptor 2 (ApoER2), CD11b/CD18 ($\alpha_M\beta_2$; Mac-1; CR3), PAR-3, and Tie2, whereas APC's ability to inactivate extracellular histones is presumably independent of APC's cell signaling effects.[85,86]

The currently prevailing paradigm for APC's direct cytoprotective actions on endothelial cells is that when PAR1 and EPCR are co-localized in caveolin-1 enriched lipid rafts or caveolae, APC binding to EPCR permits non-canonical PAR1 activation at Arg46 to initiate cytoprotective signaling.[11,87-89] It is important to realize that there are several fundamental distinctions between PAR1 activation by APC and thrombin on endothelial cells. Foremost, the functional outcome is different. Thrombin activation of PAR1 results in proinflammatory and endothelial barrier disruptive effects, whereas PAR1 activation by APC results in cytoprotective actions that include endothelial barrier stabilization. The reasons for this functional contrast become evident when considering the fundamental differences in PAR1 activation and signaling between these two proteases. PAR1 activation by thrombin occurs after cleavage of the canonical Arg41 site after which the tethered-ligand starting at Ser42 promotes G-protein dependent signaling that includes activation of barrier disruptive Ras homolog gene family member A (RhoA). In contrast, activation of PAR1 by APC occurs through cleavage of the non-canonical Arg46 site after which the tethered-ligand starting at Asn47 mediates β-arrestin 2-dependent barrier protective Ras-related C3 botulinum toxin substrate 1 (Rac1) activation (Figure 3).[89,90] Synthetic peptides representing the sequences of the different tethered ligands exposed after cleavage of PAR1 at Arg41 (thrombin receptor activating peptide, TRAP) or Arg46 (TR47) also recapitulate the remarkable differences in PAR1 signaling. TRAP induces typical phosphorylation of ERK1/2 but TR47 does not. Instead, TR47 but not TRAP, induces phosphorylation of Akt in endothelial cells that is linked to cytoprotective functions.[89] The fact that different ligands induce different signaling pathways via the same receptor, of which one employs G protein-dependent signaling (TRAP) and the other initiates β-arrestin 2-dependent signaling (TR47), is highly indicative that PAR1 can induce biased signaling

(Figure 3).[91] Presumably the thrombin generated TRAP-like tethered-ligand induces a subset of PAR1 conformations that preferentially employs G protein-dependent signaling, whereas the APC generated TR47-like tethered-ligand induces a different spectrum of PAR1 conformations that recruit β-arrestin 2-dependent signaling. Thus, non-canonical activation of PAR1 by APC at Arg46 and canonical activation of PAR1 by thrombin at Arg41 can understandably mediate the often opposite effects of thrombin and APC because each protease generates different tethered-ligand agonists that utilize different signaling pathways with different functional consequences.

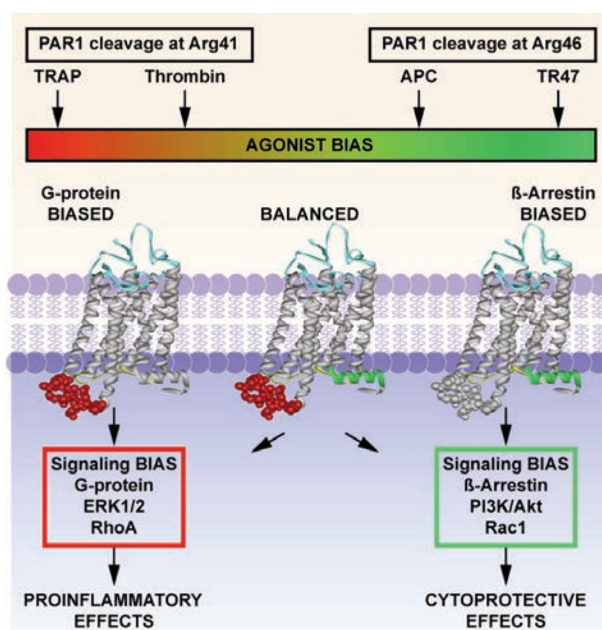

Figure 3. Induction of biased signaling by canonical and non-canonical activation of PAR1. Schematic representation of the fundamental and functional differences between APC and thrombin-mediated PAR1 activation. APC cleavage of PAR1 at Arg46 and the tethered-ligand sequence generated by this cleavage starting at Asn47 (TR47) induces a subset of PAR1 conformations that prefer signaling mediated by β-arrestin-2 involving activation of the PI3K-Akt pathway and that result in activation of barrier protective Rac1. In contrast, thrombin cleavage of PAR1 at Arg41 and the tethered-ligand sequence generated by this cleavage starting at Ser42 (TRAP) induces a subset of PAR1 conformations that prefer G protein-mediated signaling involving activation of the ERK1/2 and that result in activation of barrier disruptive RhoA. Thus, depending on the activating ligand, PAR1 can recruit different signaling pathways that result in different functional outcomes, which has been labeled 'biased agonism'. The agonist bias is thus directly related to the cleavage sites of the tethered-ligand and the new N-terminal sequence as represented by the TRAP peptide that exists after cleavage at Arg41 or the TR47 peptide that exists after cleavage at Arg46 (Mosnier *et al.* Biased agonism of protease-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46. Blood 2012;120:5237-46.© the American Society of Hematology[89]).

Relative contributions of APC's anticoagulant and cytoprotective activities

Because of its multiple biological activities, APC and the protein C pathway components have important roles in complex and challenging medical disorders, and provide potential opportunities for pharmacological treatment strategies in thrombosis, inflammation, and ischemic stroke.[15,92,93] Although APC conveys beneficial effects in numerous different *in vivo* disease models, not all APC activities are necessarily beneficial. Based on the notion that the substrates and co-factors for APC's anticoagulant activity (phospholipids, protein S and FVa) differ from APC's cytoprotective effects (EPCR and PAR1), engineered APC mutants with cytoprotective-selective activities or anticoagulant-selective activities allowed the interrogation of the differential requirements for APC beneficial activities *in vivo*.[67,94,95] Targeted disruption of the interaction of APC with protein S (PS) (Leu38Asp-APC) or with FVa results in cytoprotective-selective APC mutants such as, Arg222Cys/Asp237Cys (stabilizing the 70-80 loop, Arg229Ala/Arg230Ala-APC, Lys191Ala/Lys192Ala/Lys193Ala (a.k.a. 3K3A-APC) or a combination of the latter two (a.k.a. 5A-APC) (Figure 4).[11,65,94-97] Targeted disruption of APC binding to EPCR while leaving phospholipid binding relatively intact (Leu8Gln-APC), or disruption of a region on APC that is required for cleavage of PAR1 (Glu330Ala and Glu333Ala), yields anticoagulant-selective APC mutants.[98,99] Glu149Ala-APC, another anticoagulant-selective mutant, provides a challenging test of our current understanding of the cytoprotective protein C pathway, as its lack of cytoprotective activities remains enigmatic.[67] Cytoprotective-selective but not anticoagulant-selective APC mutants provide ben-

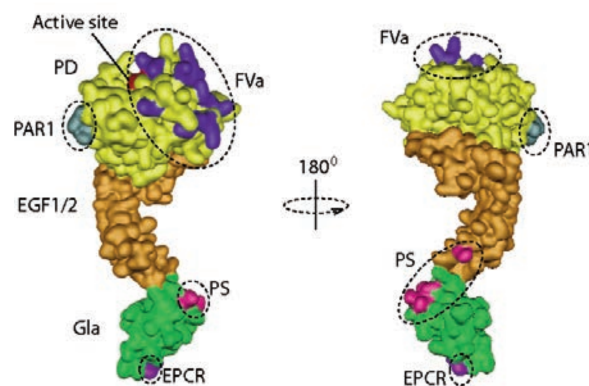

Figure 4. Schematic representation of the different structural requirements for APC's anticoagulant and cytoprotective activities. Anticoagulant activity of APC requires binding of the Gla-domain (green) to phospholipid surfaces, interaction with protein S (PS) mediated by residues on the Gla-domain and EGF1-domain (pink), and interactions of exosite residues (blue) on the APC protease domain (PD) with FVa. In contrast, cytoprotective activity of APC requires binding of the Gla-domain to EPCR (indicated by Leu8 (purple)), and interactions of a region on the opposite side of the FVa exosite on protease domain of APC that involves residues Glu330 and Glu333 (gray).

eficial effects in models of inflammation, sepsis, and ischemic stroke, whereas anticoagulant-selective but not cytoprotective-selective APC mutants prevent thrombosis, generally consistent with the concept that APC's cytoprotective activities protect cells and APC's anticoagulant activities prevent occlusive thrombosis.[67,93,94] For instance, cytoprotective-selective 3K3A-APC or 5A-APC reduce mortality in bacteremia and LPS-induced endotoxemia, but anticoagulant-selective Glu149Ala-APC fails to reduce mortality in these settings, indicating that the anticoagulant activity of APC that contributes to bleeding is dispensable for reducing mortality from sepsis.[67,94] Comparable results have been obtained for APC protection in ischemic stroke and neurodegenerative disease.[93] In contrast, cytoprotective-selective 5A-APC fails to delay time to first occlusion in an acute carotid artery thrombosis model, whereas anticoagulant-selective Glu149Ala-APC effectively delays time to first occlusion as anticipated.[67] Interestingly, and contrary to expectations, anticoagulant-selective Glu149Ala-APC but not cytoprotective-selective 5A-APC mitigates toxicity induced by lethal total body irradiation.[100] Thus, depending on insult or disease model some activities of APC mediate beneficial effects, whereas other APC activities are dispensable or even harmful.

Conclusions

A concerted effort by many has resulted in several important discoveries concerning the protein C pathway in the last decade. These important advances include novel insights into the structure-function relation of APC with its multiple co-factors, receptors and substrates. The discovery of a novel cytoprotective protein C pathway that is independent of APC anticoagulant activities and conveys activities directly on cells through interactions with cellular receptors such as EPCR and PAR1 exemplifies another major advance in the last decade. The subsequent search for molecular mechanisms to explain these remarkable effects of APC on cells has provided some initial clues as to the fundamental differences and contrasting functional effects between APC and thrombin-mediated initiation of PAR1-dependent cell signaling. Nevertheless, many unanswered questions still remain. The notion that PAR1 can initiate biased signaling with different and often opposite outcomes provides an intriguing challenge for ongoing and future basic and translational research into the protein C pathway and PAR1 structure-function. Biased signaling by PAR1 is especially relevant and helpful for interpretation of recent outcomes of large phase III clinical trials that evaluated the anti-thrombotic effects of PAR1 antagonists, as these PAR1 antagonists were associated with increased bleeding, especially intracranial bleeding.[101] In this regard, 2nd generation PAR1 compounds that antagonize PAR1-dependent G protein-mediated signaling but not β-arrestin 2-dependent signaling may provide therapeutically relevant entities, especially since APC, although an anticoagulant, prevented bleeding in the brain associated with profibrinolytic therapy.[102]

Perhaps the most striking advancement in the last decade that impacts our current view of APC's multiple activities and the regulation thereof relates to the structure-function analysis of the protein C pathway. The notion that APC's anticoagulant activity requires the APC Gla-domain to bind phospholipids and the APC protease domain to interact with FVa and FVIIIa, aided by protein S *versus* APC's cytoprotective activities that require the APC Gla-domain to bind to EPCR and the APC protease domain to interact with PAR1, led to new investigation into the extended exosite on the protease domain of APC that is required for interactions with FVa and FVIIIa. Observations that the FVa exosite on APC is not required for APC interactions with PAR1, but instead that APC interaction with PAR1 requires a negatively charged region on the other side of the protease domain, provided a way to separate APC anticoagulant activities from its cytoprotective activities. Pharmacological applications of these activity-selective APC mutants have provided unique insights into the relative contributions and requirements of anticoagulant *versus* cytoprotective activities of APC for its beneficial effects in numerous *in vivo* injury and disease models. In addition, activity-selective APC mutants allow for the exploration of new avenues in translation, pre-clinical and clinical research. For instance, the cytoprotective-selective APC 3K3A mutant has recently entered phase I clinical testing for applications in ischemic stroke.[103]

In summary, APC has multiple activities that require assembly of APC in macromolecular complexes supported by interactions of APC with co-factors and by exosite interactions on the protease domain of APC with its different substrates. These exosite interactions are overlapping or partially overlapping for some substrates, whereas for other substrates they are unique and non-overlapping. Although the novel advances of the last decade provide unique insights into how a single enzyme can mediate multiple biologically and therapeutically relevant activities, information on spatial orientation of the various ternary APC co-factor-substrate complexes is limited and much remains unknown. Overall, the protein C pathway provides plentiful opportunities for basic research on the structure-function and molecular mechanisms of its multiple activities, as well as exciting avenues for translational research with potential therapeutic applications in complex diseases, such as the treatment of thrombosis, ischemic stroke, inflammatory disease, atherosclerosis, and vascular disease.

References

1. Gailani D, Broze GJ Jr. Factor XI activation in a revised model of blood coagulation. Science. 1991;253:909-12.
2. Bouma BN, von dem Borne PA, Meijers JC. Factor XI and protection of the fibrin clot against lysis—a role for the intrinsic pathway of coagulation in fibrinolysis. Thromb Haemost. 1998;80:24-7.
3. Renne T, Schmaier AH, Nickel KF, Blomback M, Maas C. In vivo roles of factor XII. Blood. 2012;120:4296-303.
4. Morrissey JH, Choi SH, Smith SA. Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation. Blood. 2012;119:5972-9.
5. Mosnier LO, Bouma BN. Regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor, an unstable carboxypeptidase B that unites the pathways of coagulation and fibrinolysis. Arterioscler Thromb Vasc Biol. 2006;26:2445-53.
6. Bajzar L, Manuel R, Nesheim ME. Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. J Biol Chem. 1995;270:14477-84.
7. von dem Borne PA, Bajzar L, Meijers JC, Nesheim ME, Bouma BN. Thrombin-mediated activation of factor XI results

in a thrombin-activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. J Clin Invest. 1997;99:2323-7.
8. Zwaal RF, Comfurius P, Bevers EM. Lipid-protein interactions in blood coagulation. Biochim Biophys Acta. 1998;1376:433-53.
9. Rosing J, van Rijn JL, Bevers EM, van Dieijen G, Comfurius P, Zwaal RF. The role of activated human platelets in prothrombin and factor X activation. Blood. 1985;65:319-32.
10. Huntington JA. Serpin structure, function and dysfunction. J Thromb Haemost. 2011;(1 Suppl):26-34.
11. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. Blood. 2007;109:3161-72.
12. Dahlbäck B, Villoutreix BO. Regulation of blood coagulation by the protein C anticoagulant pathway. Novel insights into structure-function relationships and molecular recognition. Arterioscler Thromb Vasc Biol. 2005;25:1311-20.
13. Rezaie AR. Regulation of the protein C anticoagulant and anti-inflammatory pathways. Curr Med Chem. 2010;17:2059-69.
14. Esmon CT. Molecular events that control the protein C anticoagulant pathway. Thromb Haemost. 1993;70:29-35.
15. Esmon CT. Protein C anticoagulant system—anti-inflammatory effects. Semin Immunopathol. 2012;34:127-32.
16. Griffin JH, Evatt B, Zimmerman TS, Kleiss AJ, Wideman C. Deficiency of protein C in congenital thrombotic disease. J Clin Invest. 1981;68:1370-3.
17. Branson HE, Katz J, Marble R, Griffin JH. Inherited protein C deficiency and coumarin-responsive chronic relapsing purpura fulminans in a newborn infant. Lancet. 1983;2:1165-8.
18. Chalmers E, Cooper P, Forman K, Grimley C, Khair K, Minford A, et al. Purpura fulminans: recognition, diagnosis and management. Arch Dis Child. 2011;96:1066-71.
19. Reitsma PH, Poort SR, Allaart CF, Briet E, Bertina RM. The spectrum of genetic defects in a panel of 40 Dutch families with symptomatic protein C deficiency type I: heterogeneity and founder effects. Blood. 1991;78:890-4.
20. Dahlbäck B. The protein C anticoagulant system: inherited defects as basis for venous thrombosis. Thromb Res. 1995;77:1-43.
21. Nazarian RM, van Cott EM, Zembowicz A, Duncan LM. Warfarin-induced skin necrosis. J Am Acad Dermatol. 2009;61:325-32.
22. Yan SB, Helterbrand JD, Hartman DL, Wright TJ, Bernard GR. Low levels of protein C are associated with poor outcome in severe sepsis. Chest. 2001;120:915-22.
23. Mosnier LO, Griffin JH. Protein C, protein S, thrombomodulin and the endothelial protein C receptor pathways. In: Marder VJ, Aird WC, Bennett JS, Schulman S, White GC, Colman RW, eds. Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Philadelphia: Lippincott Williams & Wilkins; 2013:300-13.
24. Mather T, Oganessyan V, Hof P, Huber R, Foundling S, Esmon CT, et al. The 2.8 Å crystal structure of Gla-domainless activated protein C. EMBO J. 1996;15:6822-31.
25. Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin catalyzed activation of protein C. Proc Natl Acad Sci USA. 1981;78:2249-52.
26. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. J Biol Chem. 1994;269:26486-91.
27. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc Natl Acad Sci USA. 1996;93:10212-6.
28. Grinnell BW, Gerlitz B, Berg DT. Identification of a region in protein C involved in thrombomodulin- stimulated activation by thrombin: potential repulsion at anion-binding site I in thrombin. Biochem J. 1994;303(Pt 3):929-33.
29. Gale AJ, Griffin JH. Characterization of a thrombomodulin binding site on protein C and its comparison to an activated protein C binding site for factor Va. Proteins. 2004;54:433-41.
30. Knobe KE, Berntsdotter A, Shen L, Morser J, Dahlbäck B, Villoutreix BO. Probing the activation of protein C by the thrombin-thrombomodulin complex using structural analysis, site-directed mutagenesis and computer modeling. Proteins. 1999;35:218-34.
31. Schechter I, Berger A. On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun. 1967;27:157-62.
32. Ehrlich HJ, Grinnell BW, Jaskunas SR, Esmon CT, Yan SB, Bang NU. Recombinant human protein C derivatives: altered response to calcium resulting in enhanced activation by thrombin. EMBO J. 1990;9:2367-73.
33. Richardson MA, Gerlitz B, Grinnell BW. Enhancing protein C interaction with thrombin results in a clot- activated anticoagulant. Nature. 1992;360:261-4.
34. Pozzi N, Barranco-Medina S, Chen Z, Di CE. Exposure of R169 controls protein C activation and autoactivation. Blood. 2012;120:664-70.
35. Isermann B, Vinnikov IA, Madhusudhan T, Herzog S, Kashif M, Blautzik J, et al. Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. Nat Med. 2007;13:1349-58.
36. Rezaie AR. Exosite-dependent regulation of the protein C anticoagulant pathway. Trends Cardiovasc Med. 2003;13:8-15.
37. Wildhagen KC, Lutgens E, Loubele ST, Ten CH, Nicolaes GA. The structure-function relationship of activated protein C. Lessons from natural and engineered mutations. Thromb Haemost. 2011;106:1034-45.
38. Berg DT, Gerlitz B, Shang J, Smith T, Santa P, Richardson MA, et al. Engineering the proteolytic specificity of activated protein C improves its pharmacological properties. Proc Natl Acad Sci USA. 2003;100:4423-8.
39. Shen L, Villoutreix BO, Dahlbäck B. Involvement of lys 62(217) and lys 63(218) of human anticoagulant protein C in heparin stimulation of inhibition by the protein C inhibitor. Thromb Haemost. 1999;82:72-9.
40. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. J Biol Chem. 1994;269:31869-80.
41. Nicolaes GAF, Tans G, Thomassen MC, Hemker HC, Pabinger I, Varadi K, et al. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem. 1995;270:21158-66.
42. Smirnov MD, Esmon CT. Phosphatidylethanolamine incorporation into vesicles selectively enhances factor Va inactivation by activated protein C. J Biol Chem. 1994;269:816-9.
43. Bajzar L, Nesheim ME, Tracy PB. The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent. Blood. 1996;88:2093-100.
44. McDonald JF, Shah AM, Schwalbe RA, Kisiel W, Dahlbäck B, Nelsestuen GL. Comparison of naturally occurring vitamin K-dependent proteins: correlation of amino acid sequences and membrane binding properties suggests a membrane contact site. Biochemistry. 1997;36:5120-7.
45. Smirnov MD, Safa O, Regan L, Mather T, Stearns-Kurosawa DJ, Kurosawa S, et al. A chimeric protein C containing the prothrombin Gla domain exhibits increased anticoagulant activity and altered phospholipid specificity. J Biol Chem. 1998;273:9031-40.
46. Shen L, Shah AM, Dahlbäck B, Nelsestuen GL. Enhancement of human protein C function by site-directed mutagenesis of the gamma-carboxyglutamic acid domain. J Biol Chem. 1998;273:31086-91.
47. Camire RM. A new look at blood coagulation factor V. Curr Opin Hematol. 2011;18:338-42.
48. Nesheim ME, Taswell JB, Mann KG. The contribution of bovine Factor V and Factor Va to the activity of prothrombinase. J Biol Chem. 1979;254:10952-62.
49. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, et al. Mutations in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994;369:64-7.
50. Pellequer JL, Gale AJ, Getzoff ED, Griffin JH. Three-dimensional model of coagulation factor Va bound to activated protein C. Thromb Haemost. 2000;84:849-57.
51. Friedrich U, Nicolaes GAF, Villoutreix BO, Dahlbäck B. Secondary substrate-binding exosite in the serine protease domain of activated protein C important for cleavage at Arg-506 but not at Arg-306 in factor Va. J Biol Chem. 2001;276:23105-8.
52. Gale AJ, Tsavaler A, Griffin JH. Molecular characterization of an extended binding site for coagulation factor Va in the positive exosite of activated protein C. J Biol Chem. 2002;277:28836-40.
53. Lenting PJ, van Mourik JA, Mertens K. The life cycle of coagulation Factor VIII in view of its structure and function. Blood. 1998;92:3983-96.
54. van Dieijen G, Tans G, Rosing J, Hemker HC. The role of phospholipid and factor VIIIa in the activation of bovine factor X. J Biol Chem. 1981;256:3433-42.
55. Regan LM, Lamphear BJ, Huggins CF, Walker FJ, Fay PJ. Factor IXa protects factor VIIIa from activated protein C. Factor IXa inhibits activated protein C-catalyzed cleavage of factor VIIIa at Arg562. J Biol Chem. 1994;269:9445-52.
56. Manithody C, Fay PJ, Rezaie AR. Exosite-dependent regulation of factor VIIIa by activated protein C. Blood. 2003;101:4802-7.
57. O'Brien LM, Mastri M, Fay PJ. Regulation of factor VIIIa by

human activated protein C and protein S: inactivation of cofactor in the intrinsic factor Xase. Blood. 2000;95:1714-20.
58. Cramer TJ, Gale AJ. The anticoagulant function of coagulation factor V. Thromb Haemost. 2012;107:15-21.
59. Hafizi S, Dahlbäck B. Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. FEBS J. 2006;273:5231-44.
60. Hackeng TM, Maurissen LF, Castoldi E, Rosing J. Regulation of TFPI function by protein S. J Thromb Haemost. 2009;7:165-8.
61. ten Kate MK, van der Meer J. Protein S deficiency: a clinical perspective. Haemophilia. 2008;14:1222-8.
62. Rosing J, Hoekema L, Nicolaes GAF, Thomassen MC, Hemker HC, Varadi K, et al. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor Va R506Q by activated protein C. J Biol Chem. 1995;270:27852-8.
63. Norstrom EA, Tran S, Steen M, Dahlback B. Effects of factor Xa and protein S on the individual activated protein C-mediated cleavages of coagulation factor Va. J Biol Chem. 2006;281:31486-94.
64. Yegneswaran S, Wood GM, Esmon CT, Johnson AE. Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography. J Biol Chem. 1997;272:25013-21.
65. Harmon S, Preston RJ, Ainle FN, Johnson JA, Cunningham MS, Smith OP et al. Dissociation of activated protein C functions by elimination of protein S cofactor enhancement. J Biol Chem. 2008;283:30531-9.
66. Preston RJ, Ajzner E, Razzari C, Karageorgi S, Dua S, Dahlbäck B, et al. Multifunctional specificity of the protein C/activated protein C GLA domain. J Biol Chem. 2006;281:28850-7.
67. Mosnier LO, Zampolli A, Kerschen EJ, Schuepbach RA, Banerjee Y, Fernandez JA, et al. Hyper-antithrombotic, noncytoprotective Glu149Ala-activated protein C mutant. Blood. 2009;113:5970-8.
68. Ohlin AK, Landes G, Bourdon P, Oppenheimer C, Wydro R, Stenflo J. Beta-hydroxyaspartic acid in the first epidermal growth factor-like domain of protein C. Its role in Ca2+ binding and biological activity. J Biol Chem. 1988;263:19240-8.
69. Adams TE, Hockin MF, Mann KG, Everse SJ. The crystal structure of activated protein C-inactivated bovine factor Va: Implications for cofactor function. Proc Natl Acad Sci USA. 2004;101:8918-23.
70. Villoutreix BO, Teleman O, Dahlback B. A theoretical model for the Gla-TSR-EGF-1 region of the anticoagulant cofactor protein S: from biostructural pathology to species-specific cofactor activity. J Comput Aided Mol Des. 1997;11:293-304.
71. Walker FJ, Scandella D, Fay PJ. Identification of the binding site for activated protein C on the light chain of factors V and VIII. J Biol Chem. 1990;265:1484-9.
72. Krishnaswamy S, Williams EB, Mann KG. The binding of activated protein C to factors V and Va. J Biol Chem. 1986;261:9684-93.
73. Dahlbäck B, Villoutreix BO. The anticoagulant protein C pathway. FEBS Lett. 2005;579:3310-6.
74. Saller F, Villoutreix BO, Amelot A, Kaabache T, Le Bonniec BF, Aiach M, et al. The gamma-carboxyglutamic acid domain of anticoagulant protein S is involved in activated protein C cofactor activity, independently of phospholipid binding. Blood. 2005;105:122-30.
75. He XH, Shen L, Villoutreix BO, Dahlbäck B. Amino acid residues in thrombin-sensitive region and first epidermal growth factor domain of vitamin K-dependent protein S determining specificity of the activated protein C cofactor function. J Biol Chem. 1998;273:27449-58.
76. Hackeng TM, Yegneswaran S, Johnson AE, Griffin JH. Conformational changes in activated protein C caused by binding of the first epidermal growth factor-like module of protein S. Biochem J. 2000;349:757-64.
77. Andersson HM, Arantes MJ, Crawley JT, Luken BM, Tran S, Dahlbäck B, et al. Activated protein C cofactor function of protein S: a critical role for Asp95 in the EGF1-like domain. Blood. 2010;115:4878-85.
78. Ahnstrom J, Andersson HM, Canis K, Norstrom E, Yu Y, Dahlbäck B, et al. Activated protein C cofactor function of protein S: a novel function for a {gamma}-carboxyglutamic acid residue. Blood. 2011;117:6685-93.
79. Evenas P, Garcia de FP, Nicolaes GA, Dahlback B. The second laminin G-type domain of protein S is indispensable for expression of full cofactor activity in activated protein C-catalysed inactivation of factor Va and factor VIIIa. Thromb Haemost. 2000;84:271-7.
80. Heeb MJ, Kojima Y, Rosing J, Tans G, Griffin JH. C-terminal residues 621-635 of protein S are essential for binding to factor Va. J Biol Chem. 1999;274:36187-92.
81. Joyce DE, Gelbert L, Ciaccia A, DeHoff B, Grinnell BW. Gene expression profile of antithrombotic protein C defines new mechanisms modulating inflammation and apoptosis. J Biol Chem. 2001;276:11199-203.
82. Coughlin SR, Camerer E. PARticipation in inflammation. J Clin Invest. 2003;111:25-7.
83. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science. 2002;296:1880-2.
84. Zhang C, Srinivasan Y, Arlow DH, Fung JJ, Palmer D, Zheng Y, et al. High-resolution crystal structure of human protease-activated receptor 1. Nature. 2012;492:387-92.
85. Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, et al. Extracellular histones are major mediators of death in sepsis. Nat Med. 2009;15:1318-21.
86. Weiler H. Multiple receptor-mediated functions of activated protein C. Hamostaseologie. 2011;31:185-95.
87. Rezaie AR. The occupancy of endothelial protein C receptor by its ligand modulates the par-1 dependent signaling specificity of coagulation proteases. IUBMB Life. 2011;63:390-6.
88. Russo A, Soh UJ, Paing MM, Arora P, Trejo J. Caveolae are required for protease-selective signaling by protease-activated receptor-1. Proc Natl Acad Sci USA. 2009;106:6393-7.
89. Mosnier LO, Sinha RK, Burnier L, Bouwens EA, Griffin JH. Biased agonism of protease-activated receptor 1 by activated protein C caused by non-canonical cleavage at Arg46. Blood. 2012;120:5237-46.
90. Soh UJ, Trejo J. Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through beta-arrestin and dishevelled-2 scaffolds. Proc Natl Acad Sci USA. 2011;108:E1372-E1380.
91. Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. Molecular Mechanism of b-Arrestin-Biased Agonism at Seven-Transmembrane Receptors. Annu Rev Pharmacol Toxicol. 2012;52:179-97.
92. Danese S, Vetrano S, Zhang L, Poplis VA, Castellino FJ. The protein C pathway in tissue inflammation and injury: pathogenic role and therapeutic implications. Blood. 2010;115:1121-30.
93. Zlokovic BV, Griffin JH. Cytoprotective protein C pathways and implications for stroke and neurological disorders. Trends Neurosci. 2011;34:198-209.
94. Kerschen EJ, Fernandez JA, Cooley BC, Yang XV, Sood R, Mosnier LO, et al. Endotoxemia and sepsis mortality reduction by non-anticoagulant activated protein C. J Exp Med. 2007;204:2439-48.
95. Bae JS, Yang L, Manithody C, Rezaie AR. Engineering a disulfide bond to stabilize the calcium binding loop of activated protein C eliminates its anticoagulant but not protective signaling properties. J Biol Chem. 2007;282:9251-9.
96. Mosnier LO, Gale AJ, Yegneswaran S, Griffin JH. Activated protein C variants with normal cytoprotective but reduced anticoagulant activity. Blood. 2004;104:1740-5.
97. Mosnier LO, Yang XV, Griffin JH. Activated protein C mutant with minimal anticoagulant activity, normal cytoprotective activity, and preservation of thrombin activable fibrinolysis inhibitor-dependent cytoprotective functions. J Biol Chem. 2007;282:33022-33.
98. Yang L, Bae JS, Manithody C, Rezaie AR. Identification of a specific exosite on activated protein C for interaction with protease activated receptor 1. J Biol Chem. 2007;282:25493-500.
99. Preston RJ, Villegas-Mendez A, Sun YH, Hermida J, Simioni P, Philippou H, et al. Selective modulation of protein C affinity for EPCR and phospholipids by Gla domain mutation. FEBS J. 2005;272:97-108.
100. Geiger H, Pawar SA, Kerschen EJ, Nattamai KJ, Hernandez I, Liang HP, et al. Pharmacological targeting of the thrombomodulin-activated protein C pathway mitigates radiation toxicity. Nat Med. 2012;18:1123-9.
101. Morrow DA, Braunwald E, Bonaca MP, Ameriso SF, Dalby AJ, Fish MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. N Engl J Med. 2012;366:1404-13.
102. Cheng T, Petraglia AL, Li Z, Thiyagarajan M, Zhong Z, Wu Z, et al. Activated protein C inhibits tissue plasminogen activator-induced brain hemorrhage. Nat Med. 2006;12:1278-85.
103. Williams PD, Zlokovic BV, Griffin JH, Pryor KE, Davis TP. Preclinical safety and pharmacokinetic profile of 3K3A-APC, a novel, modified activated protein C for ischemic stroke. Curr Pharm Des. 2012;18:4215-22.

Evolution of thrombophilia testing

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A B S T R A C T

Thrombophilia can be identified in many patients presenting with venous thromboembolism (VTE). Whether the results of such tests help in the clinical management of patients has not been settled. Thrombophilia testing in asymptomatic relatives may be useful in families with antithrombin, protein C or protein S deficiency, or homozygosity for factor V Leiden, but is limited to women who intend to become pregnant or who would like to use oral contraceptives. Careful counseling with knowledge of absolute risks helps patients make an informed decision in which their own preferences can be taken into account. Patients who have had VTE and have thrombophilia are, at most, at a slightly increased risk for recurrence. In the absence of trials that compared routine and prolonged anticoagulant treatment in patients testing positive for thrombophilia, testing for such defects to prolong anticoagulant therapy cannot be justified. Diagnosing antiphospholipid syndrome in patients with VTE and in women with recurrent miscarriage usually leads to a change in patient management, although the evidence to support this is limited. Over the past half century there has been an increase in our knowledge and greater possibilities for genetic testing have become available. Despite this, testing for thrombophilia serves only a limited purpose and should not be performed on a routine basis.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe currently established thrombophilias;
- describe the risks of clinical manifestations associated with thrombophilias;
- discuss the pros and cons of thrombophilia testing in various clinical settings.

Introduction

To our knowledge, the term 'thrombophilia' was first used by Nygaard and Brown in 1937 when they described sudden occlusion of the large arteries, sometimes with co-existent venous thrombosis.[1] In 1956, Jordan and Nandorff extensively reviewed their own and published cases on the *familial* tendency in thrombo-embolic disease.[2] Nowadays, the term is generally used for a laboratory abnormality, most often in the coagulation system, which increases the risk of venous thromboembolism (VTE), *i.e.* venous thrombosis in any site or pulmonary embolism. In the past half century, thrombophilia has evolved from a very rare genetic disorder to a highly prevalent trait. This evolution is an immediate consequence of increasing insight into the blood coagulation system, as well as into genetic research possibilities, that made it possible to search for specific candidate abnormalities in the coagulation proteins and their encoding genes. Nowadays, some form of thrombophilia can be identified in approximately half of the patients presenting with VTE. Testing has increased tremendously for various indications,[3] but whether the results of such tests help in the clinical management of patients has not been settled.[4,5] In this educational session, we give a short overview of the history of thrombophilia research and review the currently most commonly tested thrombophilias, with a focus on an evidence-based approach to justify testing for thrombophilia in various patient groups.

A short history of thrombophilia research

Research into thrombophilia started by investigating candidate proteins or genes in highly thrombophilic families and linking abnormalities with the clinical phenotype within these families. As a next step, findings were confirmed in case control studies. These showed increased risk compared to controls, often taken from the general population. For clinicians and patients, an absolute risk estimate is more appropriate to guide decisions regarding prevention or treatment, and this was the subject of family studies of consecutive probands with a specific thrombophilic defect. The huge progress in genetic and bioinformatic techniques now allows all kinds of searches to be made, both in population-derived studies of cases with VTE and controls, and in thrombophilic families.[6-8]

In 1965, Egeberg identified a deficiency of the physiological anticoagulant antithrombin in a

Norwegian family in which several members suffered from venous thrombosis.[9] In the early 1980s, deficiencies of the other anticoagulant proteins, *i.e.* protein C and protein S, were discovered as hereditary risk factors of VTE.[10,11] At this time, the genes could be cloned, and since then numerous mutations in the genes encoding antithrombin, protein C and protein S have been identified as underlying causes of low plasma levels of the anticoagulant proteins.[12-14] Another decade later, in 1993, Dahlbäck described the phenomenon of a poor anticoagulant response to activated protein C (APC), *i.e*. APC resistance, in a Swedish family with a high tendency of venous thrombosis.[15] In the original paper, Dahlbäck proposed that APC resistance was best explained by an inherited deficiency of a previously unrecognized cofactor to APC, after having ruled out several possible mechanisms, including deficiencies of protein S, protein C, or linkage with polymorphisms in the factor VIII or Von Willebrand factor genes. He then showed that this alleged 'co-factor' was identical to coagulation factor V.[16] Soon thereafter, several laboratories independently reported the underlying genetic defect: a single G to A substitution in the gene of factor V at nucleotide position 1691, resulting in an amino acid change at position 506, the first cleavage site of factor Va for APC (FV Q506, also named FV Leiden).[17-20] In 1996, genetic analysis of candidate factor prothrombin revealed a G to A transition at position 20210 that was quite common in patients with VTE who had a family history of VTE. The mutation was linked to elevated levels of prothrombin.[21] Since then, various more common genetic variants that increase the risk of VTE to a greater or lesser extent have been identified and are included in diagnostic panels of thrombophilia testing.[22] For the more common thrombophilias that increase the risk at least 2-fold, a large number of clinical studies have led to reliable estimates of the relative and absolute risk for VTE; these will be summarized in this review.

Current thrombophilia test panel

The currently most commonly tested inherited thrombophilias include deficiencies of antithrombin, protein C, or protein S, and the gain-of-function mutations factor V Leiden and prothrombin G20210A, that impact either the anticoagulant or procoagulant pathways.[4] Lupus anticoagulant, anticardiolipin antibodies, and anti-ß2-glycoprotein1 antibodies, which are laboratory features of the acquired thrombophilic antiphospholipid syndrome, are also generally included in a thrombophilia testing panel.[23] Elevated levels of several coagulation factors, including factors VIII, IX and XI, also increase the risk of VTE.[24-26] Although the levels of coagulation factors are in part determined genetically, factor VIII also increases with age and during various inflammatory diseases including VTE. It is worthy of note that some laboratories also include other, less well-established polymorphisms in their thrombophilia panel for which clinical implications are most uncertain. Examples are MTHFR 677TT and PAI-1 4G/5G that have at most only a weak association with VTE.[6]

Epidemiology of thrombophilia

General considerations

Thrombophilic abnormalities can be either acquired or inherited. An example of acquired thrombophilia is the antiphospholipid antibody syndrome that is characterized by a tendency toward venous or arterial thrombosis, recurrent pregnancy loss or late pregnancy-related complications, in combination with persistent lupus anticoagulant or antiphospholipid antibodies. Furthermore, there are many acquired and/or transient conditions that lead to a prothrombotic state including cancer, surgery, strict immobilization, pregnancy and the postpartum period, and use of estrogen-containing medication, such as oral contraceptives and hormone replacement therapy. Although the term thrombophilia was traditionally used to apply to patients with unusual manifestations of VTE, such as recurrent spontaneous episodes, thrombosis at a young age, a strong family history, or thrombosis in an unusual site, we now know that thrombophilia tends to increase the risk for any episode of venous thrombosis or pulmonary embolism. Approximately half of the patients with inherited thrombophilia will develop their first VTE related to an acquired or transient prothrombotic risk situation. Furthermore, despite the fact that thrombosis at a young age was assumed to be a criterion for thrombophilia, and the mean age at time of a first thrombotic age is approximately ten years lower than in the general population, the vast majority of patients will have the first episode when they are over 45 years of age; a threshold that is often used to justify thrombophilia testing. The theoretical concept is that patients with thrombophilia have an intrinsic prothrombotic state which in itself is insufficient to cause thrombosis, but may lead to an event when superimposed on clinical risk factors, including increasing age.[27] It is also likely that selective testing in families with a strong history of VTE, and consequently co-segregation of known and unknown genes in the early days of thrombophilia research, has resulted in a perceived stronger risk increase than more contemporary studies have established.[28,29]

Prevalence of thrombophilia and association with various clinical conditions

Table 1 shows the prevalence of the various established thrombophilias in the general population, as well as their relationship with first and recurrent episodes of VTE, arterial thrombosis, and pregnancy complications. These defects are consistently associated with a first episode of VTE, with relative risk increases of 2 to 10.[4,30] However, inherited thrombophilias only modestly increase the risk of recurrent episodes.[4,31] Also, the association between thrombophilias and arterial thrombosis or pregnancy complications is not consistent.[32,33] Nevertheless, approximately half of all thrombophilia tests are being performed in the latter clinical settings.[3] The prevalence of persistent lupus anticoagulant or antibodies against phospholipid in the general population is not well known, since in most population-based studies these were only assessed once.[4]

Pros and cons of thrombophilia testing according to clinical factors

Testing for thrombophilia to modify the risk of a first VTE

In clinical practice, requests for thrombophilia testing often come from asymptomatic individuals with a family history of VTE, in whom the index patients may or even may not have a known specific thrombophilic defect. Having a family history of VTE is a very poor predictor of the presence of thrombophilia.[34,35] However, VTE in one or more first degree relatives increases the risk of VTE by approximately 2-fold in the absence of an inherited thrombophilic defect, but even more so when both are present.[35] Still, a potential advantage of testing patients with VTE for thrombophilia may be the identification of asymptomatic family members in order to take preventive measures if tested positive, and to withhold such measures if relatives have tested negative. An important requisite is that a test result does indeed differentiate between carriers and non-carriers in terms of their risk for a first episode of VTE.

Table 2 summarizes the absolute risks for a first episode of VTE as assessed in several retrospective and prospective family cohort studies with a similar design that have been summarized in a previous review.[4] The overall annual incidence of a first VTE in individuals with antithrombin, protein C or protein S deficiency is approximately 1.5%, whereas this risk is approximately 0.5% for carriers of the factor V Leiden or prothrombin 20210A mutation. These estimates roughly correspond with multiplying the baseline risk in the general population with the relative risk estimates as listed in Table 1. Obviously, the 2% annual major bleeding risk associated with continuous anticoagulant treatment with vitamin K antagonists outweighs the risk of VTE.[36] Table 2 also shows that during high-risk situations such as surgery, immobilization, trauma, pregnancy, the postpartum period, and during the use of oral contraceptives the absolute risk is generally low, with the exception of women with some defects who use oral contraceptives or who are pregnant.

For women who wish to use oral contraceptives and who have a positive first degree relative with VTE and a known thrombophilic defect, one can estimate the effect of avoidance of oral contraceptives on the number of prevented episodes of VTE by means of thrombophilia testing or, alternatively, by using a positive family history without thrombophilia testing. The results are listed in

Table 1. Prevalence of thrombophilia and relative risk estimates for various clinical manifestations.

	Antithrombin deficiency	Protein C deficiency	Protein S deficiency	Factor V Leiden	Prothrombin 20210A mutation	Lupus anticoagulant*	Anti cardiolipin antibodies*	Anti 2 GPI antibodies
Prevalence in the general population	0.02%	0.2%	0.03-0.13%	3-7%	0.7-4%	1-8%	5%	3.4%
Relative risk for a first venous thrombosis	5-10	4-6.5	1-10	3-5	2-3	3-10	0.7	2.4
Relative risk for recurrent venous thrombosis	1.9-2.6	1.4-1.8	1.0-1.4	1.4	1.4	2-6	1-6	-
Relative risk for arterial thrombosis	No association	No consistent association	No consistent association	1.3	0.9	10	1.5-10	-
Relative risk for pregnancy complications	1.3-3.6	1.3-3.6	1.3-3.6	1.0-2.6	0.9-1.3	No consistent data	No consistent data	-

*Figures are derived from studies that are reviewed in detail elsewhere.[4] *In most studies, presence of these thrombophilic risk factors was only assessed once.*

Table 2. Estimated incidence of a first episode of VTE in carriers of various thrombophilias (data apply to individuals with at least one symptomatic first-degree relative).

	Antithrombin, protein C, or protein S deficiency	Factor V Leiden,	Prothrombin 20210A mutation heterozygous	Factor V Leiden, homozygous
Overall (%/year, 95%CI)	1.5 (0.7-2.8)	0.5 (0.1-1.3)	0.4 (0.1-1.1)	1.8 (0.1-4.0)*
Surgery, trauma, or immobilization (%/episode, 95%CI)†	8.1 (4.5-13.2)	1.8 (0.7-4.0)	1.6 (0.5-3.8)	-
Pregnancy (%/pregnancy, 95%CI)	4.1 (1.7-8.3)	2.1 (0.7-4.9)	2.3 (0.8-5.3)	16.3[#]
During pregnancy, %, 95%CI	1.2 (0.3-4.2)	0.4 (0.1-2.4)	0.5 (0.1-2.6)	7.0[#]
Postpartum period, %, 95%CI	3.0 (1.3-6.7)	1.7 (0.7-4.3)	1.9 (0.7-4.7)	9.3[#]
Oral contraceptive use (%/year of use, 95%CI)	4.3 (1.4-9.7)	0.5 (0.1-1.4)	0.2 (0.0-0.9)	-

*Figures are derived from numerous family studies which are reviewed in detail elsewhere.[4] [†]These risk estimates reflect to a large extent the situation before thrombosis prophylaxis was routine patient care. *Based on pooled OR of 18 (8-40) and an incidence of 0.1% in non-carriers. [#]Data from family studies; risk estimates lower in other settings.*

Table 3, in which the first column shows the observed incidence of VTE during one year of oral contraceptive use in carriers and non-carriers from thrombophilic families. From the risk difference between carriers and non-carriers (second column) the number of women who need to refrain from oral contraceptive use to prevent one episode of VTE can be calculated (third column). Table 3 clearly indicates that women with antithrombin, protein C or protein S deficiency have a high absolute risk of VTE provoked by use of oral contraceptives. However, in these families, women without a deficiency also have a markedly increased risk of oral contraceptive-related VTE compared to pill users from the general population (0.7% *vs*. 0.04% per year of use), reflecting a selection of families with a strong thrombotic tendency in which yet unknown thrombophilias have co-segregated. Thus, although selective avoidance of oral contraceptive use prevents VTE episodes in deficient women, for women from these families a negative thrombophilia test may lead to false reassurance. The risk estimates are very different for the more common and less severe thrombophilias, such as factor V Leiden and the prothrombin 20210A mutation, with a large number of women needing to avoid use of oral contraceptives to avoid 1 VTE, and 666 study subjects needed to power the results. Also, from these families, women without the mutation have a higher incidence of pill-related VTE than women in the general population (0.2% *vs*. 0.04% per year of use).

Table 4 indicates the number of study subjects needed to test to initiate prophylactic measurements around pregnancy, again applicable to women from thrombophilic families. For women with antithrombin, protein C or protein S deficiency, or those who are homozygous for factor V Leiden, the risks of 4% and 16%, respectively, during pregnancy and the postpartum period may outweigh the nuisance of daily subcutaneous low molecular weight heparin (LMWH) injections with frequently occurring skin reactions, and the very small risk of severe complications of anticoagulant therapy during pregnancy.[40,42] However, the optimal dose of LMWH prophylaxis in pregnancy has not been established and the most often used regimen of low-dose LMWH is certainly not 100% effective.[42,43] Hence,

Table 3. Estimated number of asymptomatic thrombophilic women or women with a positive family history for VTE who should avoid using oral contraceptives to prevent one VTE, and estimated number needed to test.

Thrombophilia	Risk on OC per year, %	Risk difference per 100 women	N. not taking OC to prevent 1 VTE	N. of female relatives to be tested
Antithrombin, protein C, or protein S deficiency				
Deficient relatives	4.3*	3.6	28	56
Non-deficient relatives	0.7*			
Factor V Leiden or prothrombin 20210A mutation				
Relatives with the mutation	0.5*	0.3	333	666
Relatives without the mutation	0.2*			
Family history of VTE				
General population, no family history	0.04#	0.03	3333	none
General population, positive family history	0.08#	0.06	1667	none

Based on family studies as outlined in Table 2. #Based on a population baseline risk of VTE in young women of 0.01% per year,[37] a relative risk of VTE by use oral contraceptives of 4,[38] and a relative risk of 2 of VTE by having a positive family history.[35] OC: oral contraceptives.

Table 4. Estimated number of asymptomatic thrombophilic women who should use LMWH prophylaxis during pregnancy and/or the postpartum period to prevent pregnancy-related VTE, and estimated number needed to test.

Thrombophilia	Risk of VTE per pregnancy,§ %	Risk difference per 100 women	N. using prophylaxis to prevent 1 VTE^	N. of female relatives to be tested
Antithrombin, protein C, or protein S deficiency				
Deficient relatives	4.1*	3.6	28	56
Non-deficient relatives	0.5*			
Factor V Leiden or prothrombin 20210A mutation, heterozygous				
Relatives with the mutation	2.0*	1.5	66	132
Relatives without the mutation	0.5*			
Factor V Leiden or prothrombin 20210A mutation, homozygous				
Homozygous relatives	16.0	15.5	6	24
Relatives without the mutation	0.5			
Family history of VTE				
General population, no family history	0.5#	n/a	200	none
General population, positive family history	1.0#	0.5	200	none

*§Antepartum and postpartum combined. *Based on family studies as outlined in Table 2. #Based on a population risk of pregnancy-related VTE of 0.5% per pregnancy,[39] and a relative risk of 2 of VTE by having a positive family history.[35] ^These estimates apply to women with a positive family history of VTE and assume an unrealistic 100% efficacy of prophylaxis with LMWH.*

the figures in Table 4 underestimate the true number of women that need to use prophylaxis (and be tested prior to this decision) in order to avoid pregnancy-related VTE. Whether the absolute risks of pregnancy-related episodes justifies prophylaxis for eight months during pregnancy, or the shorter postpartum period of six weeks is a matter of choice for the physician and patient. The risk of pregnancy-related VTE in women from these families who do not have the inherited thrombophilic defect is approximately 0.5%, compared to 0.2% in the general population.[39] Hence, withholding prophylaxis from women from thrombophilic families who do not have the defect is supported by evidence from well-designed studies of individuals in the same clinical context.

Thrombophilia testing in patients with venous thromboembolism

Thrombophilia testing is most often considered in patients with VTE, particularly if they are young, have recurrent episodes, have thrombosis at unusual sites, or have a positive family history for the disease. However, although such a strategy may lead to an increased yield of testing, the main question is whether a positive test result should change patient management. VTE tends to recur, with a cumulative incidence of a second episode of approximately 25% in five years. Patients with a transient clinical risk factor such as surgery eliciting their first VTE have a very low risk of recurrence.[44,45] However, whether the presence of thrombophilia is able to predict recurrence is much less clear, with conflicting results in various studies that compared the prevalence of thrombophilia in patients with recurrent VTE with those in patients without recurrence.[4, 31] The relative risk of recurrent VTE for carriers of inherited thrombophilia found in most population-based cohorts is estimated to be approximately 1.5 for most defects (Table 1). In a large pooled study of thrombophilic families, we observed a cumulative incidence of VTE recurrences after ten years of 55% in relatives with a deficiency of antithrombin, protein C or protein S deficiency, as compared to 25% in those with the factor V Leiden mutation, the prothrombin 20210A mutation or high levels of FVIII.[46] For homozygous or double heterozygous carriers of factor V Leiden and/or the prothrombin 20210A mutation, the estimated risks of recurrence vary widely between studies, with a pooled estimate of 2.7 (95%CI: 1.2-6.0).[47,48] Whether such a risk increase warrants prolongation of the duration of anticoagulation, particularly after provoked VTE, is a matter of debate.[49,50] Furthermore, given the rarity of homozygous or double heterozygous thrombophilias in unselected patients with VTE, the efficiency of testing is obviously very low.[5,51]

A randomized controlled trial in which testing for thrombophilia in patients with a first episode of VTE is the intervention, and recurrent VTE is the outcome, would provide the ultimate evidence to decide whether this is justified. Testing should lead to a pre-defined strategy to prevent recurrence with, for instance, a longer or indefinite duration of anticoagulant therapy. To our knowledge, no such trials have been successfully performed.[52] In order to investigate whether testing for thrombophilia reduces the risk of recurrent VTE in patients after a first episode, for instance by prolonged use of anticoagulation, avoidance of high-risk situations, or intensified prophylaxis in high-risk situations, we selected 197 patients from the MEGA case control study who had had a recurrent event during follow up.[53] We compared the proportion of these patients who had been tested with the proportion of 324 control patients who did not have a recurrence during follow up, matched for age, sex, year of event and geographical region. Thrombophilia tests were performed in 35% of cases and in 30% of controls. The OR for recurrence was 1.2 (95%CI: 0.9-1.8) for tested *versus* non-tested patients, indicating that testing, with real-life clinical decisions based on the outcome of testing, does not reduce the risk of recurrent VTE in patients who have experienced a first episode. For patients with antiphospholipid syndrome the issue is more complicated. It is a heterogeneous syndrome, both clinically as well as due to problems in standardization of laboratory tests. There is no evidence to define the optimal treatment duration of consecutive patients with VTE and persistent laboratory criteria for antiphospholipid syndrome, although it is widely recommended to treat such patients for a prolonged period with anticoagulant medication.[54] If the prevalence of persistently positive tests in patients with VTE is 10%, 10 patients would need to be tested in order to identify one patient with antiphospholipid syndrome in whom prolonged anticoagulant treatment should be initiated. This seems to be a reasonable number, but most clinicians only test for antiphospholipid syndrome in patients with VTE in the absence of provoking risk factors, or when other clinical manifestations raise suspicion.

Vitamin K antagonists at a higher than normal INR intensity do not decrease the risk of recurrence in patients with well-defined antiphospholipid syndrome, as compared to vitamin K antagonists at a target intensity of 2.0 to 3.0.[55,56]

Thrombophilia testing in patients with arterial cardiovascular disease

Numerous studies have investigated the association between thrombophilia and arterial cardiovascular diseases, and yielded conflicting results.[32] There is no evidence that the presence of inherited thrombophilia should lead to different secondary prevention, and testing in this clinical setting is not justified.

Thrombophilia testing in women with pregnancy complications

The association between inherited thrombophilia and pregnancy complications varies depending on the type of thrombophilia and the complication (Table 1).[33] Pregnancy complications are amongst the clinical manifestations of the antiphospholipid syndrome.[57] Aspirin and heparin treatment is suggested for women with antiphospholipid syndrome and recurrent miscarriage, although the evidence that this is efficacious is very limited.[42,58]

Whether the association between pregnancy complications and inherited thrombophilia is causal is controversial, as many other factors play a role in this risk.[59,60] Therapeutic options to prevent pregnancy complications in women with inherited thrombophilia, like in antiphospholipid syndrome, include aspirin as well as LMWH. There is no current evidence supporting treatment since observational research is hampered by poor methodology or inconsistent results.[60,61] In women with unexplained recurrent miscarriage, two recent randomized controlled trials, *i.e.* the ALIFE and the SPIN studies, were unable to demonstrate a beneficial effect of anticoagulant therapy

compared to no pharmacological treatment or placebo.[62,63] The HABENOX trial also did not demonstrate a difference in live birth between three active treatment arms, *i.e.* LMWH combined with aspirin, LMWH alone, and aspirin alone, in 207 women with recurrent pregnancy loss with or without inherited thrombophilia.[62] A subgroup analysis did not suggest a differential effect amongst the 25% women with thrombophilia. Although the ALIFE study was underpowered for subgroup analyses, an *a priori* planned analysis in women with inherited thrombophilia showed a relative risk for live birth of 1.31 (95%CI: 0.74 to 2.33) for the combined intervention compared to placebo, and 1.22 (95%CI: 0.69 to 2.16) for aspirin, with corresponding absolute difference in live birth rates of 16.3% (95%CI: –18.2 to 50.8) and 11.8% (95%CI: –21.1 to 44.6), respectively.[62] The possibility that one or both of these interventions might be beneficial in such women warrants further study in adequately powered, controlled trials. We have just started recruiting in the multicenter ALIFE2 trial (www.trialregister.nl; NTR3361) that compares LMWH with standard pregnancy care in women with thrombophilia and a history of recurrent miscarriage.

Some trials have shown benefit of anticoagulant treatment for specific pregnancy complications in women with inherited thrombophilia. First, women with a single previous pregnancy loss after ten weeks' gestation and who had heterozygous factor V Leiden mutation, prothrombin G20210A mutation, or protein S deficiency, were allocated to enoxaparin 40 mg once daily (n=80) or to aspirin 100 mg (n=80).[65] Women who were treated with enoxaparin had a higher chance of a live birth than those allocated to aspirin (86% and 29%, respectively, risk difference 57%, odds ratio 15.5, 95%CI: 7 to 34). However, methodological issues were raised regarding concealment of allocation, lack of generalizability due to very stringent inclusion criteria, and an unusually high prevalence of late miscarriage in the source cohort.[66] Furthermore, women who experienced an early miscarriage after randomization were not taken into account.[67] The results of this single study have not been implemented in recent evidence-based guidelines.[42] Second, for women at moderate to high risk of preeclampsia, aspirin provides a modest benefit in reducing this risk, whereas anticoagulants are not considered useful.[42,68,69] The recently published FRUIT trial evaluated the effect of adding LMWH to standard aspirin in 139 women who had had previous early-onset preeclampsia, HELLP syndrome, eclampsia and/or small for gestational age babies and had inherited thrombophilia without antiphospholipid antibodies.[70] LMWH with aspirin reduced the incidence of early onset recurrent hypertensive disorders (risk difference 8.7%, 95%CI: 1.9-15.5%). Whether this single, relatively small trial justifies testing and subsequent treatment in all women with a history of severe preeclampsia has not yet been settled.

In conclusion, given the currently available evidence, using anticoagulant therapy to improve the prognosis of a pregnancy in women with recurrent pregnancy loss who do not have a diagnosis of antiphospholipid syndrome must be considered experimental.[42,61] Furthermore, for women with other pregnancy complications including preeclampsia, testing for antiphospholipid syndrome or inherited thrombophilia at present can not be justified.[42]

General cons of thrombophilia testing

A disadvantage of testing patients with a VTE for thrombophilia is the high cost involved. Although two studies concluded that testing for thrombophilia in some scenarios could indeed be cost-effective, the underlying assumptions from inconsistent observational studies seriously hamper their interpretation.[71,72] The psychological impact and consequences of knowing that one is a carrier of a genetic thrombophilic defect are considered to be limited, although a qualitative study described several negative effects of both psychological and social origin.[73,74] Furthermore, difficulties in obtaining life or disability insurance are frequently encountered by individuals who are known carriers of thrombophilia, regardless of whether they are symptomatic or asymptomatic.[73]

Future of thrombophilia testing

Whereas a somewhat nihilistic approach may be the result of the currently available evidence in favor of thrombophilia testing in clinical practice, this obviously should not prevent investigators from acquiring more insight. To be able to better predict risk to the point where it will enable evidence-based decisions to be made would be of particular interest for patients with all clinical indications. It is possible that in the future, multiple SNP analyses of genes inside or outside the coagulation system will further improve and become feasible in clinical practice.[75,76]

Conclusion

Despite the increasing knowledge about the etiology of VTE, testing for thrombophilia serves only a limited purpose and should not be performed on a routine basis. Thrombophilia testing in asymptomatic relatives may be useful in families with antithrombin, protein C or protein S deficiency, or for siblings of patients who are homozygous for factor V Leiden, and is limited to women who intend to become pregnant or who would like to use oral contraceptives. Careful counseling with knowledge of the absolute risks helps patients make an informed decision in which their own preferences can be taken into account. Observational studies show that patients who have had VTE and have thrombophilia are, at most, at a slightly increased risk of recurrence. Furthermore, no beneficial effect on the risk of recurrent VTE was observed in patients who had been tested for inherited thrombophilia. In the absence of trials that compare routine and prolonged anticoagulant treatment in patients testing positive for thrombophilia, testing for such defects to prolong anticoagulant therapy cannot be justified. Diagnosing antiphospholipid syndrome would potentially lead to changes in treatment in selected patients with VTE and women with recurrent miscarriage, although the evidence to support this is limited.

References

1. Nygaard KK, Brown GE. Essential thrombophilia: report of five cases. Arch Intern Med. 1937;59(1):82-106.
2. Jordan FLJ, Nandorff A. The familial tendency in thrombo-

embolic disease. Acta Med Scand. 1956;156:267-75.
3. Coppens M, van Mourik JA, Eckmann CM, Buller HR, Middeldorp S. Current practice of testing for hereditary thrombophilia in The Netherlands. J Thromb Haemost. 2007;5:1979-81.
4. Middeldorp S, van Hylckama Vlieg A. Does thrombophilia testing help in the clinical management of patients? Br J Haematol. 2008;143:321-35.
5. Baglin T, Gray E, Greaves M, et al. Clinical guidelines for testing for heritable thrombophilia. Br J Haematol. 2010;149(2):209-20.
6. Gohil R, Peck G, Sharma P. The genetics of venous thromboembolism. A meta-analysis involving approximately 120,000 cases and 180,000 controls. Thromb Haemost. 2009;102(2):360-70.
7. Bezemer ID, Bare LA, Doggen CJ, et al. Gene variants associated with deep vein thrombosis. JAMA. 2008;299(11):1306-14.
8. Lotta LA, Wang M, Yu J, et al. Identification of genetic risk variants for deep vein thrombosis by multiplexed next-generation sequencing of 186 hemostatic/pro-inflammatory genes. BMC Med Genomics. 2012;5:7.
9. Egeberg O. Inherited antithrombin III deficiency causing thrombophilia. Thromb Diath Haemorrh. 1965;13:516-30.
10. Griffin JH, Evatt B, Zimmerman TS, Kleiss AJ, Wideman C. Deficiency of protein C in congenital thrombotic disease. J Clin Invest. 1981;68(5):1370-3.
11. Comp PC, Esmon CT. Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Eng J Med. 1984;311(24):1525-8.
12. Lane DA, Bayston T, Olds RJ, et al. Antithrombin mutation database: 2nd (1997) update. For the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost. 1997;77(1):197-211.
13. Reitsma PH. Protein C deficiency: summary of the 1995 database update. Nucleic Acids Res. 1996;24(1):157-9.
14. Gandrille S, Borgel D, Sala N, et al. Protein S deficiency: a database of mutations—summary of the first update. Thromb Haemost. 2000;84(5):918.
15. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci USA. 1993;90(3):1004-8.
16. Dahlback B, Hildebrand B. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. Proc Natl Acad Sci USA. 1994;91(4):1396-400.
17. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994;369(6475):64-7.
18. Voorberg J, Roelse J, Koopman R, et al. Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. Lancet. 1994;343(8912):1535-6.
19. Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt B. Activated protein C resistance caused by Arg506Gln mutation in factor Va. Lancet. 1994;343(8909):1361-2.
20. Zoller B, Dahlback B. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. Lancet. 1994;343(8912):1536-8.
21. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. Acommon genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. 1996;88(10):3698-703.
22. Reitsma PH, Rosendaal FR. Past and future of genetic research in thrombosis. J Thromb Haemost. 2007;5(Suppl 1):264-9.
23. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4(2):295-306.
24. van Hylckama Vlieg A, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. Blood. 2000;95(12):3678-82.
25. Bank I, Libourel EJ, Middeldorp S, et al. Elevated levels of FVIII:c within families are associated with an increased risk for venous and arterial thrombosis. J Thromb Haemost. 2005;3(1):79-84.
26. Meijers JCM, Tekelenburg W, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Eng J Med. 2000;342:696-701.
27. Rosendaal FR. Venous thrombosis: a multicausal disease. Lancet. 1999;353(9159):1167-73.
28. Zoller B, Berntsdotter A, Garcia de Frutos P, Dahlback B. Resistance to activated protein C as an additional genetic risk factor in hereditary deficiency of protein S. Blood. 1995;85(12):3518-23.
29. Lensen RP, Rosendaal FR, Koster T, et al. Apparent different thrombotic tendency in patients with factor V Leiden and protein C deficiency due to selection of patients. Blood. 1996;88(11):4205-8.
30. Galli M, Barbui T. Antiphospholipid syndrome: clinical and diagnostic utility of laboratory tests. Semin Thromb Hemost. 2005;31(1):17-24.
31. Ho WK, Hankey GJ, Quinlan DJ, Eikelboom JW. Risk of recurrent venous thromboembolism in patients with common thrombophilia: a systematic review. Arch Intern Med. 2006;166(7):729-36.
32. Boekholdt SM, Kramer MH. Arterial thrombosis and the role of thrombophilia. Semin Thromb Hemost. 2007;33(6):588-96.
33. Robertson L, Wu O, Langhorne P, et al. Thrombophilia in pregnancy: a systematic review. Br J Haematol. 2006;132(2):171-96.
34. van Sluis GL, Sohne M, El Kheir DY, Tanck MW, Gerdes VE, Buller HR. Family history and inherited thrombophilia. J Thromb Haemost. 2006;4(10):2182-7.
35. Bezemer ID, van der Meer FJ, Eikenboom JC, Rosendaal FR, Doggen CJ. The value of family history as a risk indicator for venous thrombosis. Arch Intern Med. 2008;169(6):610-5.
36. Linkins LA, Choi PT, Douketis JD. Clinical impact of bleeding in patients taking oral anticoagulant therapy for venous thromboembolism: a meta-analysis. Ann Intern Med. 2003;139:893-900.
37. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrom J. Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost. 2007;5(4):692-9.
38. van Hylckama Vlieg A, Baglin CA, Bare LA, Rosendaal FR, Baglin TP. Proof of principle of potential clinical utility of multiple SNP analysis for prediction of recurrent venous thrombosis. J Thromb Haemost. 2008;6(5):751-4.
39. Heit JA, Kobbervig CE, James AH, Petterson TM, Bailey KR, Melton LJ III. Trends in the incidence of venous thromboembolism during pregnancy or postpartum: a 30-year population-based study. Ann Intern Med. 2005;143(10):697-706.
40. Bank I, Libourel EJ, Middeldorp S, van der Meer J, Buller HR. High rate of skin complications due to low-molecular-weight heparins in pregnant women. J Thromb Haemost. 2003;1(4):859-61.
41. Deruelle P, Denervaud M, Hachulla E, et al. Use of low-molecular-weight heparin from the first trimester of pregnancy: a retrospective study of 111 consecutive pregnancies. Eur J Obstet Gynecol Reprod Biol. 2006;127(1):73-8.
42. Bates SM, Greer IA, Middeldorp S, Veenstra D, Prabulus AM, Vandvik PO. Venous Thromboembolism, Thrombophilia, Antithrombotic Therapy and Pregnancy: ACCP Evidence-Based Clinical Practice Guidelines (Ninth Edition). Chest. 2012;141(Suppl 2):e691S-e736S.
43. Roeters van Lennep JE, Meijer E, Klumper FJ, Middeldorp JM, Bloemenkamp KW, Middeldorp S. Prophylaxis with low-dose low-molecular-weight-heparin during pregnancy and postpartum: is it effective? J Thromb Haemost. 2011;9:473-80.
44. Baglin T, Luddington R, Brown K, Baglin C. Incidence of recurrent venous thromboembolism in relation to clinical and thrombophilic risk factors: prospective cohort study. Lancet. 2003;362:523-6.
45. Christiansen SC, Cannegieter SC, Koster T, Vandenbroucke JP, Rosendaal FR. Thrombophilia, clinical factors, and recurrent venous thrombotic events. JAMA. 2005;293(19):2352-61.
46. Lijfering W, Brouwer JL, Veeger NJ, et al. Selective testing for thrombophilia in patients with first venous thrombosis. Results from a retrospective family cohort study on absolute thrombotic risk for currently known thrombophilic defects in 2479 relatives. Blood. 2009;113(21):5314-22.
47. Segal JB, Brotman DJ, Necochea AJ, et al. Predictive Value of Factor V Leiden and Prothrombin G20210A in Adults With Venous Thromboembolism and in Family Members of Those With a Mutation: A Systematic Review. JAMA. 2009;301(23):2472-85.
48. Lijfering WM, Middeldorp S, Veeger NJ, et al. Risk of recurrent venous thrombosis in homozygous carriers, and double heterozygous carriers of factor V Leiden and prothrombin G20210A. Circulation. 2010;121(15):1706-12.

49. Middeldorp S. Duration of anticoagulation for venous thromboembolism. BMJ. 2011;342:d2758.
50. de Jong PG, Coppens M, Middeldorp S. Duration of anticoagulant therapy for venous thromboembolism: balancing benefits and harms on the long term. Br J Haematol. 2012; 158(4):433-41.
51. EGAPP Working Group. Recommendations from the EGAPP Working Group: routine testing for Factor V Leiden (R506Q) and prothrombin (20210G>A) mutations in adults with a history of idiopathic venous thromboembolism and their adult family members. Genet Med. 2011;13(1):67-76.
52. Cohn DM, Vansenne F, de Borgie CA, Middeldorp S. Thrombophilia testing for prevention of recurrent venous thromboembolism. Cochrane Database of Systematic Reviews. 2009;(1):CD007069.
53. Coppens M, Reijnders JH, Middeldorp S, Doggen CJ, Rosendaal FR. Testing for inherited thrombophilia does not reduce recurrence of venous thrombosis. J Thromb Haemost. 2008;6:1474-7.
54. Ruiz-Irastorza G, Hunt BJ, Khamashta MA. A systematic review of secondary thromboprophylaxis in patients with antiphospholipid antibodies. Arthritis Rheum. 2007;57(8): 1487-95.
55. Crowther MA, Ginsberg JS, Julian J, et al. A comparison of two intensities of warfarin for the prevention of recurrent thrombosis in patients with the antiphospholipid antibody syndrome. N Engl J Med. 2003;349:1133-8.
56. Finazzi G, Marchioli R, Brancaccio V, et al. A randomized clinical trial of high-intensity warfarin vs. conventional antithrombotic therapy for the prevention of recurrent thrombosis in patients with the antiphospholipid syndrome (WAPS). J Thromb Haemost. 2005;3(5):848-53.
57. Opatrny L, David M, Kahn SR, Shrier I, Rey E. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmunie disease: a metaanalysis. J Rheumatol. 2006;33(11):2214-21.
58. Empson M, Lassere M, Craig JC, Scott JR. Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant. The Cochrane Database of Systematic Reviews. 2005;(2):CD002859.
59. Middeldorp S. Thrombophilia and pregnancy complications: cause or association? J Thromb Haemost. 2007;5(Suppl 1):276-82.
60. Rodger MA, Paidas MJ, Mclintock C, et al. Inherited thrombophilia and pregnancy complications revisited: association not proven causal and antithrombotic prophylaxis is experimental. Obstet Gynecol. 2008;112(2):320-4.
61. Kaandorp SP, Di Nisio M, Goddijn M, Middeldorp S. Aspirin or anticoagulants for treating recurrent miscarriage in women without antiphospholipid syndrome. The Cochrane Library. 2009;(1):CD004734.
62. Kaandorp SP, Goddijn M, van der Post JA, et al. Aspirin plus Heparin or Aspirin Alone in Women with Recurrent Miscarriage. N Engl J Med. 2010;362(17):1586-96.
63. Clark P, Walker ID, Langhorne P, et al. SPIN: the Scottish Pregnancy Intervention Study: a multicentre randomised controlled trial of low molecular weight heparin and low dose aspirin in women with recurrent miscarriage. Blood. 2010;115(21):4162-7.
64. Visser J, Ulander VM, Helmerhorst FM, et al. Thromboprophylaxis for recurrent miscarriage in women with or without thrombophilia. HABENOX: a randomised multicentre trial. Thromb Haemost. 2011;105(2):295-301.
65. Gris JC, Mercier E, Quere I, et al. Low-molecular-weight heparin versus low-dose aspirin in women with one fetal loss and a constitutional thrombophilic disorder. Blood. 2004;103:3695-9.
66. Lissalde-Lavigne G, Fabbro-Peray P, Cochery-Nouvellon E, et al. Factor V Leiden and prothrombin G20210A polymorphisms as risk factors for miscarriage during a first intended pregnancy: the matched case-control 'NOHA first' study. J Thromb Haemost. 2005;3(10):2178-84.
67. Rodger M. Important publication missing key information. Blood. 2004;104(10):3413.
68. Askie LM, Duley L, Henderson-Smart DJ, Stewart LA. Antiplatelet agents for prevention of pre-eclampsia: a meta-analysis of individual patient data. Lancet. 2007;369(9575): 1791-8.
69. Dodd JM, McLeod A, Windrim RC, Kingdom J. Antithrombotic therapy for improving maternal or infant health outcomes in women considered at risk of placental dysfunction. Cochrane Database Syst Rev. 2010;(6):CD006780.
70. de Vries JI, van Pampus MG, Hague WM, Bezemer PD, Joosten JH. Low-Molecular-Weight Heparin Added to Aspirin in the Prevention of Recurrent Early-Onset Preeclampsia in women with Inheritable Thrombophilia: the FRUIT-RCT. J Thromb Haemost. 2012;10(1):64-72.
71. Marchetti M, Pistorio A, Barosi G. Extended anticoagulation for prevention of recurrent venous thromboembolism in carriers of factor V Leiden: cost-effectiveness analysis. Thromb Haemost. 2000;84:752-7.
72. Wu O, Robertson L, Twaddle S, et al. Screening for thrombophilia in high-risk situations: systematic review and cost-effectiveness analysis. The Thrombosis: Risk and Economic Assessment of Thrombophilia Screening (TREATS) study. Health Technol Assess. 2006;10(11):1-110.
73. Bank I, Scavenius MPRB, Buller HR, Middeldorp S. Social aspects of genetic testing for factor V Leiden mutation in healthy individuals and their importance for daily practice. Thromb Res. 2004;113:7-12.
74. Cohn DM, Vansenne F, Kaptein AA, de Borgie CA, Middeldorp S. The psychological impact of testing for thrombophilia: a systematic review. J Thromb Haemost. 2008;6: 1099-104.
75. van Hylckama Vlieg A, Baglin CA, Bare LA, Rosendaal FR, Baglin TP. Proof of principle of potential clinical utility of multiple SNP analysis for prediction of recurrent venous thrombosis. J Thromb Haemost 2008;6(5):751-4.
76. de Haan HG, Bezemer ID, Doggen CJ, et al. Multiple SNP testing improves risk prediction of first venous thrombosis. Blood 2012;120(3):656-63.

Optimal duration of venous thrombosis treatment

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A B S T R A C T

Deciding on the optimal duration of anticoagulation is based on the risk of recurrent venous thromboembolism (VTE) and of bleeding during anticoagulation. The duration of anticoagulation should be at least three months as shorter courses double the recurrence rates. At three months, anticoagulation can be stopped in patients with a VTE provoked by a transient risk factor, as the recurrence risk is expected to be lower than the bleeding risk during anticoagulation. Patients with unprovoked VTE are at higher risk of recurrence and prolonged anticoagulation is currently recommended. However, attempts are made to stratify these patients according to their recurrence risk and to identify those with a low recurrence risk who would not benefit from extended anticoagulation. Novel approaches to optimize the management of patients with unprovoked VTE are the use of prediction models which link clinical patients' characteristics with laboratory testing to discriminate between patients with a low risk (who may discontinue anticoagulation) and those with high risk (in whom long-term anticoagulation is justified). Moreover, new antithrombotic concepts including new oral anticoagulants or aspirin, both of which potentially confer a lower bleeding risk and are more convenient for the patients, have been explored for extended thromboprophylaxis.

Learning goals

At the conclusion of this activity, participants should be able to:
- assess the recurrence risk of patients with venous thrombosis;
- reflect on options to prevent recurrence;
- consider benefits and risks of antithrombotic drugs to prevent recurrence;
- decide on the optimal duration of anticoagulation after venous thrombosis.

Introduction

Venous thromboembolism (VTE), a syndrome comprising deep vein thrombosis and pulmonary embolism, is a common and potentially fatal disease. The incidence ranges between 1-2 per 1000 person-years and the short-term mortality rate is approximately 25%.[1-3] The major goals of treatment of VTE are to immediately restore perfusion of the vessel, to inhibit progression and embolization of the thrombus, and to prevent recurrence. The recurrence risk is highest during the first few weeks after the acute event, but never subsides. It may remain as high as 10% per year with a case-fatality rate of 3.6-12%.[4-6]

Recurrence can be prevented in more than 95% of patients through antithrombotic treatment.[7] The minimum duration of anticoagulation for all patients with VTE is three months. Reducing the duration to 4-6 weeks doubles the risk of recurrence during the next year.[8,9]

Considering a potentially high recurrence rate, prolonged and even indefinite anticoagulation may be required in some patients. Several interventional trials have investigated the value of extending anticoagulation from 3 to 6, 12, or 24 months, or even indefinitely.[10-16] While recurrence is effectively prevented during treatment, the recurrence risk increases again once anticoagulation is stopped and is not lower than what would have been expected after a shorter anticoagulation period.[17] Thus, after a VTE, if a high risk of recurrence is suspected, patients should either be treated for three months or for an indefinite period of time.

The bleeding risk is increased during anticoagulant treatment, and has to be taken into account when deciding on its optimal duration. In addition, the patient's preference and adherence issues should be considered before making a decision on long-term treatment.[18]

Treatment of acute and subacute venous thromboembolism (first 3 months)

Once the diagnosis of VTE has been objectively confirmed, immediate anticoagulation is required. Up to now, this has only been achieved by parenteral administration of heparin or fondaparinux. Since the risk of thrombus progression, embolization and recurrence does not subside within a few days, anticoagulation beyond the acute phase is necessary. Usually, for that purpose, a vitamin K antagonist is started simultaneously with

heparin or fondaparinux. Parenteral anticoagulation can be discontinued after a minimum of five days provided that the international normalized ratio (INR) is 2.0 or higher for at least 24 h. In patients with cancer and VTE, the risk of recurrence, and also the bleeding risk, are particularly high.[19] There is evidence from randomized interventional trials that extended anticoagulation with low molecular weight heparin at therapeutic dose followed by a 25% dose reduction after four weeks is more effective than, and as safe as, treatment with vitamin K antagonists at conventional intensity.[20,21] The optimal duration of secondary thromboprophylaxis is less well defined. Since cancer patients have a high risk of recurrence, low molecular weight heparin should be given for at least six months.[22]

For the treatment of acute VTE in pregnant women, fixed-dose, weight-adjusted subcutaneous low molecular weight heparin is the anticoagulant of choice and should be given at a therapeutic dose throughout pregnancy.[23] Studies on the optimal duration and intensity of anticoagulation are lacking. Low molecular weight heparin should be discontinued 24 h before induction of labor or caesarean section, re-started at a reduced dose when it is safe to do so, and continued for an additional 6-8 weeks.

Recently, new oral anticoagulants have been tested in patients with acute VTE. Rivaroxaban, a direct factor Xa inhibitor, is already licensed for the treatment of acute deep vein thrombosis and pulmonary embolism. Treatment consists of an oral dose of 15 mg twice daily for three weeks followed by 20 mg once daily. No initial parenteral anticoagulant drug is required. Rivaroxaban has similar efficacy and a lower rate of major bleeding compared to conventional treatment with a parenteral anticoagulant followed by vitamin K antagonist.[24,25] The results of a phase III study with dabigatran, an oral direct thrombin inhibitor, for treatment of acute VTE shows similar efficacy and safety compared to standard treatment,[26] and licensing of the drug for this indication is awaited. The results of interventional trials with apixaban or edoxaban, other oral direct factor Xa inhibitors, on safety and efficacy for treatment of acute VTE are also awaited.

All new oral anticoagulants are contraindicated during pregnancy and breastfeeding, and have not been adequately studied for the treatment of VTE in cancer patients.

Who should stop anticoagulation after three months?

Patients in whom VTE occurs in association with a temporary risk condition have a low risk of recurrence. This is particularly true for patients with venous thrombosis after surgery or while using hormone contraceptives.[8,17,27-30] The risk of recurrence is less well studied in patients who had their initial venous thrombosis provoked by trauma, pregnancy, immobilization or long-distance travel, but is also regarded as low. As the recurrence risk in these patients is expected to be lower than the bleeding risk in case of extended anticoagulation, stopping anticoagulation after three months is recommended for all patients with VTE in association with a temporary risk factor.[7]

Also in patients with a high risk of bleeding and/or poor adherence to treatment, anticoagulation may be stopped after three months regardless of the presence or absence of a transient triggering risk condition.

Who should continue anticoagulation beyond three months?

Patients with unprovoked VTE, *i.e.* an event that cannot be explained by a temporary triggering event, may have an annual recurrence risk of as high as 10%.[4] These patients would be candidates for extending anticoagulation beyond three months. However, despite an overall high recurrence risk, many patients with a first unprovoked VTE will stay recurrence free but are exposed to an unnecessary risk of bleeding in case of extended anticoagulation. For vitamin K antagonists, the annual risk of bleeding ranges between 1% and 3%,[7,31,32] and the case-fatality rate is approximately 13%.[6] Moreover, some patients dislike anticoagulation because of the prospect of long-term medical treatment or inconvenience in their professional life.

There are basically two approaches that may help to optimize the long-term management of patients with unprovoked VTE: 1) discriminating between patients with a low risk of recurrence and related fatalities in whom anticoagulation can be stopped from patients with a high risk who may indeed benefit from indefinite anticoagulation; or 2) use of alternative antithrombotic concepts which have a low risk of bleeding and/or are more convenient to manage.

Strategies to identify patients with unprovoked venous thromboembolism with a low or high recurrence risk

Thrombophilia screening

Screening for laboratory markers of thrombophilia has been widely used with the primary aim of identifying patients at high risk of recurrence. This so-called thrombophilia screening has been abandoned as the relevance of these parameters with regard to the risk of recurrence is either only moderate, unknown or is seen as controversial. Importantly, most patients carry more than one risk factor and their combined effect on the recurrence risk is largely unknown.[4] In addition, we recently reported that a large proportion of patients with two unprovoked events have a normal thrombophilia screening result indicating that the absence of laboratory defects in patients with a history of VTE does not necessarily mean that their recurrence risk is low.[4]

Clinical features

Some years ago, the risk of recurrent VTE was estimated on the basis of only a few patients' characteristics, including absence or presence of a triggering factor, concomitant pulmonary embolism, previous venous thrombosis or a positive family history. More recently, high quality clinical studies led to a better understanding of the importance of clinical conditions and patients' characteristics for predicting the risk of recurrence. Accepted clinical features associated with a high risk of recurrence are now male sex, proximal deep vein thrombosis or pulmonary embolism (as compared to distal DVT), multiple thrombotic events, presence of the postthrombotic syndrome and being overweight.[28,33-41] Some studies[28,41-43] but not others[44-47] report an association between advancing age and an increased risk of recurrent venous thrombosis.

The usefulness of residual vein thrombosis as a predic-

tor of recurrence is under debate. In a systematic review and meta-analysis, residual vein thrombosis was associated with a modestly increased risk of recurrent VTE in patients with DVT. However, residual vein thrombosis was not a predictor of recurrent VTE in patients with unprovoked DVT.[48] Moreover, association with recurrence risk is strongly dependent on how residual vein thrombosis is defined.[49] The definition of vein recanalization lacks standardization, and assessment requires a high degree of expertise. Thus, it is premature to make clinical decisions on the basis of residual vein thrombosis measurements.

Global markers of fibrin and thrombin generation

VTE is a multicausal disease that may be driven by multifactorial thrombophilia. Several coagulation markers that provide a more global measurement combining effects of clotting or fibrinolytic disorders have been tested. Among these, D-Dimer is the most promising and allows discrimination into groups of high and low risk of recurrence. In an Italian study, patients with a low D-Dimer after stopping anticoagulation had a low risk of recurrence (4.4 recurrences per 100 patient years). Patients with high D-Dimer in whom anticoagulation was discontinued after six months had a 5-fold higher risk of recurrence than those who received anticoagulation for a longer period of time (10.9 *vs.* 2.0 recurrences per 100 patient years).[50] We found that patients with a first unprovoked VTE and D-Dimer levels less than 250 ng/mL had a 60% lower recurrence rate than those with higher levels.[51,52] In a Canadian study, the 250 ng/mL cut off during warfarin treatment proved to be particularly useful to identify women at low recurrence risk.[28]

In a meta-analysis of patients with a first unprovoked VTE, the timing of post-anticoagulation D-Dimer testing (<3 weeks, 3-5 weeks, >5 weeks), patient age (<65 or >65 years), and the assay cut-off point used (500 ng/mL and 250 ng/mL) did not affect the ability of D-Dimer to distinguish between patients with a higher or lower risk for recurrent VTE.[53] The high negative predictive value of recurrence of D-Dimer is independent of the presence or absence of hereditary thrombophilia.[54]

Until we have the results of ongoing large clinical studies further investigating the usefulness of D-Dimer to predict recurrence, measuring D-Dimer to guide the duration of anticoagulation in patients with unprovoked VTE cannot be recommended for routine patient care.

Prediction models

A novel approach for assessing the risk of recurrent VTE consists of linking clinical patients' characteristics with laboratory testing. Thus far, three such scoring models have been developed. In a multicenter prospective cohort study from Canada, 69 potential clinical and laboratory predictors of recurrent VTE were assessed in 646 participants with a first, unprovoked VTE while they were taking oral anticoagulation therapy.[28] Women with less than 2 specific characteristics (hyperpigmentation, edema or redness of either leg, D-Dimer ≥250 μg/L or over while taking warfarin, body mass index ≥30 kg/m^2, age ≥65 years) had an annual risk of recurrent VTE of 1.6% (95%CI: 0.3-4.6%), whereas women with 2 or more of these findings had a much higher recurrence risk (14.1%; 95%CI: 10.9-17.3%). None of the combination of predictors was useful for identifying a low-risk group of men.

Within the frame of the Austrian Study on Recurrent Venous Thromboembolism (AUREC), we developed the "Vienna Prediction Model" to calculate the recurrence risk in patients with unprovoked deep vein thrombosis and/or pulmonary embolism. We prospectively followed 929 patients after discontinuation of anticoagulation.[55] Among several pre-selected variables, patient's sex, thrombus location and D-Dimer but not body mass index, factor V Leiden, or the prothrombin mutation, were relevant predictors of the recurrence risk. Based on these variables, we developed a nomogram that can be used to calculate risk scores and to estimate the cumulative probability of recurrence in an individual patient. A web-based risk calculator is available for ease of calculation (www.meduniwien.ac.at/user/georg.heinze/zipfile/).

Tosetto and colleagues analyzed data of seven prospective studies enrolling patients with a first objectively diagnosed VTE in an individual patient data meta-analysis.[56] The patient population consisted of 1818 cases with unprovoked VTE. Abnormal D-Dimer after stopping anticoagulation, age under 50 years, male sex and VTE not associated with hormonal therapy (in women) were the main predictors of recurrence. The score is: +2 points for positive (abnormal) post-anticoagulation D-Dimer, +1 point for age 50 years or under, +1 point for male sex, -2 points for hormone use in women at time of initial VTE. The annualized recurrence risk was 3.1% (95%CI: 2.3-3.9) in patients with a score of 1 or under, 6.4% (95%CI: 4.8-7.9) in patients with a score of 2, and 12.3% (95%CI: 9.9-14.7) in patients with a so-called DASH score of 3 or over. We believe that prediction models are suitable for identifying patients with unprovoked VTE and a recurrence risk that is low enough to justify discontinuation of anticoagulation after three months. We should wait for the results of well-designed ongoing validation studies before applying these models in routine care.

Alternative antithrombotic concepts

The safety and efficacy of new oral anticoagulants for extended anticoagulation after VTE have been tested in phase III studies. Dabigatran has been studied in this indication in two separate trials in comparison to either placebo or warfarin, and publication of the complete data is awaited. For the two factor Xa inhibitors, rivaroxaban and apixaban, placebo-controlled data have been reported.[24,57] Table 1 provides an overview of the two trials which have already been published. Compared to placebo, all new oral anticoagulants are highly effective in preventing recurrent VTE. Rivaroxaban is already licensed for extended thromboprophylaxis after deep vein thrombosis or pulmonary embolism. Although bleeding rates were generally low in all these trials, none of the studies was powered to adequately assess the actual bleeding risk over time. Observation time in all the trials was limited to approximately 12 months and no data on long-term risk are available.

Thus far, the use of aspirin has had a negligible role in the prevention of VTE. Recently, results of two randomized placebo controlled trials on the use of aspirin for extended thromboprophylaxis after VTE have been published. In the "Warfarin and Aspirin" (WARFASA) study, an interventional, multicenter, double-blind study which

was run predominantly in Italy, patients with a first unprovoked VTE who had completed 6-18 months of oral anticoagulant treatment were randomly assigned to aspirin 100 mg daily or placebo.[58] During a median treatment period of 23.9 months, 28 of 205 patients taking aspirin and 43 of 197 taking placebo had a recurrence (6.6% *vs.* 11.2%/year; HR 0.58; 95%CI: 0.36-0.93). One patient in each treatment group had a major bleeding episode.

In the Australian "Aspirin to Prevent Recurrent Venous Thromboembolism" (ASPIRE) trial studied 822 patients who had completed initial anticoagulant therapy after a first unprovoked VTE receiving either aspirin 100 mg daily or placebo.[59] Median follow up was 37.2 months. Venous thromboembolism recurred in 73 of 411 patients assigned to placebo and in 57 of 411 assigned to aspirin (6.5% *vs.* 4.8%/year; HR 0.74; 95%CI: 0.52-1.05; *P*=0.09). Aspirin reduced the rate of the pre-specified secondary composite outcome (rate of VTE, myocardial infarction, stroke, or cardiovascular death) by 34% (8.0%/year with placebo *vs.* 5.2%/year with aspirin; HR 0.66; 95%CI: 0.48-0.92; *P*=0.01). There was no significant between-group difference in the rates of major or clinically relevant non-major bleedings (0.6%/year with placebo *vs.* 1.1%/year with aspirin; *P*=0.22). Table 2 shows the combined results of the two studies with regard to number of VTE events, major vascular events, and clinically relevant bleeding. The results show a significant reduction of 32% in the rate of recurrence of VTE and a reduction of 34% in the rate of major vascular events without an excess of bleeding.

Table 1. Recurrence and bleeding rates of new oral anticoagulants during thromboprophylaxis beyond three months after venous thromboembolism.

	EINSTEINext[24]	AMPLIFYext[57]
N. of patients	1197	2486
Compound	Rivaroxaban	Apixaban
Dose	1 x 20 mg	2 x 2.5 mg 2 x 5 mg
Control	Placebo	Placebo
Duration of treatment	12 months	12 months
Recurrent VTE		
Study drug, n/n	8/602	32/840 (2.5 mg) 34/813 (5 mg)
Placebo, n/n	42/594	96/829
Hazard ratio (95%CI)	0.18 (0.09-0.4)	0.33 (0.22-0.48) (2.5 mg) 0.36 (0.25-0.53) (5 mg)
Bleedings (major and CRNM)		
Study drug, n/n	36/602	27/840 (2.5 mg) 35/813 (5 mg)
Placebo, n/n	7/594	22/829
Hazard ratio (95%CI)	5.19 (2.3-11.7)	1.20 (0.69-2.10) (2.5 mg) 1.62 (0.96-2.73) (5 mg)

n/n: events/total patient number; CRNM: clinically relevant non-major; HR: hazard ratio; CI: confidence interval.

Table 2. Combined results of the WARFASA and ASPIRE trials.

	Placebo	Aspirin	Hazard ratio (95%CI)	*P*
Venous thromboembolism				
WARFASA	43/197	28/205	0.58 (0.36-0.93)	0.02
ASPIRE	73/411	57/411	0.74 (0.52-1.05)	0.09
Pooled data	116/608	85/616	0.68 (0.51-0.90)	0.007
Major vascular events*				
WARFASA	48/197	36/205	0.67 (0.43-1.03)	0.06
ASPIRE	88/411	62/411	0.66 (0.48-0.92)	0.01
Pooled data	136/608	98/616	0.66 (0.51-0.86)	0.002
Major or clinically relevant non-major bleeding				
WARFASA	4/197	4/205	0.98 (0.24-3.96)	0.97
ASPIRE	8/411	14/411	1.72 (0.72-4.11)	0.22
Pooled data	12/608	18/616	1.47 (0.70-3.08)	0.31

**Composite of venous thromboembolism, myocardial infarction, stroke, or cardiovascular death.*

Questions to be answered

Duration of anticoagulation in special patient populations

Patients with multiple episodes of VTE have a higher risk of recurrence.[18,19] Recurrence is effectively prevented in these patients by indefinite anticoagulation but the bleeding risk is substantially increased. It is not known whether patients with multiple provoked venous thrombosis also have a high risk of recurrence. The optimal treatment, particularly with regard to duration of anticoagulation in patients with subsegmental pulmonary embolism, incidentally detected pulmonary embolism, or in patients with small isolated calf vein thrombosis or muscle vein thrombosis has not been fully studied and remains, therefore, a subject of debate.

Choice of anticoagulant for extended anticoagulation

Anticoagulation with a vitamin K antagonist is standard for patients requiring extended thromboprophylaxis after VTE. However, data on efficacy and safety of new oral anticoagulants or aspirin are promising. With regard to new oral anticoagulants, there are still some caveats and issues that need to be addressed before clear recommendations regarding their use for extended thromboprophylaxis after VTE can be given. In all the trials, the decision as to the duration of anticoagulation was left to the discretion of the treating physician. Hence, the group of patients included in the extended treatment studies was 'pre-selected' and, therefore, extremely heterogeneous. To improve the decision as to who will truly benefit from extending anticoagulation, the patient profile needs to be better described in subgroup analyses. In addition, more data on the bleeding risk and other potential side effects (e.g. risk of myocardial infarction, gastrointestinal bleeding) of the new anticoagulants are needed to make decisions on a more individualized treatment. Similar considerations apply when deciding on the use of aspirin for long-term antithrombotic therapy in patients with VTE. Overall, aspirin reduces the risk of recurrence by approximately 50%. In the two studies discussed, the annual recurrence rates in the aspirin group were 6.6% and 4.8%, respectively. In ASPIRE, major vascular events, including myocardial infarction, stroke and cardiovascular death were significantly lower in the patients treated with aspirin.[59] Aspirin could, therefore, be an attractive option particularly in patients with VTE and risk of cardiovascular events.

References

1. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrøm J. Incidence and mortality of venous thromboembolism: a population-based study. J Thromb Haemost. 2007;5:692-99.
2. Kyrle PA, Eichinger S. Deep vein thrombosis. Lancet. 2005;365:1163-74.
3. Murin S, Romano PS, White RH. Comparison of outcomes after hospitalization for deep venous thrombosis or pulmonary embolism. Thromb Haemost. 2002;88:407-14.
4. Kyrle PA, Rosendaal FR, Eichinger S. Risk assessment for recurrent venous thrombosis. Lancet. 2010;376:2032-9.
5. Douketis JD, Gu CS, Schulman S, Ghirarduzzi A, Pengo V, Prandoni P. The risk for fatal pulmonary embolism after discontinuing anticoagulant therapy for venous thromboembolism. Ann Intern Med. 2007;147:766-74.
6. Carrier M, Le Gal G, Wells PS, Rodger MA. Systematic review: case-fatality rates of recurrent venous thromboembolism and major bleeding events among patients treated for venous thromboembolism. Ann Intern Med. 2010;152:578-89.
7. Kearon C, Akl EA, Comerota AJ, Prandoni P, Bounameaux H, Goldhaber SZ, et al. American College of Chest Physicians. Antithrombotic therapy for venous thromboembolic disease. Chest. 2012;141:e419S-e494S.
8. Kearon C, Ginsberg JS, Anderson DR, Kovacs MJ, Wells P, Julian JA, et al; SOFAST Investigators. Comparison of 1 month with 3 months of anticoagulation for a first episode of venous thromboembolism associated with a transient risk factor. J Thromb Haemost. 2004;2:743-9.
9. Schulman S, Rhedin AS, Lindmarker P, Carlsson A, Lärfars G, Nicol P, et al. A comparison of six weeks with six months of oral anticoagulant therapy after a first episode of venous thromboembolism. Duration of Anticoagulation Trial Study Group. N Engl J Med. 1995;332:1661-5.
10. Agnelli G, Prandoni P, Becattini C, Silingardi M, Taliani MR, Miccio M, et al; Warfarin Optimal Duration Italian Trial Investigators. Extended oral anticoagulant therapy after a first episode of pulmonary embolism. Ann Intern Med. 2003;139:19-25.
11. Agnelli G, Prandoni P, Santamaria MG, Bagatella P, Iorio A, Bazzan M, et al; Warfarin Optimal Duration Italian Trial Investigators. Three months versus one year of oral anticoagulant therapy for idiopathic deep vein thrombosis. N Engl J Med. 2001;345:165-9.
12. Campbell IA, Bentley DP, Prescott RJ, Routledge PA, Shetty HG, Williamson IJ. Anticoagulation for three versus six months in patients with deep vein thrombosis or pulmonary embolism, or both: randomised trial. BMJ. 2007;334:674-80.
13. Kearon C, Gent M, Hirsh J, Weitz J, Kovacs MJ, Anderson DR, et al. A comparison of three months of anticoagulation with extended anticoagulation for a first episode of idiopathic venous thromboembolism. N Engl J Med. 1999;340:901-7.
14. Pinede L, Ninet J, Duhaut P, et al. for the Investigators of the Duree Optimale du Traitement Antivitamines K (DOTAVK) study. Comparison of 3 and 6 months of oral anticoagulant therapy after a first episode of proximal deep vein thrombosis or pulmonary embolism and comparison of 6 and 12 weeks of therapy after isolated calf deep vein thrombosis. Circulation. 2001;103:2453-60.
15. Ridker PM, Goldhaber SZ, Danielson E, Rosenberg Y, Eby CS, Deitcher SR, et al; PREVENT Investigators. Long-term, low-intensity warfarin therapy for prevention of recurrent venous thromboembolism. N Engl J Med. 2003;348:1425-34.
16. Schulman S, Granqvist S, Holmström M, Carlsson A, Lindmarker P, Nicol P, et al. The duration of oral anticoagulant therapy after a second episode of venous thromboembolism. The Duration of Anticoagulation Trial Study Group. N Engl J Med. 1997;336:393-8.
17. Boutitie F, Pinede L, Schulman S, Agnelli G, Raskob G, Julian J, et al. Influence of preceding length of anticoagulant treatment and initial presentation of venous thromboembolism on risk of recurrence after stopping treatment: analysis of individual participants' data from seven trials. Br Med J. 2011;24: 342:d3036.
18. MacLean S, Mulla S, Akl EA, et al. Patient values and preferences for decision making in antithrombotic therapy: antithrombotic therapy and prevention of thrombosis, 9th ed. American College of Chest Physicians evidence based clinical practice guidelines. Chest. 2012;141(Suppl):e1S-e23S.
19. Prandoni P, Lensing AW, Piccioli A, Bernardi E, Simioni P, Girolami B, et al. Recurrent venous thromboembolism and bleeding complications during anticoagulant treatment in patients with cancer and venous thrombosis. Blood. 2002;100:3484-8.
20. Lee AY, Levine MN, Baker RI, Bowden C, Kakkar AK, Prins M, et al; Randomized Comparison of Low-Molecular-Weight Heparin versus Oral Anticoagulant Therapy for the Prevention of Recurrent Venous Thromboembolism in Patients with Cancer (CLOT) Investigators. Low-molecular-weight heparin versus a coumarin for the prevention of recurrent venous thromboembolism in patients with cancer. N Engl J Med. 2003;349:146-53.
21. Meyer G, Marjanovic Z, Valcke J, Lorcerie B, Gruel Y, Solal-Celigny P, et al. Comparison of low-molecular-weight heparin and warfarin for the secondary prevention of venous thromboembolism in patients with cancer: a randomized controlled study. Arch Intern Med. 2002;162:1729-35.
22. Farge D, Debourdeau P, Beckers M, et al. International clinical practice guidelines for the treatment and prophylaxis of venous thromboembolism in patients with cancer. J Thromb Haemost. Dec 8 2012. [Epub ahead of print].
23. Arya R. How I manage venous thromboembolism in pregnancy. Br J Haematol. 2011;153:698-708.
24. EINSTEIN Investigators. Oral rivaroxaban for symptomatic venous thromboembolism. N Engl J Med. 2010;363:2499-510.
25. EINSTEIN–PE Investigators. Oral rivaroxaban for the treatment of symptomatic pulmonary embolism. N Engl J Med. 2012;366:1287-97.
26. Schulman S, Kearon C, Kakkar AK, Mismetti P, Schellong S, Eriksson H, et al; RE-COVER Study Group. Dabigatran versus warfarin in the treatment of acute venous thromboembolism. N Engl J Med. 2009;361:2342-52.
27. Baglin T, Luddington R, Brown K, Baglin C. Incidence of recurrent venous thromboembolism in relation to clinical and thrombophilic risk factors: prospective cohort study. Lancet. 2003;362:523-6.
28. Rodger MA, Kahn SR, Wells PS, Anderson DA, Chagnon I, Le Gal G, et al. Identifying unprovoked thromboembolism patients at low risk for recurrence who can discontinue anticoagulant therapy. CMAJ. 2008;179:417-26.
29. Christiansen SC, Cannegieter SC, Koster T, Vandenbroucke JP, Rosendaal FR. Thrombophilia, clinical factors, and recurrent venous thrombotic events. JAMA. 2005;293:2352-61.
30. Le Gal G, Kovacs MJ, Carrier M, Do K, Kahn SR, Wells PS, et al. Risk of recurrent venous thromboembolism after a first oestrogen-associated episode. Data from the REVERSE cohort study. Thromb Haemost. 2010;104:498-503.
31. Linkins LA, Choi PT, Douketis JD. Clinical Impact of bleeding in patients taking oral anticoagulant therapy for venous thromboembolism. Ann Intern Med. 2003;139:893-900.
32. Fanikos J, Grasso-Correnti N, Shah R, Kucher N, Goldhaber SZ. Major bleeding complications in a specialized anticoagulation service. Am J Cardiol. 2005;96:595-8.
33. Kyrle PA, Minar E, Bialonczyk C, Hirschl M, Weltermann A, Eichinger S. The risk of recurrent venous thromboembolism in men and women. N Engl J Med. 2004;350:2558-63.
34. McRae S, Tran H, Schulman S, Ginsberg J, Kearon C. Effect of patient´s sex on risk of recurrent venous thromboembolism: a meta-analysis. Lancet. 2006;368:371-8.
35. Douketis J, Tosetto A, Marcucci M, Baglin T, Cosmi B, Cushman M, et al. Risk of recurrence after venous thromboembolism in men and women: patient level meta-analysis. BMJ. 2011;342.
36. Baglin T, Douketis J, Tosetto A, Marcucci M, Cushman M, Kyrle P, et al. Does the clinical presentation and extent of venous thrombosis predict likelihood and type of recurrence? A patient-level meta-analysis. J Thromb Haemost. 2010;8: 2436-42.
37. Eichinger S, Weltermann A, Minar E, et al. Symptomatic pulmonary embolism and the risk of recurrent venous thromboembolism. Arch Intern Med. 2004;164:92-6.
38. Schulman S, Granqvist S, Holmström M, et al. The duration of oral anticoagulant therapy after a second episode of venous thromboembolism. The Duration of Anticoagulation Trial Study Group. N Engl J Med. 1997;336:393-8.
39. Stain M, Schönuer V, Minar E, et al. The post-thrombotic syndrome: risk factors and impact on the course of thrombotic disease. J Thromb Haemost. 2005;3:2671-6.
40. Eichinger S, Hron G, Bialonczyk C, et al. Overweight, obesity, and the risk of recurrent venous thromboembolism. Arch Intern Med. 2008;168:1678-83.
41. Heit JA, Silverstein MD, Mohr DN, et al. The epidemiology of venous thromboembolism in the community. Thromb Haemost. 2001;86:452-63.

42. Prandoni P, Noventa F, Ghirarduzzi A, et al. The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism. A prospective cohort study in 1,626 patients. Haematologica. 2007;92:199-205.
43. Schulman S, Lindmarker P, Holmström M, et al. Post-thrombotic syndrome, recurrence, and death 10 years after the first episode of venous thromboembolism treated with warfarin for 6 weeks or 6 months. J Thromb Haemost. 2006;4:734-42.
44. Beyth RJ, Cohen AM, Landefeld CS. Long-term outcomes of deep-vein thrombosis. Arch Intern Med. 1995;155:1031-7.
45. White RH, Zhou H, Romano PS. Length of hospital stay for treatment of deep venous thrombosis and the incidence of recurrent thromboembolism. Arch Intern Med. 1998;158: 1005-10.
46. Hansson PO, Sörbo J, Eriksson H. Recurrent venous thromboembolism after deep vein thrombosis: incidence and risk factors. Arch Intern Med. 2000;160:769-74.
47. Eischer L, Eichinger S, Kyrle PA. Age at first venous thromboembolism and risk of recurrence: a prospective cohort study. Medicine. 2009;88:366-70.
48. Carrier M, Rodger MA, Wells PS, Righini M, Le Gal G. Residual vein obstruction to predict the risk of recurrent venous thromboembolism in patients with deep vein thrombosis: a systematic review and meta-analysis. J Thromb Haemost. 2011;9:1119-25.
49. Tan M, Mos IC, Klok FA, Huisman MV. Residual venous thrombosis as predictive factor for recurrent venous thrombembolim in patients with proximal deep vein thrombosis: a sytematic review. Br J Haematol. 201;153:168-78
50. Palareti G, Cosmi B, Legnani C, et al; PROLONG Investigators. D-dimer testing to determine the duration of anticoagulation therapy. N Engl J Med. 2006;355:1780-9.
51. Eichinger S, Minar E, Bialonczyk C, et al. D-dimer levels and risk of recurrent venous thromboembolism. JAMA. 2003;290:1071-4.
52. Eichinger S, Hron G, Kollars M, Kyrle PA. Prediction of recurrent venous thromboembolism by endogenous thrombin potential and D-dimer. Clin Chem. 2008;54:2042-8.
53. Douketis J, Tosetto A, Marcucci M, Baglin T, Cushman M, Eichinger S, et al. Patient-level meta-analysis: effect of measurement timing, threshold, and patient age on ability of D-dimer testing to assess recurrence risk after unprovoked venous thromboembolism. Ann Intern Med. 2010;153:523-31.
54. Palareti G, Legnani C, Cosmi B, et al. Predictive value of D-dimer test for recurrent venous thromboembolism after anticoagulation withdrawal in subjects with a previous idiopathic event and in carriers of congenital thrombophilia. Circulation. 2003;108:313-8.
55. Eichinger S, Heinze G, Jandeck LM, Kyrle PA. Risk assessment of recurrence in patients with unprovoked deep vein thrombosis or pulmonary embolism: the Vienna prediction model. Circulation. 2010;121:1630-6.
56. Tosetto A, Iorio A, Marcucci M, Baglin T, Cushman M, Eichinger S, et al. Predicting disease recurrence in patients with previous unprovoked venous thromboembolism. A proposed prediction score (DASH). J Thromb Haemost. 2012;10: 1019-25.
57. Agnelli G, Buller HR, Cohen A, Curto M, Gallus AS, Johnson M, et al; the AMPLIFY-EXT Investigators. Apixaban for Extended Treatment of Venous Thromboembolism. N Engl J Med. 2012 Dec 8. [Epub ahead of print].
58. Becattini C, Agnelli G, Schenone A, Eichinger S, Bucherini E, Silingardi M, et al; WARFASA Investigators. Aspirin for preventing the recurrence of venous thromboembolism. N Engl J Med. 2012;366:1959-67.
59. Brighton TA, Eikelboom JW, Mann K, Mister R, Gallus A, Ockelford P, et al; ASPIRE Investigators. Low-dose aspirin for preventing recurrent venous thromboembolism. N Engl J Med. 2012;367:1979-87.

Biochemistry of red cell aging in vivo and storage lesions

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A B S T R A C T

The study of *in vivo* and *in vitro* (storage conditions) aging of red blood cells has recently taken advantage of the introduction of mass spectrometry-based "-omics" disciplines, such as proteomics, metabolomics and lipidomics.
In vivo and *in vitro* aging are characterized by shared features, including altered cation homeostasis, alteration of metabolic fluxes via decreased enzymatic activity and progressive depletion of high energy phosphates, increased susceptibility to oxidative stress, which in turn promotes oxidative lesions to proteins (carbonylation, fragmentation, hemoglobin glycation) and lipids (peroxidation), morphological changes (membrane blebbing, vesiculation). Most of these mechanisms closely resemble apoptosis-like phenomena.
On the other hand, the closed system of blood bank storage in plastic bags and additive solutions results in particular *in vitro* alterations to red blood cells, such as hypothermically-depressed metabolism, the exacerbation of oxidative stress-related phenomena, the progressive leakage of DEHP-plasticizers, the accumulation of microvesicles shed from red blood cells in the supernatant. These phenomena underlie the difficulties related to the extension of the shelf-life of red blood cell concentrates *in vitro* from the currently allowed threshold (42 days) up to the actual life-span of red blood cells *in vivo* (120 days).
Meanwhile, retrospective clinical and basic science evidence suggests that red blood cells stored longer than 14 days might not be as safe and effective as fresh ones.

Learning goals

At the conclusion of this activity, the participant should understand that:
- mass spectrometry-based "-omics" (such as proteomics and metabolomics) strategies have contributed to recent developments in this field of research;
- ageing of red blood cells *in vivo* and *in vitro* promotes the accumulation of reversible and irreversible lesions;
- *in vitro* storage of red blood cells (closed plastic bag system, hypothermia, additive solutions) exacerbates oxidative stress and accelerates aging;
- storage lesions accumulating *in vitro* soon after 14 days of storage are only partly reversible.

Introduction

Aging of red blood cells (RBCs) *in vivo* and *in vitro* represents a key biomedical issue.

Human RBCs have an approximate lifespan of 120 days *in vivo*. In most countries, the shelf-life of RBC concentrates stored under blood bank conditions is limited to 42 days.

Normal human RBCs all survive to about the same age, which implies the existence of a molecular countdown that triggers a series of changes leading to removal by the reticuloendothelial system (Table 1, Figure 1).[1,2] These changes share some distinct features with programmed cell death of nucleated cells, which prompted Lang's group to coin the term 'eryptosis', *i.e.* referring to erythrocyte-specific apoptosis.[3]

Storage under blood bank conditions results in the exacerbation of most of these changes and a shortening of RBC lifespan, a phenomenon referred to as the 'storage lesions'.[4-6] However, it should be remembered that an RBC concentrate unit already contains normally distributed (partly aged) RBC populations.

It is still a matter of debate as to whether and to what extent transfusion of RBC concentrate units older than 14 days might cause untoward effects in certain categories of recipients (e.g. traumatized, peri-operative and critically ill patients).[7,8] Prospective clinical trials (summarized in[9]) currently underway may shed light on this delicate issue, though results in very low birthweight infants have shown no effect of older RBC transfusions on clinical outcomes.[10]

From a biochemical standpoint, storage lesions are only partly reversible and might affect RBC viability and functionality on transfusion. They might, therefore, at least theoretically, affect the safety and effectiveness of the transfusion therapy with older units.[4-6]

This paper will attempt to review the major aspects of RBC aging *in vivo* and *in vitro*, while focusing on recent findings with novel

technologies, such as mass spectrometry (MS)-based metabolomics, proteomics, and lipidomics. These disciplines fit within the framework of "-omics" technologies, whereby specific classes of biomolecules (e.g. metabolites, proteins and lipids) are qualitatively and quantitatively investigated together.

Since aging RBCs have been shown to undergo dehydration with increased density and decreased size, RBC senescence has so far been investigated through the isolation of cell populations of different mean ages based on the increasing density of older cells.[11] It has also been argued that density might not represent a good criterion to determine RBC age, and alternative approaches, such as biotin labeling, which allows age-dependent separation of normal RBCs in animals, have been proposed.[12]

RBC aging and physiology *in vivo* and *in vitro*

The main biological role of RBCs is to deliver oxygen to peripheral tissues. Therefore, investigators have long been concerned to know if stored human erythrocytes could still handle oxygen delivery efficiently.[13] Among factors determining hemoglobin-oxygen affinity *in vivo*, aged erythrocytes show a decreased content of organic phosphate compounds (adenosine triphosphate-ATP and 2,3-diphosphoglycerate, DPG)[14] and an internal pH of approximately 0.2 pH units more alkaline than the younger cells.[15] These results suggest that *in vivo* aged RBCs may show increased hemoglobin-oxygen affinity.[15] Although ATP levels influence membrane stability and thus RBC survival,[16] *in vitro* alterations to DPG, ATP and cation imbalances are rapidly restored upon transfusion of RBCs in the bloodstream of the recipients.[17]

Analogous observations were reported for *in vitro* stored RBCs,[18] possibly reflecting the concomitant decline in DPG (98% decline by 2 weeks). Since pH is inversely related to oxygen off-loading capacity (Bohr effect), it is important to note that RBC storage under blood bank conditions also results in lower intracellular pH[15] as a result of glycolysis in a closed system, though lower pH will have a negative feedback on glycolysis itself.[19] Cation transport is negatively influenced by RBC age *in vivo*[20] and *in vitro*.[21] Sodium influx and potassium efflux become dysregulated in senescent RBCs and in erythrocyte concentrates that have been stored longer when they are also affected by hypothermia.[18,19,21] Supernatant accumulation of potassium may be dangerous for infants receiving large volume transfusion.

Altered potassium homeostasis is linked to an increase in intracellular ionic calcium.[22] *In vivo*[22] and *in vitro*[23-25] aging of RBCs have been related to intracellular increases of Ca^{2+}, which can lead to Ca^{2+} pump proteolysis and opening of the Ca^{2+}-dependent K^+ channel. Increases in intracellular calcium levels are consistent with activation of calcium-activated proteases (i.e. μ-calpain) and apoptosis-like phenomena,[3] though *in vitro* eryptosis mechanisms can still be triggered by starvation (high energy phosphate consumption) in the absence of calcium.[26] Calcium loading in rabbit erythrocytes results in dose-dependent decreases in reduced glutathione (GSH) levels.[27]

Cation perturbation, metabolic decay and oxidative damage are all interrelated in the erythrocyte aging process.[27]

Table 1. List of the main biochemical changes of aging red blood cells *in vivo* and in *vitro*.

Potassium leakage to the supernatant
Loss of metabolic modulation and depletion of DPG and ATP stores and pH lowering
Accumulation of intracellular calcium and activation of Ca2+-mediated signaling cascades (*e.g.* kinases, calpains)
Reduced oxygen off-loading capacity
Decreased S-nitrosothiohemoglobin;
Increased susceptibility to oxidative stress and alteration to the GSH homeostasis and Pentose Phosphate Pathway metabolism
Alteration of lipids (phospholipid loss, phospatidylserine exposure to the outer membrane leaflet, accumulation of ceramide)
Alteration of membrane proteins (membrane protein fragmentation and migration to the membrane and/or vesiculation of subsets of structural or cytosolic antioxidant proteins)
miR-96, miR-150, miR-196a, and miR-197 increase up to Day 20 and subsequently decreased during storage in vitro
Decreased desialiation, increased glycosylation and carbonylation of proteins; increased non-enzymatic glycation of hemoglobin (HbA1c)
Increased lipid oxidation (storage duration-dependent accumulation of malondialdehyde and 8-isoprostane)
Increased non-enzymatic glycation of hemoglobin and protein carbonylations
Oligomerization of band 3 and enzyme/ROS-mediated fragmentation
Accumulation of protein biomarkers at the membrane level (CD47, Apo-J/Clusterin, peroxiredoxin 2, RH and rheology markers)
Progressive leaching of DEHP plasticizers (in vitro) that intercalates into the membrane
More rigid cell structures (reduced deformability and increased osmotic fragility)
Increased vesiculation rate (shedding of nano- and micro-vesicles)
Loss of the discocytic shape towards the acquisition of the echinocytic, spheroechinocytic and utterly echinocytic phenotype

From physiology to metabolism

Owing to the lack of nuclei and organelles, including mitochondria, mature RBCs are incapable of generating energy via the (oxidative) Krebs cycle. They rely upon a limited network of metabolic pathways for energy production and redox homeostasis:[28,29]

- the Embden-Meyerhof pathway (glycolysis), in which 90% of the ATP is generated through the anaerobic breakdown of glucose;
- the pentose phosphate pathway, which is responsive to oxidative stress;
- the Rapoport-Lubering shunt, for DPG production;
- the purine salvage pathway, to salvage purine substrates for replenishing high energy purine reservoirs (*de novo* synthesis of purines is not present in RBCs);

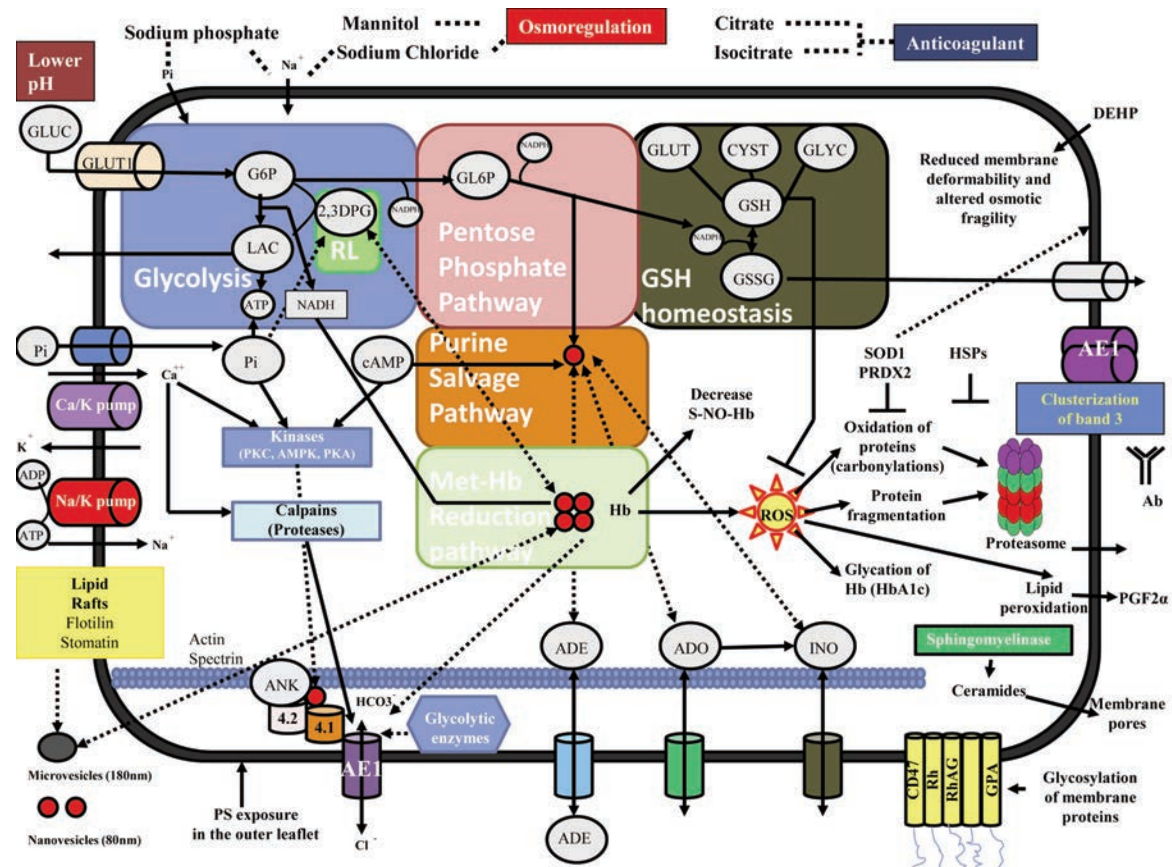

Figure 1. The figure can be read from the upper-left corner in an anti-clockwise direction. An overview of the main biochemical changes of *in vitro* aging red blood cells (RBCs) under blood bank conditions. Cation homeostasis dysregulation (K+, Ca^{2+}) is influenced by low temperatures and progressive depletion of high-energy phosphate reservoirs (adenosine triphosphate – ATP and 2,3-diphosphoglycerate - DPG). Glucose (additive solution) is internalized through GLUT transporters and consumed through the Emden-Meyerhof glycolytic pathway, in order to produce ATP, lactate (LAC) and promote pH lowering. Besides, storage results in progressive decrease of S-nitrosothiol-hemoglobin (Hb). However, low temperatures and the progressive accumulation of oxidative stress (likely triggered by Hb–mediated Fenton reactions) promote a metabolic divertion towards the pentose phosphate pathway, in order to produce oxidized glutathione (GSSG)-reducing NADPH from glucose 6-phosphate (G6P). Pentose phosphate pathway intermediates can re-enter glycolysis or rather proceed towards the purine salvage pathway (also influenced by adenosine and inosine in the additive/rejuvenation solution). Alterations to calcium (Ca^{2+}) homeostasis (and of other second messenger signaling molecules, such as cAMP and AMP) promote the activation of specific kinases (*e.g.* PKC, PKA, AMPK) or rather activate proteolytic enzymes (such as calpains) that start digesting structural and functional proteins at the cytosol and membrane level, above all band 3 (AE1). Anion exchanger 1/band 3 (AE1) is indeed responsible for the chloride shift, whereby bicarbonate (HCO3-) is exchanged for chloride (Cl-), thus modulating anion homeostasis, intracellular pH and, indirectly, Hb-oxygen affinity and thus gas exchanges. Fragmentation of the cytosolic domain of AE1 (also mediated by reactive oxygen species, ROS) promotes displacement of glycolytic enzymes (thereby bound/inhibited) and structural proteins (ankyrin, ANK, band 4.2 and 4.1). Enhanced oxidation of cytosolic proteins is partly challenged by antioxidant defenses (SOD1, PRDX2) and chaperone molecules (heat shock proteins, HSPs), while they progressively result in the accumulation of redox modifications to proteins (carbonylations, glycation of hemoglobin (HbA1c), protein fragmentation) and lipids (lipid peroxidation, accumulation of prostaglandins in the supernatant). A role in the process is also mediated by alternative degradation strategies to proteins (proteasome, eventually extruded in the supernatant) and lipids (sphingomyelinase-dependent accumulation of ceramides). Progressive leaching of plasticizers (DEHP) from the plastic bag results in the local accumulation at the membrane. At the membrane level, AE1 clusters, exposure of phosphatidylserine (PS) in the outer leaflet, lipid raft formation alter RBC pro-immunogenic potential. Taken together, these alterations affect membrane deformability, increase osmotic fragility and promote vesiculation events, a process where micro- and nanovesicles are shed in order to eliminate irreversibly altered proteins (among which traces of glycolytic enzymes), enriched with hemoglobin and lipid raft proteins, membrane portions (also exposing common rheological antigens - CD47, Rh, RhAG, glycophorin A-GPA).

- glutathione (GSH) homeostasis;
- the methemoglobin (met-Hb) reduction pathway, which reduces ferric heme iron to the ferrous form to prevent Hb denaturation via the enzyme NADH-cytochrome b5 reductase.

Energy metabolism

RBC aging *in vivo* corresponds to a steep decline in the activity of key metabolic enzymes, including hexokinase and pyruvate kinase (Embden Meyerhof).[30] Using novel MS-based metabolomics, optimized for RBC investigations,[31] we recently confirmed and expanded these data by demonstration of decreased levels of the main glycolytic intermediate and end-product metabolites (glucose 6-phosphate, glyceraldehyde 3-phosphate, phosphoenolpyruvate and lactate) in density gradient-separated senescent RBCs.[32]

RBC storage under blood bank conditions also results in loss of metabolic activity, with decreased rates of ATP and DPG production, also favored by the negative effect of hypothermic storage temperature on enzyme activity rates, lactate accumulation in the supernatants and altered glycolysis/pentose phosphate fluxes.[15,33] MS-based approaches revealed consistent trends for RBCs stored in two different storage solutions, namely mannitol-adenine-phosphate (MAP)[34] and citrate-phosphate-dextrose–saline-adenine-glucose-mannitol (CPD-SAGM).[23,35] In particular, in CPD-SAGM-stored erythrocyte concentrates we showed increased levels of glycolytic metabolites over the first two weeks of storage, while from Day 14 onwards, we observed a significant consumption of all metabolic species, and diversion towards the oxidative phase of the pentose phosphate (NADPH and 6-phosphogluconic acid), in response to an exacerbation of oxidative stress.[23,35]

Redox metabolism

Senescent RBCs display increased susceptibility to oxidative stress and altered glutathione homeostasis.[36] The activity of the key anti-oxidant enzyme glutathione S-transferase is independent of erythrocyte age, but aged erythrocytes have decreased activities of the rate limiting enzyme for the oxidative phase of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase, and of NADH-cytochrome b5 reductase.[37,38] GSH levels fall in senescent RBCs *in vivo* with accumulation of GSSG.[32,39] GSH depletion *in vivo* is paralleled by significant decreases in the rate of GSH synthesis (-45±8%)[40] and increased GSSG[23,35] levels under *in vitro* blood bank conditions, both being largely attributable to reduced amino acid transport (reduced levels of glutamate, glutamate-precursor glutamine, glycine and cysteine), secondary to decreased ATP concentration.[32,41]

Direct measurement of reactive oxygen species (ROS) during RBC storage under blood bank conditions evidenced a significant increase in oxidative stress after 14 days of storage of either leukofiltered or non-leukofiltered erythrocyte concentrates.[22,35]

From metabolism to proteomics: the transport metabolon

Both *in vivo* and *in vitro*, cation and metabolic modulation of RBCs is largely dependent on ultra-structural complexes of cytosolic enzymes and protein-protein interactions, of which those involving the anion exchanger 1-band 3(AE1) membrane protein represent a structural example.

AE1, the major integral membrane protein of RBCs, is involved in the 'chloride shift' (exchange of cellular HCO_3^- with plasma Cl-), a process that promotes the conversion of the weak acid H_2CO_3 to the strong acid HCl, thereby rendering the intracellular pH acidic. Acidification increases the dissociation of molecular oxygen (O_2) from oxyhemoglobin, and the dissociated O_2 is supplied to tissues that metabolically produce CO_2. Protons formed in RBCs are accepted by the groups of deoxy-hemoglobin participating in the 'Bohr Effect'. By means of the transient acidification triggered by the anion exchange activity, tissues producing more CO_2 are supplied with more O_2 from oxy-hemoglobin.

The N-terminal cytosolic domain of AE1 is a docking site for several enzymes of the glycolytic pathway, including phosphofructokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase[40,42], as well as deoxy-hemoglobin[40,42,43] and the anti-oxidant enzyme peroxiredoxin 2,[44] a non-catalytic scavenger of low-level hydrogen peroxide in the erythrocyte.[45] Competitive binding of deoxy-hemoglobin to the cytosolic domain of AE1 results in the displacement of glycolytic enzymes from the RBC membrane and promotes their activation. In this way, RBCs are able to undergo an oxygen-linked modulation of metabolism.[40,42,43]It has recently been observed that phosphorylation of the tyrosine residues of the AE1 protein at position 8 and 21 modulates the binding of glycolytic enzymes and deoxy-hemoglobin to the N-terminal domain. The underlying mechanism seems to involve the phosphorylation-dependent increase in the number of negative charges at the N-terminal domain of AE1, which increases deoxy-hemoglobin binding to AE1 (similar to the stabilizing T-state of deoxy-Hb bound to negatively charged DPG), displaces otherwise bound/inhibited glycolytic enzymes, and results in an increased (+45%) glycolytic flux and reduced shift towards the pentose phosphate pathway (-66%).[42,46] Notably, deoxygenation seems to promote phosphorylation of the N-terminal domain of AE1.[44]

An understanding of the central role of AE1 as an actual 'transport metabolon' in the physiology of RBCs will help us widen our knowledge of the importance of the long documented *in vivo* and *in vitro* aging-triggered lesions to AE1.[47,48] The most widely accepted models for RBC senesce *in vitro* and *in vivo* involve either: i) enzyme and oxidative stress-mediated proteolysis of AE1 (promoting the formation of a 24 and 34kDa fragment, respectively)[49]; or ii) the formation of AE1 oligomeric clusters[47,49] which display pro-immunogenic properties and mediate recognition through naturally-occurring antibodies and RBC removal by resident spleen and liver macrophages.

Compared to the involvement of calcium-modulated proteases in red cell aging and apoptosis,[3] the involvement of oxidative stress-mediated proteolysis of the cytosolic domain of AE1 is a recent suggestion which fits well with recent theories of a central role for oxidative stress in RBC-storage lesions, most particularly in the blood bank.[32] Clusterization of AE1 proteins might be indirectly dependent upon oxidative stress, since oxidized and poor-

ly-glycosylated AE1 is selectively phosphorylated by Syk kinase to form large membrane clusters in normal and glucose 6-phosphate dehydrogenase-deficient RBCs.[50]

Evidence of increased levels of membrane peroxiredoxin-2 in long-stored RBCs[32,51] further support the rationale above. In the light of these findings, we recently proposed targeted assays as quality control tests for long-stored erythrocyte concentrates.[52]

Oxidative stress to proteins: proteomics of RBC aging *in vivo* and *in vitro*

Aging of RBCs results in the accumulation of oxidative stress modifications to RBC proteins. So far, two main oxidative stress-mediated modifications to RBC proteins have been investigated: glycation of hemoglobin and carbonylation of RBC proteins. Glycation of hemoglobin (HbA1c) is a non-enzymatic irreversible process that is promoted by the prolonged exposure of erythrocytes to high glucose concentrations,[53] a condition that is known to occur *in vivo* in diabetic patients, and *in vitro* during blood bank storage where additive solutions expose RBCs to higher than normal glycemic levels (*e.g.* 50 mM). While the process has been widely documented for senescent erythrocytes *in vivo*,[53] measurements on *in vitro* stored RBCs give conflicting results.[41,54] Recent MS-based evidence from our group[55] seems to support early observations of an increase in the levels of HbA1C in RBCs that have been stored longer.

Other than glycation, enzyme-mediated glycosylations may play a role in the alteration of rheological properties and RBC recognition by macrophages during RBC aging both *in vivo* and *in vitro*.[56] Membrane-associated carbohydrate changes act as signals for removal of senescent and damaged RBCs from the circulation, and could play a role in the RBC storage lesion and survival after transfusion. A recent experiment with fluorescein-labeled lectins in young and senescent RBC populations and RBCs stored for long periods of time, indicated that both *in vivo* and *in vitro* aging were associated with progressively increased binding of lectins specific for galactose and N-acetylglucosamine residues.[57]

Carbonylation is a hallmark of protein oxidative lesions. Carbonylation in the cytoskeletal membrane fraction increases significantly after the third week of storage in CPD-SAGM,[35,58] and in particular between Day 29 and Day 42 of storage.[58] Leukodepletion of erythrocyte concentrate appears to ameliorate oxidative stress, reducing the degree of measured carbonylation.[35,56,59]

Aging of RBCs *in vivo* is characterized by alternative oxidation and post-translational modification phenomena, such as desialiation[60] or the progressive deamidation of Asn478 and 502 of the band 4.1b protein which results in altered electrophoretic mobility, and thus different apparent molecular weight in SDS-PAGE runs.[32]

During the last five years, great progress in the field of proteomics and sample pre-fractionation strategies has led to the simultaneous identification of 1578 distinct cytosolic proteins;[61] when added to known membrane proteins, this gives a current total of 1989 RBC proteins.[62,63]

Alterations of the RBC membrane and cytosol proteome during *in vitro* storage have been analyzed by several groups.[35,64-66] Storage-induced changes to the proteome include fragmentation of membrane structural proteins (spectrin, ankyrin, AE1, band 4.1), membrane accumulation of hemoglobin, antioxidant enzymes (peroxiredoxin-2) and chaperones, and decrease in cytosolic transglutaminase-2, beta actin, and copper chaperone for superoxide dismutase. Proteomics can provide a snapshot of cytoskeletal reorganization by highlighting the relocation of SNAP proteins[35] and the decrease in RBC membrane content of lipid raft-associated proteins flotillins and stomatin.[65]

Alterations to the RBC membrane proteome are dependent on the tested additive solution. The storage induced increase in the overall spot number on 2D-gel electrophoresis, a measure of protein fragmentation events, was less in AS-3 stored RBCs than in SAG-M.[67]

Interestingly, RBCs do have a functional proteasome-based protein degradation system, while cell aging *in vitro* corresponds to proteasome 20S accumulation in the supernatants.[68] Membrane remodeling *in vivo* results in the impairment of proper ubiquitination of specific structural proteins, such as spectrin.[69,70] This phenomenon might be affected to some extent by phosphorylation of structural proteins (spectrin and band 4.1),[71] a post-translational modification that deserves further investigations within the framework of RBC aging *in vivo* and *in vitro* through the application of innovative Omics approaches such as electron transfer dissociation MS.

Oxidative stress: effects on the lipidome

Aging of RBCs also results in the progressive accumulation of oxidative stress markers in the lipid fraction. Thiobarbituric acid-reactivity assays show malondialdehyde accumulation in senescent RBCs *in vivo*[72] and *in vitro*.[35,73] Glucose autoxidation from excess glucose in RBC storage solutions may contribute to this accumulation.[74] Consistent with this possibility, we recently detected ferrous-conjugated lactone dimer derivatives of glucose autoxidation in the supernatants of RBC concentrates stored for longer periods of time.[23]

Oxidative stress under prolonged storage *in vitro* also promoted the accumulation of peroxidized lipids in the supernatant, in the form of prostaglandins (such as 8-isoprostane, $PGF_{2\alpha}$).[6,23]

Oxidative stress-induced alterations to the RBC lipidome are relevant in that mature erythrocytes are devoid of any *de novo* lipid synthesis capacity, owing to an incomplete long chain fatty acid synthesizing system.[75]

RBC membrane properties are largely affected by lipid composition, which in turn is influenced by diet.[76] Early studies[77-79] have demonstrated membrane phospholipid asymmetry in senescent RBCs with externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane recalling apoptosis-like phenomena.[3] However, a recent study found no evidence for elevated external PS in senescent RBCs, even though older RBCs had significantly lower activity of aminophospholipid translocase.[80] Increased externalization of PS has been shown in long-stored RBCs *in vitro*.[81]

Like apoptotic cells,[3] senescent and long-stored RBCs display higher levels of ceramides which may be produced from cell membrane sphingomyelins by an acid sphingomyelinase.[82,83] The sphingomyelinase is stimulated by

platelet-activating factor PAF which is generated from cell membrane lipids by a phospholipase, that is in turn activated during osmotic erythrocyte shrinkage (reviewed in[3]). Ceramides and sphingosines might also be responsible for the formation of specific rafts/membrane domains, which underlie hot cold-hemolysis (cells pre-incubated at 37°C in the presence of certain agents undergo rapid hemolysis when transferred to 4°C).[84]

Osmotic fragility, morphology changes and vesiculation

Senescent RBCs are characterized by increased osmotic fragility and impaired deformability, as measured through viscoelastic time constant indexes.[85] In other words, shape recovery following membrane deformation is delayed in old RBC, which compromises their functionality *in vivo* where they should be able to traverse passage ways as narrow as 1 μm in diameter (capillaries and splenic slits) and survive periodic high turbulence and shear stresses, along with extremely hypertonic conditions. Increased osmotic fragility has also been reported for RBCs stored for long periods of time under blood bank conditions.[86]

In vitro storage results in the prolonged exposure to plasticizers that might affect membrane deformability and thus osmotic fragility.[87,88] Plastic bags were first introduced in the 1950s when their lighter weight and greater resistance to breakage in comparison to glass bottles made them more suitable for military logistics. Prolonged RBC storage in plastic bags under blood bank conditions is also accompanied by the progressive leaching and membrane intercalation of the plasticizer di-2-ethylhexyl phthalate (DEHP), a common component in medical plastics, which promotes a 4-fold improvement of weekly measurements of hemolysis values over other plasticizers.[87] Owing to the potential toxicity of DEHP, novel plasticizers are continuously under evaluation.[88]

Proportional to RBC age *in vivo* and *in vitro*, the red cell phenotype changes from a biconcave disc, towards an echinocyte, spheroechinocyte and, finally, spherocytic phenotype.[87,89-91] Data acquired on tens of thousands of red cells showed that nearly as much membrane area is lost during the 1-2 days of reticulocyte maturation (10-14%) as in the subsequent four months of erythrocyte aging (approx. 16-17%).[90] Surface/volume ratio constantly increases as RBCs shed one microvesicle per hour during their lifespan *in vivo*.[92] *In vitro*, irreversible morphology phenotypes accumulate significantly after the first two weeks of storage.[86,91] By storage Day 21, more than 50% of RBCs displayed a non-discocyte shape.[86] Several biological inputs (*e.g.* calcium signaling, ATP depletion, ceramide accumulation) and physico-chemical constraints (*e.g.* alterations to surface charge density and surface/volume ratio minimization in the model proposed by Gov[92]) trigger the acquisition of the spheroechinocytic-spherocytic phenotype.[92]

Alterations in RBC morphology related to storage age recall *in vivo* erythrocyte senescence whereby membrane blebbing and vesiculation represent the conclusive step towards apoptosis.[3] Over the years, concerns have arisen about the possible untoward consequences of transfusion of exocytic micro- and nano-vesicles (180 and 80 nm, respectively).[93] Several research groups have studied the rheological properties and molecular content of these vesicles, mainly through flow-cytometry and proteomic approaches.[94-99] Importantly, leukofiltration affects RBC-shed vesicle quantity and content.[22,95] RBC-derived vesicles can be separated from white blood cell counterparts by the presence of membrane markers including blood group antigens from the RH, KEL, JK, FY, MNS, LE and LU systems and plasma protein S-antigen (PS).[99] On the other hand, the presence of M(MNS1), N(MNS2) and s(MNS4) antigens could not be demonstrated by flow-cytometry, despite the fact that glycophorin A and B were identified on microparticles using anti-CD235a and anti-MNS3.[99]

Generation of vesicles during blood bank storage accounts for a considerable part of the cellular hemoglobin loss.[96] These vesicles expose PS and are also targeted by immunoglobulins and various complement proteins, which may contribute to the adverse effects upon transfusion. The identification of human immunoglobulins on vesicles,[95] especially upon exposure to plasma rich media,[97] supports the hypothesis that vesicles might serve to remove membrane patches that have a high content of removal signals (such as the marker of self and molecular switch for erythrocyte phagocytosis CD47[98]).

Vesicles are also enriched in ankyrin, AE1, spectrin beta, lipid raft-associated proteins (flotillin and stomatin), while relatively low amounts of glyceraldehyde 3-phosphate dehydrogenase have been detected.[99] Proteomic analyses of RBC-shed vesicles have shown a resemblance to older RBC membranes, which has prompted suggestions of a possible role of vesiculation as a mechanism to remove damaged proteins. Supporting this, extracellular 20S proteasome subunits have been found to accumulate in the supernatants of packed RBC units.[68]

miRNAs

Though investigations are at an early stage, profiling of RBCs for 52 micro-RNAs (miRNAs, negative regulators of mRNAs) revealed that miR-96, miR-150, miR-196a, and miR-197 increase up to Day 20 and subsequently decreased during *in vitro* storage.[100]

Conclusion

In the present review, we have summarized the major biochemical changes related to RBC aging both *in vivo* and *in vitro*.

Future developments in the field will be fueled by the introduction of novel storage strategies (e.g. new additive or rejuvenation solutions, anaerobiosis, pathogen inactivation protocols) and the further application of integrated "-omics" approaches and mathematical models,[28] and the use of nanotechnology-based assays, such as atomic force microscopy.

References

1. Shinozuka T. Changes in human red blood cells during aging in vivo. Keio J Med. 1994;43(3):155-63.
2. Bosman GJ, Lasonder E, Groenen-Döpp YA, Willekens FL, Werre JM, Novotný VM. Comparative proteomics of erythrocyte aging in vivo and in vitro. J Proteomics. 2010;73(3):396-

402.
3. Lang E, Qadri SM, Lang F. Killing me softly - suicidal erythrocyte death. Int J Biochem Cell Biol. 2012;44(8):1236-43.
4. D'Alessandro A, Liumbruno G, Grazzini G, Zolla L. Red blood cell storage: the story so far. Blood Transfus. 2010;8(2):82-8.
5. Lion N, Crettaz D, Rubin O, Tissot JD. Stored red blood cells: a changing universe waiting for its map(s). J Proteomics. 2010;73(3):374-85.
6. Karon BS, Van Buskirk CM, Jaben EA, Hoyer JD, Thomas DD. Temporal sequence of major biochemical events during Blood Bank storage of packed red blood cells. Blood Transfus. 2012;28:1-9.
7. Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihaljevic T, et al. Duration of red-cell storage and complications after cardiac surgery. N Engl J Med. 2008;358:1229-39.
8. Lelubre C, Piagnerelli M, Vincent JL. Association between duration of storage of transfused red blood cells and morbidity and mortality in adult patients: myth or reality? Transfusion. 2009;49(7):1384-94.
9. Grazzini G, Vaglio S. Red blood cell storage lesion and adverse clinical outcomes: post hoc ergo propter hoc? Blood Transfus. 2012;10(2):4-6.
10. Fergusson DA, Hébert P, Hogan DL, LeBel L, Rouvinez-Bouali N, Smyth JA, et al. Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birthweight infants: the ARIPI randomized trial. JAMA. 2012;308(14):1443-51.
11. Bosch FH, Werre JM, Roerdinkholder-Stoelwinder B, Huls TH, Willekens FL, Halie MR. Characteristics of red blood cell populations fractionated with a combination of counterflow centrifugation and Percoll separation. Blood. 1992;79(1):254-60.
12. Suzuki T, Dale GL. Biotinylated erythrocytes: In vivo survival and in vitro recovery. Blood. 1987;791-5.
13. Valtis DJ. Defective gas-transport function of stored red bloodcells. Lancet. 1954;266(6803):119-24.
14. Samaja M, Rovida E, Motterlini R, Tarantola M, Rubinacci A, di Prampero PE. Human red cell age, oxygen affinity and oxygen transport. Respir Physiol. 1990;79(1):69-79.
15. Romero PJ, Romero EA. Determinant factors for an apparent increase in oxygen affinity of senescent human erythrocytes. Acta Cient Venez. 2004;55(1):83-5.
16. Nakao K, Wada T, Kamiyama T, Nakao M, Nagano K. A direct relationship between adenosine triphosphate-level and in vivo viability of erythrocytes. Nature. 1962;194:877-8.
17. Valeri CR, Hirsch NM. Restoration in vivo of erythrocyte adenosine triphosphate, 2,3-diphosphoglycerate, potassium ion, and sodium ion concentrations following the transfusion of acid-citrate-dextrose-stored human red blood cells. J Lab Clin Med. 1969;73(5):722-33.
18. Bennett-Guerrero E, Veldman TH, Doctor A, Telen MJ, Ortel TL, Reid TS, et al. Evolution of adverse changes in stored RBCs. Proc Natl Acad Sci USA. 2007;104(43):17063-8.
19. Burr MJ. The relationship between pH and aerobic glycolysis in human and canine erythrocytes. Comp Biochem Physiol B. 1972;41(4):687-94.
20. Hentschel WM, Wu LL, Tobin GO, Anstall HB, Smith JB, Williams RR, et al. Erythrocyte cation transport activities as a function of cell age. Clin Chim Acta. 1986;157(1):33-43.
21. Wallas CH. Sodium and potassium changes in blood bank stored human erythrocytes. Transfusion. 1979;19(2):210-5.
22. Romero PJ, Romero EA. The role of calcium metabolism in human red blood cell ageing: a proposal. Blood Cells Mol Dis. 1999;25(1):9-19.
23. Wiley JS, McCulloch KE, Bowden DS. Increased calcium permeability of cold-stored erythrocytes. Blood. 1982;60(1):92-8.
24. Antonelou MH, Tzounakas VL, Velentzas AD, Stamoulis KE, Kriebardis AG, Papassideri IS. Effects of pre-storage leukoreduction on stored red blood cells signaling: a time-course evaluation from shape to proteome. J Proteomics. 2012;76:220-38.
25. Gevi F, D'Alessandro A, Rinaldducci S, Zolla L. Alterations of red blood cell metabolome during cold liquid storage of erythrocyte concentrates in CPD-SAGM. J Proteomics. 2012;76:168-80.
26. Pompeo G, Girasole M, Cricenti A, Boumis G, Bellelli A, Amiconi S. Erythrocyte death in vitro induced by starvation in the absence of Ca(2+). Biochim Biophys Acta. 2010;1798(6):1047-55.
27. Kurata M, Suzuki M. Glutathione regeneration in calcium-loaded erythrocytes: a possible relationship among calcium accumulation, ATP decrement and oxidative damage. Comp Biochem Physiol B Biochem Mol Biol. 1994;109(2-3):305-12.
28. Jamshidi N, Palsson BØ. Systems biology of the human red blood cell. Blood Cells Mol Dis. 2006;36:239-47.
29. Messana I, Misiti F, el-Sherbini S, Giardina B, Castagnola M. Quantitative determination of the main glucose metabolic fluxes in human erythrocytes by 13C- and 1H-MR spectroscopy. J Biochem Biophys Methods. 1999;39(1-2):63-84.
30. Jansen G, Koenderman L, Rijksen G, Cats BP, Staal GE. Characteristics of hexokinase, pyruvate kinase, and glucose-6-phosphate dehydrogenase during adult and neonatal reticulocyte maturation. Am J Hematol. 1985;20(3):203-15.
31. D'Alessandro A, Gevi F, Zolla L. A robust high resolution reversed-phase HPLC strategy to investigate various metabolic species in different biological models. Mol Biosyst. 2011;7(4):1024-32.
32. D'Alessandro A, Blasi B, D'Amici GM, Marrocco C, Zolla L. Red blood cell subpopulations in freshly drawn blood: application of proteomics and metabolomics to a decades-long biological issue. Blood Transfus. 2013;11:1-13.
33. Messana I, Ferroni L, Misiti F, Girelli G, Pupella S, Castagnola M, et al. Blood bank conditions and RBCs: the progressive loss of metabolic modulation. Transfusion. 2000;40(3):353-60.
34. Nishino T, Yachie-Kinoshita A, Hirayama A, Soga T, Suematsu M, Tomita M. In silico modeling and metabolome analysis of long-stored erythrocytes to improve blood storage methods. J Biotechnol. 2009;144(3):212-23.
35. D'Alessandro A, D'Amici GM, Vaglio S, Zolla L. Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics. Haematologica. 2012;97(1):107-15.
36. Sass MD, Caruso CJ, O'Connell DJ. Decreased glutathione in aging red cells. Clin Chim Acta. 1965;11:334.
37. Strange RC, Johnson PH, Lawton A, Moult JA, Tector MJ, Tyminski RJ, et al. Studies on the variability of glutathione S-transferase from human erythrocytes. Clin Chim Acta. 1982;120(2):251-60.
38. Brajovich ML, Rucci A, Acosta IL, Cotorruelo C, García Borrás S, Racca L, et al. Effects of aging on antioxidant response and phagocytosis in senescent erythrocytes. Immunol Invest. 2009;38(6):551-9.
39. Ghashghaeinia M, Cluitmans JC, Akel A, Dreischer P, Toulany M, Köberle M, et al. The impact of erythrocyte age on eryptosis. Br J Haematol. 2012;157(5):606-14.
40. Lewis IA, Campanella ME, Markley JL, Low PS. Role of band 3 in regulating metabolic flux of red blood cells. Proc Natl Acad Sci USA. 2009;106(44):18515-20.
41. Whillier S, Raftos JE, Sparrow RL, Kuchel PW. The effects of long-term storage of human red blood cells on the glutathione synthesis rate and steady-state concentration. Transfusion. 2011;51(7):1450-9.
42. D'Alessandro A, Gevi F, Zolla L. Red blood cell metabolism under prolonged anaerobic storage. Molecular Biosystems. 2013;doi:10.1039/c3mb25575a
43. Castagnola M, Messana I, Sanna MT, Giardina B. Oxygen-linked modulation of erythrocyte metabolism: state of the art. Blood Transfus. 2010;8(3):53-8.
44. Matte A, Bertoldi M, Mohandas N, An X, Bugatti A, Brunati AM, et al. Membrane association of peroxiredoxin-2 in red cells is mediated by the N-terminal cytoplasmic domain of band 3. Free Radic Biol Med. 2012;55C:27-35.
45. Low FM, Hampton MB, Peskin AV, Winterbourn CC. Peroxiredoxin 2 functions as a noncatalytic scavenger of low-level hydrogen peroxide in the erythrocyte. Blood. 2007;109(6):2611-7.
46. Rogers SC, Said A, Corcuera D, McLaughlin D, Kell P, Doctor A. Hypoxia limits antioxidant capacity in red blood cells by altering glycolytic pathway dominance. FASEB J. 2009;23:3159-70.
47. Lutz HU, Fasler S, Stammler P, Bussolino F, Arese P. Naturally occurring anti-band 3 antibodies and complement in phagocytosis of oxidatively-stressed and in clearance of senescent red cells. Blood Cells. 1988;14(1):175-203.
48. Rinalducci S, Ferru E, Blasi B, Turrini F, Zolla L. Oxidative stress and caspase-mediated fragmentation of cytoplasmic domain of erythrocyte band 3 during blood storage. Blood Transfus. 2012;10(Suppl 2):s55-62.
49. Karon BS, Hoyer JD, Stubbs JR, Thomas DD. Changes in Band 3 oligomeric state precede cell membrane phospholipid loss during blood bank storage of red blood cells. Transfusion. 2009;49:1435-42.
50. Pantaleo A, Ferru E, Giribaldi G, Mannu F, Carta F, Matte A, et al. Oxidized and poorly glycosylated band 3 is selectively phosphorylated by Syk kinase to form large membrane clusters in normal and G6PD-deficient red blood cells. Biochem J. 2009;418(2):359-67.
51. Rinalducci S, D'Amici GM, Blasi B, Vaglio S, Grazzini G, Zolla L. Peroxiredoxin-2 as a candidate biomarker to test

oxidative stress levels of stored red blood cells under blood bank conditions. Transfusion. 2011;51(7):1439-49.

52. Marrocco C, Pallotta V, D'Alessandro A, Alves G, Zolla L. Red blood cell populations and membrane levels of peroxiredoxin 2 as candidate biomarkers to reveal blood doping. Blood Transfus. 2012;10(2):s71-7.
53. Bunn HF, Haney DN, Kamin S, Gabbay KH, Gallop PM. The biosynthesis of human hemoglobin Alc. Slow glycosylation of hemoglobin in vivo. J Clin Invest. 1976;57:1652-9.
54. Szelényi JG, Földi J, Hollàn SR. Enhanced nonenzymatic glycosylation of blood proteins in stored blood. Transfusion. 1983;23:11-14.
55. D'Alessandro A, Mirasole C, Zolla L. Hemoglobin alpha glycation (Hb1Ac) increases during red blood cell storage: a MALDI-TOF mass spectrometry-based investigation. Vox Sanguinis. 2013;doi:10.1111/vox.12029.
56. Sparrow RL, Veale MF, Healey G, Payne KA. Red blood cell (RBC) age at collection and storage influences RBC membrane-associated carbohydrates and lectin binding. Transfusion. 2007;47(6):966-8.
57. Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells. J Cell Mol Med. 2007;11:148-55.
58. Delobel J, Prudent M, Rubin O, Crettaz D, Tissot JD, Lion N. Subcellular fractionation of stored red blood cells reveals a compartment-based protein carbonylation evolution. J Proteomics. 2012;76:181-93.
59. D'Amici GM, Rinalducci S, Zolla L. Proteomic analysis of RBC membrane protein degradation during blood storage. J Proteome Res. 2007;6(8):3242-55.
60. Jakubowska-Solarska B, Solski J. Sialic acids of young and old red blood cells in healthy subjects. Med Sci Monit. 2000;6(5):871-4.
61. Roux-Dalvai F, Gonzalez de Peredo A, Simó C, Guerrier L, Bouyssié D, Zanella A, et al. Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry. Mol Cell Proteomics. 2008;7(11):2254-69.
62. D'Alessandro A, Righetti PG, Zolla L. The red blood cell proteome and interactome: an update. J Proteome Res. 2010;9(1):144-63.
63. Goodman SR, Kurdia A, Ammann L, Kakhniashvili D, Daescu O. The human red blood cell proteome and interactome. Exp Biol Med. 2007;232(11):1391-408.
64. Antonelou MH, Kriebardis AG, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol. Transfusion. 2010;50(2):376-89.
65. Bosman GJ, Lasonder E, Luten M, Roerdinkholder-Stoelwinder B, Novotný VM, Bos H, et al. The proteome of red cell membranes and vesicles during storage in blood bank conditions. Transfusion. 2008;48(5):827-35.
66. Walpurgis K, Kohler M, Thomas A, Wenzel F, Geyer H, Schänzer W, et al. Storage-induced changes of the cytosolic red blood cell proteome analyzed by 2D DIGE and high-resolution/high-accuracy MS. Proteomics. 2012;12(21):3263-72.
67. D'Amici GM, Mirasole C, D'Alessandro A, Yoshida T, Dumont LJ, Zolla L. Red blood cell storage in SAGM and AS3: a comparison through the membrane two-dimensional electrophoresis proteome. Blood Transfus. 2012;10(2):46-54.
68. Geng Q, Romero J, Saini V, Patel MB, Majetschak M. Extracellular 20S proteasomes accumulate in packed red blood cell units. Vox Sang. 2009;97(3):273-4.
69. Corsi D, Paiardini M, Crinelli R, Bucchini A, Magnani M. Alteration of α-spectrin ubiquitination due to age-dependent changes in the erythrocytes membrane. Eur J Biochem. 1999;261:775-83.
70. Park Y, Best CA, Auth T, Gov NS, Safran SA, Popescu G, et al. Metabolic remodeling of the human red blood cell membrane. Proc Natl Acad Sci USA. 2010;107(4):1289-94.
71. Manno S, Takakuwa Y, Nagao K, Mohandas N. Modulation of erythrocyte membrane mechanical function by protein 4.1 phosphorylation. J Biol Chem. 2005;280:7581-7.
72. Jain SK. Evidence for membrane lipid peroxidation during the in vivo aging of human erythrocytes. Biochim Biophys Acta. 1988;937(2):205-10.
73. Dumaswala UJ, Zhuo L, Jacobsen DW, Jain SK, Sukalski KA. Protein and lipid oxidation of banked human erythrocytes: role of glutathione. Free Radic Biol Med. 1999;27(9-10):1041-9.
74. Virgili F, Battistini N, Canali R, Vannini V, Tommasi A. High glucose-induced membrane lipid peroxidation on intact erythrocytes and on isolated erythrocyte membrane (ghosts). J Nutr Biochem. 1996;151-61.
75. Pittman JG, Martin DB. Fatty acid biosynthesis in human erythrocytes: evidence in mature erythrocytes for an incomplete long chain fatty acid synthesizing system. J Clin Invest. 1966;45(2):165-72.
76. Ways P, Hanahan DJ. Characterization and quantification of red cell lipids in normal man. J Lipid Res. 1964;5(3):318-28.
77. Van Gastel C, Van Den Berg D, de Gier J, Van Deenen LLM. Some lipid characteristics of normal red blood cells of different age. Brit J Haematol. 1965;11:193-9.
78. Percy AK, Schmell E, Earles BJ, Lennarz WJ. Phospholipid biosynthesis in the membranes of immature and mature red blood cells. Biochemistry. 1973;12(13):2456-61.
79. Schroit AJ, Madsen JW, Tanaka Y. In vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes. J Biol Chem. 1985;260(8):5131-8.
80. Franco RS, Puchulu-Campanella ME, Barber LA, Palascak MB, Joiner CH, Low PS, et al. Changes in the properties of normal human red blood cells during in vivo aging. Am J Hematol. 2013;88(1):44-51.
81. Bosman GJ, Cluitmans JC, Groenen YA, Werre JM, Willekens FL, Novotný VM. Susceptibility to hyperosmotic stress-induced phosphatidylserine exposure increases during red blood cell storage. Transfusion. 2011;51(5):1072-8.
82. Dinkla S, Wessels K, Verdurmen WP, Tomelleri C, Cluitmans JC, Fransen J, et al. Functional consequences of sphingomyelinase-induced changes in erythrocyte membrane structure. Cell Death Dis. 2012;3:e410.
83. Bicalho B, Holovati JL, Acker JP. Phospholipidomics reveals differences in glycerophosphoserine profiles of hypothermically stored red blood cells and microvesicles. Biochim Biophys Acta. 2013;1828(2):317-26.
84. Montes LR, López DJ, Sot J, Bagatolli LA, Stonehouse MJ, Vasil ML, et al. Ceramide-enriched membrane domains in red blood cells and the mechanism of sphingomyelinase-induced hot-cold hemolysis. Biochemistry. 2008;47(43):11222-30.
85. Linderkamp O, Meiselman HJ. Geometric, osmotic, and membrane mechanical properties of density-separated human red cells. Blood. 1982;59(6):1121-7.
86. Blasi B, D'Alessandro A, Ramundo N, Zolla L. Red blood cell storage and cell morphology. Transfus Med. 2012;22(2):90-6.
87. Hill HR, Oliver CK, Lippert LE, Greenwalt TJ, Hess JR. The effects of polyvinyl chloride and polyolefin bags on red blood cells stored in a new additive solution. Vox Sang. 2001;81:161-6.
88. Dumont LJ, Baker S, Dumont DF, Herschel L, Waters S, Calcagni K, et al. Exploratory in vitro study of red blood cell storage containers formulated with an alternative plasticizer. Transfusion. 2012;52(7):1439-45.
89. Bessis M. Red cell shapes. An illustrated classification and its rationale. Nouv Rev Fr Hematol. 1972;12(6):721-45.
90. Gifford SC, Derganc J, Shevkoplyas SS, Yoshida T, Bitensky MW. A detailed study of time-dependent changes in human red blood cells: from reticulocyte maturation to erythrocyte senescence. Br J Haematol. 2006;135(3):395-404.
91. Berezina TL, Zaets SB, Morgan C, Spillert CR, Kamiyama M, Spolarics Z, et al. Influence of storage on red blood cell rheological properties. J Surg Res. 2002;102(1):6-12.
92. Sens P, Gov N. Force balance and membrane shedding at the red-blood-cell surface. Phys Rev Lett. 2007;98(1):018102.
93. Greenwalt TJ, Dumaswala UJ. Effect of red cell age on vesiculation in vitro. Br J Haematol. 1988;68(4):465-7.
94. Greenwalt TJ, McGuinness CG, Dumaswala UJ. Studies in red blood cell preservation: 4. Plasma vesicle hemoglobin exceeds free hemoglobin. Vox Sang. 1991;61:14-7.
95. Dinkla S, Novotný VM, Joosten I, Bosman GJ. Storage-induced changes in erythrocyte membrane proteins promote recognition by autoantibodies. PLoS One. 2012;7(8):e42250.
96. Anniss AM, Glenister KM, Killian JJ, Sparrow RL. Proteomic analysis of supernatants of stored red blood cell products. Transfusion. 2005;45(9):1426-33.
97. Burger P, Hilarius-Stokman P, de Korte D, van den Berg TK, van Bruggen R. CD47 functions as a molecular switch for erythrocyte phagocytosis. Blood. 2012;119(23):5512-21.
98. Bosman GJ, Lasonder E, Groenen-Döpp YA, Willekens FL, Werre JM. The proteome of erythrocyte-derived microparticles from plasma: new clues for erythrocyte aging and vesiculation. J Proteomics. 2012;76:203-10.
99. Canellini G, Rubin O, Delobel J, Crettaz D, Lion N, Tissot JD. Red blood cell microparticles and blood group antigens: an analysis by flow cytometry. Blood Transfus. 2012;10(2):s39-45.
100. Kannan M, Atreya C. Differential profiling of human red blood cells during storage for 52 selected microRNAs. Transfusion. 2010;50(7):1581-8.

The impact of storage on the red cell physiology, function, and survival in vivo

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A B S T R A C T

The ultimate goal of red blood cell (RBC) transfusions in anemic patients is to provide oxygen-delivery to the microcirculation to improve or to preserve tissue oxygenation. There is little scientific evidence, however, as to whether this goal is actually achieved in patients. Experimental studies brought the efficacy of blood transfusions into question, and in particular, prolonged storage of RBCs was suggested to be associated with failure to preserve or improve tissue oxygenation. Several clinical studies have reported blood transfusion-related complications associated with the aging of blood, such as an increase in mortality, multiple organ failure, infections, and hospital length of stay. Other studies, in contrast, have not found any differences in outcome following transfusion of fresh blood or aged blood. So far, the studies on transfusion medicine have been mainly focused on storage-related biochemical, physical and hemorrheological changes, commonly referred to as storage lesions, and these are held responsible for many of the deleterious effects of RBC transfusions. Leukocytes and their by-products in stored blood may be another factor affecting *in vivo* function of transfused blood. Taken together, these so-called storage lesions may adversely affect the ability of transfused RBCs to deliver oxygen-rich blood to the microcirculation.

Learning goals

At the conclusion of this activity, participants should know that:
- RBCs undergo several biochemical, physical and hemorrheological changes during their storage;
- experimental studies provide scientific evidence for the impact of the so-called storage lesion on efficacy of blood transfusion;
- clinical studies have reported conflicting results regarding the efficacy and potential harm of transfusing aged RBCs;
- leukocytes and their by-products in stored blood may be another factor affecting *in vivo* function of non-leukodepleted transfused blood.

Introduction

The development of blood storage made a dramatic change in transfusion practice. Originally, the patient and the donor were directly connected to each other, whereas today blood can be stored in solutions for up to 35-42 days with a post-transfusion 24-h red cell survival of 75%-80%. Liquid preservation is the most common way to preserve blood for transfusion and prolonged duration of liquid preservation has been shown to affect RBC structure and its functional properties, which may interfere with its oxygen transporting capacity.[1,2] To prevent transfusion of dysfunctional RBCs and standardize transfusion practice, several criteria have been set. However, these criteria are based on the physical properties of RBC units, such as the mean hemoglobin mass per unit, 24-h survival of 75%, and 1% hemolysis, and do not reflect the clinical oxygenation efficacy of blood transfusion.[3]

Several clinical and pre-clinical studies have shown storage to have deleterious effects on RBC functions and storage.[1-4] However, the clinical importance of this so-called storage lesion is not well known. When exactly storage diminishes the red blood cell structural and functional properties, and how often more harm than good is encountered when stored RBCs are transfused in daily practice, remain uncertain. In a large study cohort, the ABC study,[1] the mean age of blood was 16±7 days, whereas in the CRIT study,[2] the mean age of blood was 21±11 days. Interestingly, the age of blood was found not to be related to any clinical outcome. In another study, Raat and colleagues analyzed the age of stored RBC concentrates in 74,084 units in an academic hospital in Netherlands between the years 1997-2001. They found that the mean storage time was 19.4±7 days with 37% of blood being older than three weeks.[5] The above data, in a total number of 90,000 RBC units, showed that most RBC units being used in critically ill patients are between 16-21 days old. Since one-third of the patients received blood transfusions older than 21 days, it can be stated that there may be a clinical problem which needs to be addressed if storage-related RBC dysfunction indeed occurs in these RBC units. In this paper, we focus on the effect of storage of RBC on oxygen supply in the microcirculation as estimated in animal models.

Biochemical and hemorrheological changes during storage

RBCs undergo several biochemical, physical and hemorheological changes during their storage.[6] These changes are commonly referred to as the storage lesion and are held responsible for alleged deleterious effects of RBC transfusion. The biochemical changes include a decrease in 2-3 DPG and adenosine triphosphate (ATP) levels,[7] a decrease in membrane sialic acid,[8] RBC membrane lipid peroxidation,[9] a loss of intrinsic RBC membrane proteins,[10] a loss of cellular antioxidant capability,[11] a decrease in pH,[12] an increase in free hemoglobin due to hemolysis,[13] and a decrease in S-nitrosohemoglobin concentrations.[14]

The initial studies on the loss of oxygen delivery ability of RBCs during storage were mostly focused on 2,3-diphosphoglycerate (DPG). 2,3-DPG is a metabolite and allosteric modifier of hemoglobin and decreases to very low levels during the first 2-3 weeks of storage. This decrease leads to an increase in hemoglobin oxygen affinity, which may explain the decrease in RBC oxygen delivery ability during storage. However, 2,3-DPG levels recover within hours after transfusion.[15] Additionally, a recent experimental study showed that, although RBCs were stored for 2-3 weeks and were completely devoid of 2,3-DPG, their oxygen delivery capacity to the intestinal microcirculation was no different to that of fresh (2-6 days) RBCs.[7] An additional biochemical change that occurs in stored RBC is the decrease in intracellular ATP levels. ATP, as well as playing a role in membrane deformability, is crucial for RBC function due to its role as a vasodilator under hypoxic conditions.[16,17] Raat *et al*. showed that ATP levels remained unchanged in RBCs stored for 2-3 weeks, but dropped to 60% in RBCs stored for 5-6 weeks.[7] The loss of ATP was inversely associated with oxygen delivery ability of the RBCs; old (5-6 weeks) RBCs had a reduced oxygen delivery capacity compared to fresh (2-6 days) and intermediate (2-3 weeks) groups. This finding supports the idea that ATP, suggested to be a vasodilator released by RBC in the presence of hypoxia, is related to the oxygen delivery capacity of RBCs, and may be negatively affected by storage duration.[5] Another possible mechanism that may account for alterations in the oxygen transport capabilities of transfused RBCs is nitric oxide. Nitric oxide and its products, besides their many other roles inside the organisms, can be regarded as one of the major compounds accounting for vasodilatory regulation of blood vessels. Recent studies have shown that RBCs are able to release nitric oxide in the presence of hypoxia and that this nitrite-mediated function accounts for hypoxia-induced vasodilation.[18,19] The further identification of functional eNOS on RBC membranes has made the red cell a central player, not only in oxygen transport, but also in vascular control mechanisms.[20] It could well be that this NO mediated function of RBCs may be affected during storage.[14]

Furthermore, in a very elegant study by Donadee *et al*., it was found that storage of human RBCs resulted in the accumulation of cell-free and microparticle-encapsulated hemoglobin which scavenges the vasodilator nitric oxide approximately a thousand times faster than intact erythrocytes.[13] The authors showed that cell-free and microparticle-encapsulated hemoglobin is a highly potent vasoconstrictor *in vivo*, and that even the infusion of the plasma from stored RBC units produced significant vasoconstriction in the rat due to storage-related hemolysis.

Hemorrheological alterations such as RBC shape changes, decreased membrane deformability and increased aggregability are a number of effects which can occur during storage which may possibly disturb RBC flow through the microcirculation and influence its functional activity of transporting oxygen to the tissue cells. The loss of phospholipids from RBCs is seen both in storage and physiological red cell aging and may contribute to the formation of echinocytes with protrusions and spheroechinocytes during storage.[21,22] These changes can occur parallel to decreases in surface-volume ratio, increased mean cell hemoglobin concentration, and osmotic fragility and decreased deformability. The storage-related decrease in RBC membrane deformability has been suggested to be associated with reduced ATP level.[23]

Other mechanisms such as membrane phospholipids loss or redistribution, protein and lipid oxidations have been suggested to contribute to the storage-dependant alterations of RBC membranes. The formation of microvesicals, causing the loss of membrane phospholipids, was identified by Rumsby *et al*.[24] An alternative mechanism that was proposed was the internalization of phosphotidylserine (PS) and phosphoethanolamine (PE) from the membrane into the cytosol,[25] and loss of asymmetry in the red cell membrane. These biomechanical alterations may account for less deformable RBCs, and may cause more problems for a microcirculation already under stress. However, biomechanical alterations are probably not the only problem occurring during storage. This suggestion is supported by a study by Verhoeven *et al.,* in which they compared two different methods to change the RBC asymmetry. They used flippase, which moves the PS from the outer to inner leaflet of membrane, compared to phospholipid scrambling which will move PS from the inner leaflet to the outer leaflet. They showed a decrease in flippase activity starting after 21 days of storage in SAGM and further decreased over time. The authors also showed that the correction of storage-induced metabolic changes, restores flippase activity.[26]

RBCs and the microcirculation

RBCs are primarily responsible for the oxygen and carbon dioxide exchange and transport from the lungs to the tissues. This exchange is facilitated through synergistic effects of hemoglobin, carbonic anhydrase, and band 3 protein, and followed by carbon dioxide delivery to the lungs for release. Within the organs, in order to deliver oxygen to the tissues, RBCs need to travel through a fine network of vessels with diameters smaller than 100 micrometer: the so-called microcirculation. Normally, erythrocytes have a flexible membrane and can reversibly alter their biconcave, discoid shape, which allows them to pass through capillaries smaller in diameter (2-6 micrometer) than an RBC (8 micrometer). It is obvious that RBC membrane properties are of great importance for entering the capillaries and thereby oxygen delivery to the tissues.

Under normal physiological conditions, this finely regulated system of capillaries, arterioles and venules is able to supply oxygen in excess of oxygen demand, so that the tissue cells can continue their function under changing metabolic demands. The microcirculation has an oxygen-dependent regulation system that is connected to the systemic circulation, but also able to regulate and direct blood flow to the tissues where it is needed. The flow of blood in the microcirculation, even under normal conditions, has a heterogeneous

nature that actually ensures the even distribution of oxygen in the tissues so that each cell receives the oxygen it needs. Therefore, hypoxia-detecting mechanisms are required in the tissues to produce vasoactive compounds affecting blood flow and thereby oxygen transport. Besides endothelial factors, the RBC themselves play a central role in this process.

Experimental studies

The main goal of RBC transfusion is to improve tissue oxygenation. Over the last two decades, technological advances made it possible to investigate tissue oxygenation at a microvascular level, where actual oxygen transport between RBCs and cells take place. However, these techniques were relatively invasive and were not appropriate for clinical use in human studies or for monitoring patients. This was one of the main limitations of clinical research in the field of transfusion medicine. This led to indirect measures to monitor efficacy of RBC transfusion, such as epidemiological end points, intensive care unit (ICU) and hospital mortality, and morbidity.

Given this, Van Bommel *et al.* showed that transfusion of rat blood stored for four weeks was not as effective in improving intestinal microcirculatory oxygenation following hemorrhage as compared to fresh rat blood by measuring intestinal microvascular PO_2 with O_2-dependent quenching of palladium porphyrin phosphorescence technique.[23] Furthermore, the authors showed that the type of preservation solution used to store the RBCs can significantly affect the RBC rheological properties, and consequently the efficacy of RBC transfusion with respect to improving microvascular oxygenation. A limitation of this model is described by d'Almeida *et al.*[27] and Raat *et al.;*[7] that rat RBCs age faster that human red blood cells and can not regenerate 2,3-DPG, unlike human red blood cells. Raat *et al*. developed a model to overcome this limitation by transfusing fresh (2-6 days), intermediate (2-3 weeks) and old (5-6 weeks) stored human red blood cells to improve gut microcirculatory oxygenation in anaemic oxygen-supply-dependent rats. They have shown that oxygen delivery capacity was diminished in the old (5-6 weeks) group compared to the fresh and intermediate groups by using O_2-dependent quenching of palladium porphyrin phosphorescence technique.

Visualization of microvasculature is another technique that was used by different groups. In a hamster window chamber model, Tsai *et al.* showed in 2004 that transfusion of stored RBCs resulted in significantly malperfused and underoxygenated skin microvasculature in severely hemodiluted hamsters by measuring functional capillary density (FCD), blood flow and high-resolution oxygen distribution.[28] Interestingly, these impairments of microvascular perfusion and oxygenation were not detectable at the systemic level, highlighting the importance of studying the effects of RBC transfusions at the microcirculatory level. It must be noted, however, that the blood was hamster blood stored for 28 days, which may correspond to much older human blood than is conventionally used (for example, see d'Almeida *et al*.[27]) and, in addition, the blood was not leuko-depleted so the results of this study should be evaluated with care.

These findings were confirmed by other groups. Gonzales *et al.* in 2007[29] studied intravital microcirculatory hemodynamics in the rat cremaster muscle flap. However, in contrast to other groups, they compared 2-week stored RBCs with fresh RBCs. The authors suggested that fresh blood transfusion is more effective in relieving effects of microcirculatory hypoxia compared to 2-week stored blood. Arslan *et al*.[30] provided similar results suggesting that stored blood may have a deleterious effect on the microcirculation.

Most recently, Hu *et al.*[31] studied preventive effects of RBC transfusion in a myocardial infarct model by coronary artery ligation in anemic rats. Twenty-four hours after myocardial infarction, cardiac function, infarct size, and apoptosis were determined. The authors suggested that fresh blood transfusion reduces infarct size and myocardial apoptosis, and leads to improvements in cardiac function and short-term survival in this animal model. In contrast, transfusion of blood with prolonged storage negatively affected these beneficial effects.

With the aim of studying the effects of storage duration on the efficacy of RBC transfusions to reach the microcirculation, our group performed a prospective randomized clinical pilot study. Twenty anemic hematologic outpatients were randomized into 10 patients receiving transfusion of leuko-depleted RBCs in Saline-adenine-glucose-mannitol (SAGM) stored for less than one week, and 10 patients receiving transfusion of leuko-depleted RBCs in SAGM stored for 3-4 weeks. We were able to show a parallel increase in both systemic Hb and Ht values and microvascular perfused vessel density and oxygen saturation, and found no differences between the two groups.[32]

In conclusion, the pre-clinical studies demonstrated the harmful effects of prolonged storage on RBC functions. Most of these studies suggest that prolonged storage over four weeks is associated with impaired oxygen carrying capacity of RBCs. Whether this impairment is clinically relevant for all patients remains uncertain. The results from clinical studies are confusing, and the answer to the question of how important these storage-induced alterations are *in vivo*, and especially under clinical conditions, remains uncertain.

Leukocytes

Leukocytes and their by-products in stored blood may be another confounding factor affecting *in vivo* efficacy of transfused blood. It is generally assumed that the cytokines, enzymes, and inflammatory mediators derived from leukocytes during blood storage may worsen the RBC storage lesion and cause transfusion-related immunomodulation in host. Leukoreduction is performed to reduce some of the negative immunosuppressive effects of blood transfusions and to mitigate the RBC storage lesion.[33,34] However, there are conflicting data in the literature regarding the clinical impact of transfusion of non-leukoreduced blood. Anniss *et al.* examined endothelial RBC adherence and compared non-leukodepleted blood, buffy-coat-poor blood, and leukodepleted blood, and demonstrated that leukodepleted blood showed significantly lower adhesiveness to vascular endothelial cells, an effect which would be beneficial for the microvascular perfusion of transfused blood.[35] Consistent with the idea that leukoreduction provides a better quality of stored blood, Van de Watering *et al.* reported increased survival rates in cardiac surgery patients receiving leuko-reduced RBC units compared to patients transfused with buffy-coat-removed packed RBCs units.[36] In addition, Netzer *et al.* reported an association between leuko-reduced RBC transfusion and decreased mortality rates in patients

with acute lung injury.[37] In contrast, several other randomized prospective studies comparing patients with various clinical conditions receiving either leuko-reduced or non-leuko-reduced RBC units showed no beneficial effect of leukoreduction on clinical outcome, including mortality, length of ICU stay, and readmission rate.[38,39]

Conclusions

RBCs undergo several biochemical, physical and hemorrheological changes during their storage that may affect their ability to improve tissue oxygenation in anemic patients. Leukocytes and their by-products in stored blood may be another factor affecting in vivo function of transfused RBCs. Experimental studies demonstrate failure to preserve or correct tissue oxygenation after transfusion of aged RBCs. However, using mostly epidemiological endpoints clinical studies have not confirmed this conclusion. The development of less invasive techniques to monitor the efficacy of blood transfusion in clinical setting seems essential to confirm these results in clinical studies.

References

1. Vincent JL, Baron JF, Reinhart K, Gattinoni L, Thijs L, Webb A, et al; ABC (Anemia and Blood Transfusion in Critical Care) Investigators. Anemia and blood transfusion in critically ill patients. JAMA. 2002;288(12):1499-507.
2. Cooper HA, Rao SV, Greenberg MD, Rumsey MP, McKenzie M, Alcorn KW, et al. Conservative versus liberal red cell transfusion in acute myocardial infarction (the CRIT Randomized Pilot Study). Am J Cardiol. 2011;108(8):1108-11.
3. Scott KL, Lecak J, Acker JP. Biopreservation of red blood cells: past, present, and future. Transfus Med Rev. 2005;19 (2):127-42.
4. Hébert PC, Wells G, Tweeddale M, et al. Does transfusion practice affect mortality in critically ill patients? Transfusion Requirements in Critical Care (TRICC) Investigators and the Canadian Critical Care Trials Group. Am J Respir Crit Care Med. 1997;155:1618-23.
5. Raat NJ, Berends F, Verhoeven AJ, de Korte D, Ince C. The age of stored red blood cell concentrates at the time of transfusion. Transfus Med. 2005;15(5):419-23.
6. Hess JR. Red cell changes during storage. Transfus Apher Sci. 2010;43:51-9.
7. Raat NJ, Verhoeven AJ, Mik EG, et al. The effect of storage time of human red cells on intestinal microcirculatory oxygenation in a rat isovolemic exchange model. Crit Care Med. 2005;33:39-45.
8. Bratosin D, Leszczynski S, Sartiaux C, et al. Improved storage of erythrocytes by prior leukodepletion: flow cytometric evaluation of stored erythrocytes. Cytometry. 2001;46:351-6.
9. Knight JA, Voorhees RP, Martin L, Anstall H. Lipid peroxidation in stored red cells. Transfusion. 1992;32:354-7.
10. Messana I, Ferroni L, Misiti F, et al. Blood bank conditions and RBCs: the progressive loss of metabolic modulation. Transfusion. 2000;40:353-60.
11. Racek J, Herynková R, Holecek V, Jerábek Z, Sláma V. Influence of antioxidants on the quality of stored blood. Vox Sang. 1997;72:16-9.
12. Högman CF. Preparation and preservation of red cells. Vox Sang. 1998;74(2 Suppl):177-87.
13. Donadee C, Raat NJ, Kanias T, et al. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. Circulation. 2011;124:465-76.
14. Reynolds JD, Ahearn GS, Angelo M, Zhang J, Cobb F, Stamler JS. S-nitrosohemoglobin deficiency: a mechanism for loss of physiological activity in banked blood. Proc Natl Acad Sci USA. 2007;104:17058-62.
15. Heaton A, Keegan T, Holme S. In vivo regeneration of red cell 2,3-diphosphoglycerate following transfusion of DPG-depleted AS-1, AS-3 and CPDA-1 red cells. Br J Haematol. 1989;71 (1):131-6.
16. Ellsworth ML. The red blood cell as an oxygen sensor: what is the evidence? Acta Physiol Scand. 2000;168(4):551-9.
17. Dietrich HH, Ellsworth ML, Sprague RS, Dacey RG Jr. Red blood cell regulation of microvascular tone through adenosine triphosphate. Am J Physiol Heart Circ Physiol. 2000;278 (4):H1294-8.
18. Crawford JH, Isbell TS, Huang Z, et al. Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. Blood. 2006;107(2):566-74.
19. Cosby K, Partovi KS, Crawford JH, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. Nat Med. 2003;9(12):1498-505.
20. Kleinbongard P, Schulz R, Rassaf T, et al. Red blood cells express a functional endothelial nitric oxide synthase. Blood. 2006;107(7):2943-51.
21. Card RT. Red cell membrane changes during storage. Transfus Med Rev. 1988;2:40-7,
22. Hess JR, Greenwalt TJ. Storage of red blood cells: New approaches. Transfus Med Rev. 2002;16:283-95.
23. van Bommel J, de Korte D, Lind A, et al. The effect of the transfusion of stored RBCs on intestinal microvascular oxygenation in the rat. Transfusion. 2001;41:1515-23.
24. Rumsby MG, Trotter J, Allan D, Michell RH. Recovery of membrane micro-vesicles from human erythrocytes stored for transfusion: a mechanism for the erythrocyte discocyte-to-spherocyte shape transformation. Biochem Soc Trans. 1977;5(1):126-8.
25. Brunauer LS, Moxness MS, Huestis WH. Hydrogen peroxide oxidation induces the transfer of phospholipids from the membrane into the cytosol of human erythrocytes. Biochemistry. 1994;33(15):4527-32.
26. Verhoeven AJ, Hilarius PM, Dekkers DW, Lagerberg JW, de Korte D. Prolonged storage of red blood cells affects aminophospholipid translocase activity. Vox Sang. 2006;91 (3):244-51.
27. d'Almeida MS, Jagger J, Duggan M, White M, Ellis C, Chin-Yee IH. A comparison of biochemical and functional alterations of rat and human erythrocytes stored in CPDA-1 for 29 days: implications for animal models of transfusion. Transfus Med. 2000;10:291-303.
28. Tsai AG, Cabrales P, Intaglietta M. Microvascular perfusion upon exchange transfusion with stored red blood cells in normovolemic anemic conditions. Transfusion. 2004;44:1626-34.
29. Gonzalez AM, Yazici I, Kusza K, Siemionow M. Effects of fresh versus banked blood transfusions on microcirculatory hemodynamics and tissue oxygenation in the rat cremaster model. Surgery. 2007;141:630-9.
30. Arslan E, Sierko E, Waters JH, Siemionow M. Microcirculatory hemodynamics after acute blood loss followed by fresh and banked blood transfusion. Am J Surg. 2005;190:456-62.
31. Hu H, Xenocostas A, Chin-Yee I, Lu X, Feng Q. Effects of anemia and blood transfusion in acute myocardial infarction in rats. Transfusion. 2010;50:243-51.
32. Yürük K, Milstein DM, Bezemer R, Bartels SA, Biemond BJ, Ince C. Transfusion of banked red blood cells and the effects on hemorrheology and microvascular hemodynamics in anemic hematology outpatients. Transfusion. 2012. In press.
33. Hébert PC, Fergusson D, Blajchman MA, et al. Clinical outcomes following institution of the Canadian universal leukoreduction program for red blood cell transfusions. JAMA. 2003;289:1941-9.
34. Shapiro MJ. To filter blood or universal leukoreduction: what is the answer? Crit Care. 2004;8(2 Suppl):S27-S30.
35. Anniss AM, Sparrow RL. Storage duration and white blood cell content of red blood cell (RBC) products increases adhesion of stored RBCs to endothelium under flow conditions. Transfusion. 2006;46:1561-7.
36. van de Watering LM, Hermans J, Houbiers JG, et al. Beneficial effects of leukocyte depletion of transfused blood on postoperative complications in patients undergoing cardiac surgery: a randomized clinical trial. Circulation. 1998;97:562-8.
37. Netzer G, Shah CV, Iwashyna TJ, et al. Association of RBC transfusion with mortality in patients with acute lung injury. Chest. 2007;132:1116-23.
38. Dzik WH, Anderson JK, O'Neill EM, Assmann SF, Kalish LA, Stowell CP. A prospective, randomized clinical trial of universal WBC reduction. Transfusion. 2002;42:1114-22.
39. Nathens AB, Nester TA, Rubenfeld GD, Nirula R, Gernsheimer TB. The effects of leukoreduced blood transfusion on infection risk following injury: a randomized controlled trial. Shock. 2006;26:342-7.

Do clinical studies support a deleterious role of stored red blood cell transfusions?

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A B S T R A C T

The red blood cell (RBC) storage interval has been extended progressively from less than a week when blood was collected into a citrate/glucose solution to the currently approved storage interval of 42 days. The criteria for extending storage rely upon a set of *in vitro* studies, and specify a minimal degree of hemolysis, and *in vivo* 24-h posttransfusion recovery of over 75% of cells labeled with radiochromium at the end of shelf life. Concerns have arisen that RBC at the end of storage ('old blood') not only lose efficacy, but may develop a 'storage lesion' that results in previously unrecognized toxicity. Several mechanisms for such toxicity have been proposed. Data supporting and refuting the supposed toxicity of old blood are derived from four sources: 1) retrospective analyses; 2) volunteer studies; 3) animal models; 4) controlled clinical trials. Current evidence suggests that old blood may have adverse effects, but that the patient's clinical status as well as the age, volume and method of preparation of the RBC, may be critical variables. Several large randomized controlled clinical trials are currently in progress to answer some of these questions; however, design limitations may compromise interpretation of their results.

Learning goals

At the conclusion of this activity, the participant should understand that:
- describe the kinds of studies that support or refute the supposed toxicity of transfused stored red blood cells (RBC);
- give possible explanations why some retrospective analyses suggest that old RBC are toxic and others do not;
- describe several animal models that have been used to determine the effects of RBC transfusion;
- list and discuss several prospective transfusion trials that are in progress or have been completed

Introduction

A few things improve with age: vintage wine, firewood, and one's memory of past triumphs. Blood stored at refrigerated temperatures is not among them. Blood preservative solutions were developed to eliminate reliance on vein-to-vein transfusion and to improve blood supply and logistics.[1] With the development of new anticoagulant-preservative solutions, the red blood cell (RBC) storage interval has been extended progressively from less than a week when blood was collected into a citrate-glucose solution to the currently approved storage interval of 42 days.[2] The criteria for extending shelf life as new storage systems were developed relied upon a panel of *in vitro* studies, although without precise acceptance criteria, and specified a minimal degree of hemolysis (1% in the US and 0.8% in Europe). The gold standard remains *in vivo* 24-h posttransfusion recovery of over 75% of cells labeled with radiochromium at the component outdate.[3,4] Clinical trials of safety and efficacy have never been required (or performed) for licensure.

Stored blood does not age gracefully. Changes in percent hemolysis, osmotic fragility, hematologic indexes, and gross morphology were among the earliest observations that came to be known as the 'storage lesion'. Detailed assessments of RBC metabolism and quality, including changes in cellular biochemistry, lipid concentration, membrane loss, carbohydrate alterations, oxidative injury to lipids and proteins, oxygen affinity and delivery, adhesion of RBCs to endothelial cells, as well as the secondary risks of accumulating concentrations of potassium and plasticizer, and shedding of active proteins, lipids and microvesicles have been reviewed elsewhere.[5] The principal concern is whether these changes result in clinical sequellae. Whereas no one expected that RBC would perform at the end of several weeks of refrigerated storage exactly as they do when they exit the donor's vein, neither did clinicians anticipate that stored blood would harm their patients. This conviction has recently been challenged.

Many clinicians have harbored a belief that 'fresh' blood is superior to stored RBC and that fresh blood will benefit their patients who require transfusion. However no definition of 'fresh' is generally accepted (a vocal minority request that it be warm to the touch) and, until recently, evidence for a clinical benefit for fresher RBC has been surprisingly difficult to

establish. At present, most adult patients requiring transfusion receive blood of their specific type with the oldest compatible unit available given first. This first-in, first-out principle was designed to manage blood inventories. During the last 15 years, an ever increasing number of published retrospective and prospective studies have raised concern that patients receiving older blood have an increased morbidity and mortality risk compared to patients receiving newer stored units.[6-12] A smaller number of reports have failed to confirm such an association.[13-16] If older RBC do pose a hazard, are there particularly vulnerable patient groups or clinical situations that pose a special risk?

Clinical studies comparing fresh and old stored RBC fall into four general categories. The largest number of publications involves observational studies of different patient populations that demonstrate a statistical association between prolonged storage of allogeneic blood and disease state. All of these studies have been criticized for limitations in size, design, or methodology. Some studies examine mortality, others morbidity, while still others have reported on such surrogate measures as length of hospital or ICU stay, recurrence of cancer, changes in gastric intramucosal pH, serum lactate levels or decreased oxygen delivery to different organs.[17-21] Three studies involve autologous transfusion of healthy normal volunteers with their own fresh or stored RBC. Studies in animal models provide an opportunity to design trials that cannot be performed in patients or in normal volunteers. Finally, several large prospective randomized controlled trials have either been completed recently or are in progress. Examples of each of these studies will be reviewed.

The most widely publicized observational analysis was that of Koch *et al.* published in 2008.[22] This study compared clinical and transfusion data collected from cardiac surgery patients at the Cleveland Clinic in the United States from 1998 to 2006. The authors analyzed 2872 patients who received 8802 units of RBCs that were stored for less than 14 days (fresher blood) and compared them with 3130 patients transfused with 10,782 RBCs stored for more than14 days (older blood). In-hospital mortality, need for prolonged ventilator support, development of renal failure and multi-organ dysfunction were all statistically more frequent among the patients who received blood stored for more than 14 days. Using a Kaplan-Meier survival statistic, the authors concluded that among patients undergoing cardiac surgery, transfusion of RBCs stored for more than 14 days was associated with more complications and with reduced short-term and long-term survival. The study has been justly criticized for controversial statistical treatment, numerous clinical differences (heterogeneity) between the two patient groups, an almost inevitable consequence of retrospective analyses, and the 'unadjusted comparison' in the Kaplan-Meyer curve.[23]

Following Koch's publication, we undertook our own meta-analysis of published studies.[24] We concluded that previous meta-analyses had incurable flaws, so we decided to use a methodology that allows the end points (primarily mortality and morbidity) to be comparable across the studies selected. We identified 93 unique article citations published from 2001 to 2010, including 13 observational studies (3 prospective and 10 retrospective) and 3 randomized controlled trials. Trials were included if they compared survival rates after being transfused with blood that was stored over different durations in days, one having longer storage times ('old blood') and the other having a shorter storage time ('new blood'). We excluded studies that did not have mortality data, or that did not refer to the age of the stored blood. A total of 21 trials met inclusion criteria (Figure 1).

There were a total of 374 deaths among 5185 patients transfused with new blood (7.2%) and a total of 523 deaths among 5853 patients transfused with old blood (8.9%) from ten studies that provided such data. There was no significant heterogeneity among these 21 studies for the mortality end point. The overall estimate of mortality with transfusion of old blood compared to new stored blood was highly significant (Figure 2). We also found a highly significant increase in adverse events associated with old stored blood. In our meta-analysis, approximately one-third of the studies were in trauma patients, one-third in cardiac surgery patients, and one-third in a mix of varied populations. The results were similar comparing these three subgroups and very consistent with the overall increase in mortality found with old blood *versus* new blood. In studies having 500 or under or over 500 patients, as well as in studies of patients receiving on average 3 units or under *versus* more than 3 units per patient, the results in all these subgroups were likewise similar to the overall findings of our study showing old blood *versus* new blood increases mortality. Although the published

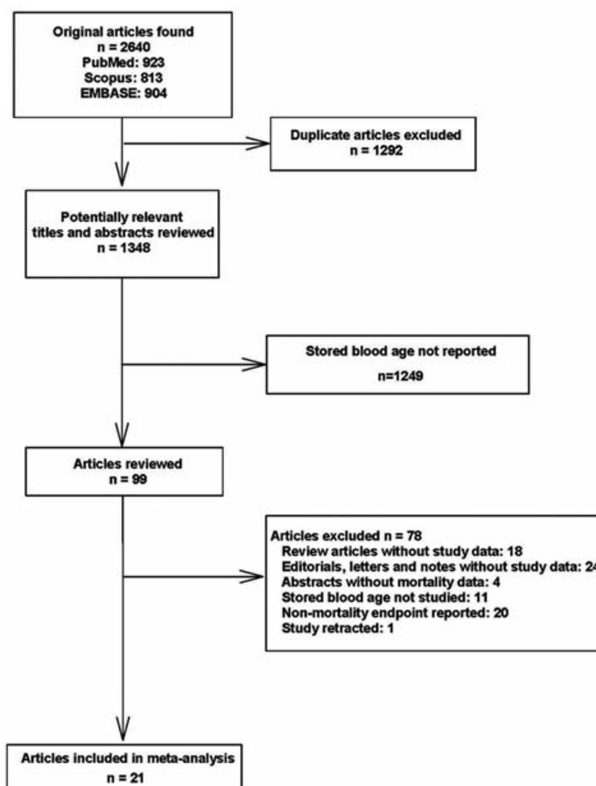

Figure 1. Flow diagram of the published studies evaluated for inclusion in the meta-analysis.

clinical experience to date suggests that newer blood, if used exclusively, might save lives, most of the data we analyzed derive from observational studies and small prospective studies, so conclusions must be interpreted cautiously. Observational studies serve the purpose of posing questions. Even a cursory reading of the several referenced studies that involve numerous confounding variables (disease state, patient demographics, nature and number of the blood components, concurrent treatments, and definition of old blood and of adverse events) suggests that additional observational studies will not answer this question regardless of the statistical legerdemain that is used.

Normal volunteers

Hod *et al.* studied 14 healthy human volunteers who donated standard leuko-reduced, double RBC units and received one unit transfused 'fresh' (3-7 days of storage), and the other 'older' unit transfused after 40-42 days of storage.[25] Significant differences between fresh and older transfusions were observed in iron parameters and markers of extravascular hemolysis. Volunteers tolerated all transfusions without incident or evidence of adverse reactions. Compared with fresh RBCs, mean serum total bilirubin increased at 4 h after transfusion of older RBCs. In addition, after the older transfusion, transferrin saturation increased progressively over 4 h to a mean of 64%, and non-transferrin-bound iron appeared, reaching a mean of 3.2 μM. The increased concentrations of non-transferrin bound iron correlated with enhanced proliferation *in vitro* of a pathogenic strain of Escherichia coli. The authors concluded that circulating non-transferrin-bound iron derived from rapid clearance of transfused, older stored RBCs may enhance transfusion-related complications, such as infection in hospitalized patients.

In a series of imaginative experiments, Weiskopf *et al.* showed that acute, severe isovolemic anemia degrades neurocognitive function in young healthy volunteers, and that this deficit can be reversed by transfusion of autologous RBCs stored in citrate-phosphate-dextrose-adenine for fewer than 4 h.[26] Using the same model in which cerebral function is oxygen-delivery dependent, these investigators further demonstrated in 9 young (21-25 years) volunteers that a sensitive, reproducible test of neurocognitive function, the digit-symbol substitution test, and a secondary physiological end point (heart rate) were both completely and equivalently reversed by RBCs stored for either 3.5 h ('fresh') or 23 days ('old').[15] These results suggest that RBCs that are fresh and those that are old deliver oxygen equivalently and normally. This was the first prospective randomized trial in humans investigating the hypothesis that erythrocytes stored for at least three weeks, with a markedly increased hemoglobin affinity for oxygen (decreased P50), are as efficacious as are erythrocytes with hemoglobin with a normal affinity for oxygen. There are several limitations to this study: the subjects are healthy young volunteers so that these results cannot be generalized to critically ill patients; observations using neurocognitive function cannot necessarily be translated to compromised organ systems; storage for 23 days in

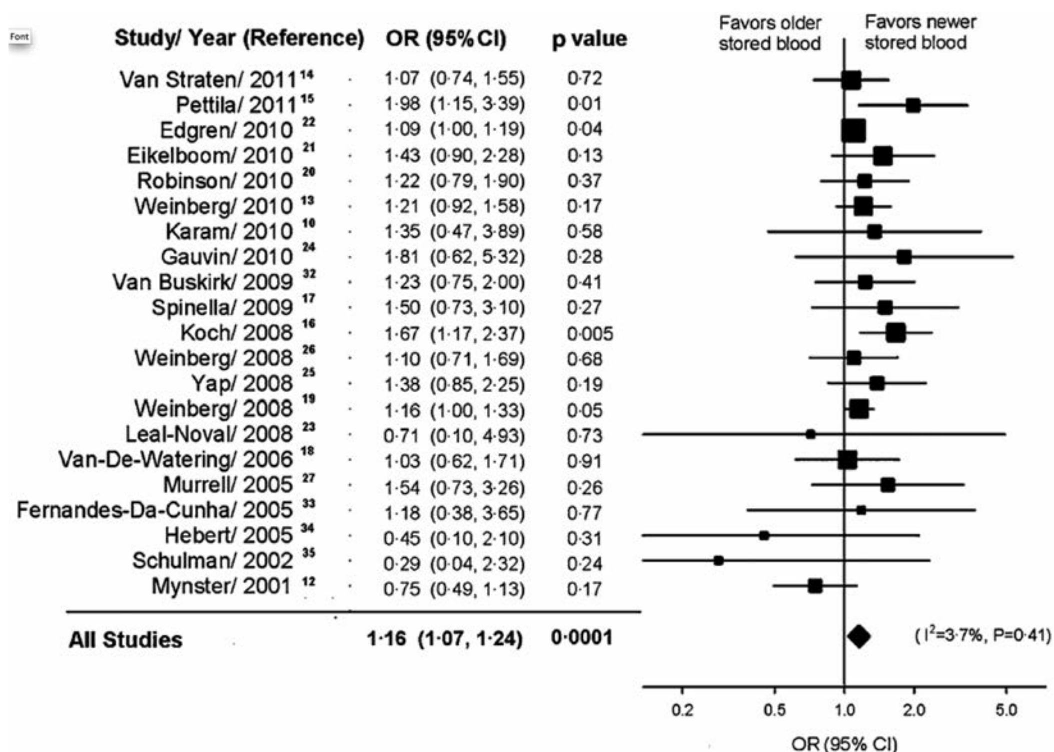

Figure 2. Mortality end point in 21 trials. The size of the data markers is proportional to the inverse variance of each point estimate. References refer to the original publication of Wang *et al.*[24]

CPDA-1 may not be equivalent to longer storage in other anticoagulant preservative solutions; the number of study subjects was small, although the power calculations were performed *a priori*.

Using a similar design but without severe anemia and with pulmonary gas exchange deficit as an end point, Weiskopf *et al.* studied 35 healthy, normal volunteers who donated one unit of blood four weeks and another three weeks prior to two study days separated by one week.[27] On study days, two units of blood were withdrawn while maintaining isovolemia, and the volunteers were transfused with either autologous fresh RBCs or autologous stored RBCs in random order. The following week, each volunteer was crossed over and studied again. RBC transfusion was found to cause subtle pulmonary dysfunction, as evidenced by impaired gas exchange; however, there was no evidence that RBCs stored for more than 21 days caused more harm than the fresh RBCs. Some of the same limitations apply to interpretation of these studies regarding their clinical meaning as to the earlier studies of cognitive function.

Animal models

Clinical studies comparing 1-day old blood with blood at the end of its storage life are operationally difficult and ethically challenging. RBCs must be processed, tested and shipped prior to issue for transfusion and this extends the interval of storage. Relatively few 5-7 day old units are available. Most blood in developed countries is used within three weeks of collection; the mean age is approximately two weeks, especially for group O RBC. Few such units are available at the end of the approved shelf life. Blood stored for 42 days deteriorates by most measures of red blood cell function, and because most of the older studies have associated old blood with increased morbidity and mortality; it is hard to imagine that an informed patient would consent to receive only blood at the very end of its shelf life. Therefore, ongoing human clinical trials have been designed to compare newer blood with current transfusion practice or with older RBCs of mixed age available in the hospital inventory. It might be better to use an appropriate animal model to assess the clinical differences, if any, between the very youngest and oldest units. If older blood is found to increase risks, animal studies can help define dose response, critical storage interval, and high-risk clinical circumstances.

Rodent models have the advantage of cost and scale for transfusion studies, although the translation of results to human clinical transfusion situations remains a concern. Hod *et al.* used a murine RBC storage and transfusion model to show that transfusion of stored RBCs, or washed stored RBCs, increases plasma nontransferrin bound iron (NTBI), produces acute tissue iron deposition, and initiates inflammation.[28] In contrast, the transfusion of fresh RBCs, or the infusion of stored RBC-derived supernatant, ghosts, or stroma-free lysate, does not produce these effects. In these studies, the insult induced by transfusion of stored RBC enhanced subclinical endotoxinemia and produced clinical effects in the animals. The increased plasma NTBI also enhanced bacterial growth in an *in vitro* culture system. These results suggest that, in a mouse model, the cellular component of leuko-reduced, stored RBC may contribute to the harmful events that occur after transfusion of RBC with prolonged storage.

Baek *et al.* have used a guinea pig model of transfusion to show that older but not newer stored blood led to hemolysis, vasoconstriction, vascular injury, and kidney dysfunction.[29] Those effects were attenuated by complexing cell-free hemoglobin (CFH) with haptoglobin, thus isolating these injuries to release of CFH.

We have used a canine model of 2-old purpose-bred beagles infected with a validated dose of *Staphyllococcus aureus* to produce pneumonia in one lung.[30] Dogs were randomized in a blinded fashion for exchange transfusion with either 7- or 42-day old canine universal donor blood (80 mL/kg) in four divided doses. Canines were chosen as a model since blood banking procedures for this species are similar to those for humans, making such a study both feasible and clinically relevant. Canine hemoglobin is functionally and immunologically indistinguishable from human hemoglobin. Given the widespread use of RBC transfusion over decades, and the relatively sparse evidence of toxicity, we chose to study critically ill animals near death and compare transfusion of large volumes of the freshest practical blood with blood at the very end of its shelf-life (42 days) to maximize the chances of finding a clinical effect if one existed.

After transfusion, the concentration of plasma CFH increased progressively for days in the animals receiving older blood; CFH release from ongoing intravascular hemolysis of older blood was observed along with significantly decreased haptoglobin levels. Transfusion of older blood resulted in a highly significant increase in mortality, arterial alveolar oxygen gradient (24-48 h post-infection, systemic and pulmonary pressures during transfusion (4-16 h) and pulmonary pressures for 8 h afterward (Figure 3). Furthermore, older blood caused more severe lung damage as evidenced by increased necrosis, hemorrhage, and thrombosis at the infection site at post-mortem exam-

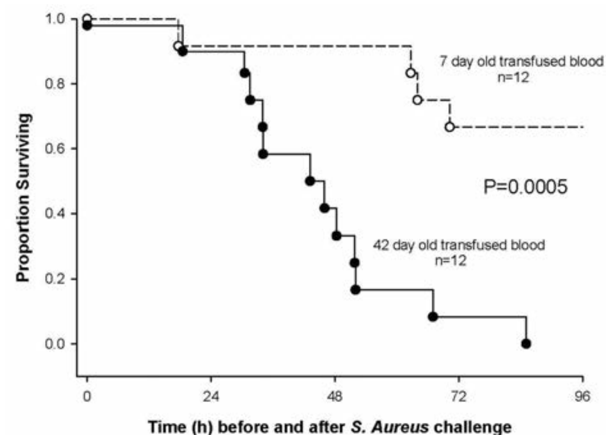

Figure 3. Survival curves. (A) Kaplan-Meier plot over the 96-h study comparing animals challenged with intrapulmonary S. aureus and exchange transfused with 42-day old (solid circle, solid line) or 7-day old (open circle, dashed line) stored blood.

ination. Plasma CFH and nitric oxide (NO) consumption capability were elevated, and haptoglobin levels were decreased with older blood during and for 32 h post-transfusion. The low haptoglobin and high NO consumption levels at 24 h were associated with poor survival. Plasma non-transferrin bound and labile iron (the toxic moiety) were significantly elevated only during transfusion, but not associated with survival. We interpret these findings to indicate that older blood after transfusion has a propensity to hemolyze *in vivo*, releases iron intravascularly early after transfusion, and CFH and possibly iron moieties over several days, worsens pulmonary hypertension, gas exchange and ischemic vascular damage in the infected lung, and thereby increases the risk of death. It is not clear from our experiment whether the 'first pass' of hemoglobin through the lung makes this organ particularly sensitive, or whether pre-existing infection, pulmonary damage, or staphylococcal sepsis are necessary for all of the effects we reported.

Randomized controlled trials

Evidence that blood transfusions are effective therapy remains largely empiric. Large-scale clinical trials of RBC safety and efficacy have never been required prior to extending the RBC storage interval, nor would such studies likely be useful or practical. However, once observational studies suggest that stored blood is harmful, carefully controlled prospective trials to confirm or refute the results become imperative. Several large randomized controlled clinical trials are studying different patient populations in different parts of the world; one such trial has been completed. The Age of Blood Evaluation (ABLE) study, supported by the Canadian Institutes of Health Research is randomizing about 2500 intensive care unit patients to receive either less than 8-day old RBC or standard-issue RBC (2-42 days) should they require transfusion.[31] More than 1500 patients have already been entered. The Red Cell Storage Duration and Outcomes in Cardiac Surgery is randomizing 2800 cardiac surgery patients who are 18 years or older to receive RBC that are either less than 14 or more than 20 days old.[32] The National Heart, Lung and Blood Institute's Red Cell Storage Duration Study (RECESS) plans to randomize approximately 1800 cardiac surgery patients to receive, if transfused, RBCs that have been stored for ten days or less or units that have been stored for 21 days or more; the composite end point includes measures of multiorgan dysfunction seven days after surgery.[33] The INFORM multicenter trial in Canada, a comparative effectiveness study, will randomize all patients requiring transfusion to receive either the freshest possible RBCs or the oldest in inventory with an in-hospital mortality end point; 1320 patients are planned to be registered. A pilot study has been successfully completed and published.[34] The Australian/New Zealand TRANSFUSE study has a similarly pragmatic approach. ICU patients are randomly assigned to receive blood at the 'front of the refrigerator' (older) or blood at the 'back of the refrigerator' (fresher), reflecting the real-world age of stored blood and standard transfusion practice. The end point is 90-day mortality.[35] Finally, a single institution, randomized, controlled trial of cardiac surgery patients at the Cleveland Clinic (Red Cell Storage Duration and Outcomes in Cardiac Surgery) plans to enroll 1800 heart surgery patients.[36] This is the follow-on study based on the findings of the retrospective analysis by Koch *et al.* that was discussed above.

In the recently-published double-blind, randomized controlled study of premature infants analogous to the ABLE trial in adults (Age of Red Blood Cells in Premature Infants, ARIPI), 377 premature infants with birth weight less than 1250 g admitted to six Canadian tertiary neonatal intensive care units received aliquots of RBC either less than 8-day or standard-issue RBC (2-42 days).[37] The primary end point was a composite measure of major neonatal morbidities, including necrotizing enterocolitis, retinopathy of prematurity, bronchopulmonary dysplasia, and intraventricular hemorrhage, as well as death. The primary outcome was measured within the entire neonatal intensive care unit stay up to 90 days after randomization. The rate of nosocomial infection was a secondary outcome. Acutely ill premature babies who received fresher blood did not fare better than those who received the current standard of care. There was no difference between the two approaches with respect to major organ injury, mortality or infection. However, the age difference between the fresher units (5.1 days) and the older units (14.6 days) was relatively small and determining the average age of blood for those neonates transfused more than once was challenging; there was a wide range of RBC age in each transfusion.

Conclusion

There is currently no consensus regarding the toxic effects of 'older' RBC. Most retrospective studies have flaws in their design or introduce bias that results in an overestimation of the association between untoward effects and the age of the blood. Studies in normal volunteers are somewhat reassuring in that no evidence of clinical toxicity has been observed. However, the number of subjects studied is small, the study end points relate more to physiology than to pathology, and the findings in these healthy subjects should not be applied to patients with a variety of illnesses of differing severity. Studies in animals, while not definitive, suggest that at the extremes of illness severity and RBC shelf life, the 'storage lesion' may be clinically relevant. It may be too smug to say that fresher is always better; it is after all well-known that certain complications (transmission of spirochetes, infection with some cell-associated viruses, and transfusion-associated graft-*versus*-host disease) are more common with fresh blood. The transfusion medicine community maintains a high degree of optimism, if not confidence, that the outcome of the ongoing randomized trials, still several years away, will resolve the issue of the risk of stored blood. It is certainly possible that these trials will prove to be underpowered to provide a definitive result, or, if negative, will fail to identify clinical situations in which stored blood might prove a substantial enough additional insult to result in an adverse outcome. It is unlikely that either animal models or additional retrospective analyses (and certainly not *in vitro* studies) will provide sufficient evidence to change blood collection and transfusion practice. A series of large, comparative effectiveness studies may be required to resolve this issue.

References

1. Robertson OH. Transfusion with preserved red blood cells. Brit Med J. 1818;1:691-5.
2. Klein HG, Anstee DJ. Mollison's Blood Transfusion in Clinical Medicine. 11th ed. Oxford: Blackwell Publishing; 2005:365-80.
3. Dumont LJ, AuBuchon JP. Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. Transfusion. 2008;48(6):1053-60.
4. Luten, M, Roerdinkholder-Stoelwinder B, Schaap NP, de Grip WJ, Bos HJ, Bosman GJ. Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods. Transfusion. 2008;48:1478-85.
5. Beutler E. Back to the future in RBC preservation. Transfusion. 2000;40:893-5.
6. Purdy ER, Tweeddale MG, Merrick PM. Association of mortality with age of blood transfused in ICU patients. Can J Anaesth. 1997;44:1256-61.
7. Zallen, G, Offner PJ, Moore EE. Age of transfused blood is an independent risk factor for postinjury multiple organ failure. Am J Surg. 1999;178:570-2.
8. Mynster T, Nielsen HJ. Storage time of transfused blood and disease recurrence after colorectal cancer surgery. Diseases of the Colon and Rectum. 2001;44(7):955-64.
9. Offner PJ, Moore EE, Biffl WL, Johnson JL, Silliman CC. Increased rate of infection associated with transfusion of old blood after severe injury. Arch Surg. 2002;137:711-6.
10. Leal-Noval SR, Jara-López I, García-Garmendia JL, Marín-Niebla A, Herruzo-Avilés A, Camacho-Laraña P, et al. Influence of red blood cell concentrates (RBCs) storage time on postsurgical morbidity in cardiac surgery patients. Anesthesiology. 2003;98:815-22.
11. Basran S, Frumento RJ, Cohen A. The association between duration of storage of transfused red blood cells and morbidity and mortality after reoperative cardiac surgery. Anesthesia and Analgesia. 2006;103:15-20.
12. Weinberg JA, McGwin G Jr, Griffin RL, et al. Age of transfused blood: an independent predictor of mortality despite universal leukoreduction. J Trauma. 2008;65(2):279-82.
13. Vamvakas EC. Meta-analysis of clinical studies of the purported deleterious effects of "old" (versus "fresh") red blood cells: Are we at equipoise? Transfusion. 2010;50(3):600-10.
14. Van De Watering L, Lorinser J, Versteegh M, Westendord R, Brand A. Effects of storage time of red blood cell transfusions on the prognosis of coronary artery bypass graft patients. Transfusion. 2006;46(10):1712-8.
15. Weiskopf RB, Feiner J, Hopf H, et al. Fresh blood and aged stored blood are equally efficacious in immediately reversing anemia-induced brain oxygenation deficits in humans. Anesthesiology. 2006;104(5):911-20.
16. Fergusson DA, Hébert P, Hogan DL, et al. Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial. JAMA. 2012;308(14):1443-51.
17. Murrell Z, Haukoos JS, Putnam B, Klein SR. The effect of older blood on mortality, need for ICU care, and the length of ICU stay after major trauma. American Surgeon. 2005;71(9):781-5.
18. Mynster T, Nielsen HJ. Storage time of transfused blood and disease recurrence after colorectal cancer surgery. Diseases of the Colon and Rectum. 2001;44(7):955-64.
19. Marik PE, Sibbald WJ. Effect of stored-blood transfusion on oxygen delivery in patients with sepsis. JAMA. 1993;269:3024-30.
20. Kiraly LN, Underwood S, Differding JA, Schreiber MA. Transfusion of aged packed red blood cells results in decreased tissue oxygenation in critically injured trauma patients. Journal of Trauma - Injury, Infection and Critical Care. 2009;67(1):29-32.
21. Walsh TS, McArdle F, McLellan SA, et al. Does the storage time of transfused red blood cells influence regional or global indexes of tissue oxygenation in anemic critically ill patients? Critical Care Medicine. 2004;32(2):364-71.
22. Koch CG, Li L, Sessler DI. Duration of red-cell storage and complications after cardiac surgery. N Engl J Med. 2008;358:1229-39.
23. Dzik W. Fresh blood for everyone? Balancing availability and quality of stored RBCs. Transfus Med. 2009;18:260-65.
24. Wang D, Sun J, Solomon SB, Klein HG, Natanson C. Transfusion of older stored blood and risk of death: a meta-analysis. Transfusion. 2012;52(6):1184-95.
25. Hod EA, Brittenham GM, Billote GB, Francis RO et al. Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron. Blood. 2011;5;118(25):6675-82.
26. Weiskopf RB, Kramer JH, Viele M, Neumann M, Feiner J, Watson JJ, et al. Acute severe isovolemic anemia impairs cognitive function and memory and humans. Anesthesiology. 2000;92:1646-52.
27. Weiskopf RB, Feiner J, Toy P, et al. Fresh and stored red blood cell transfusion equivalently induce subclinical pulmonary gas exchange deficit in normal humans. Anesth Analg. 2012;114(3):511-9.
28. Hod EA, Zhang N, Sokol SA, et al. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. Blood. 2010;115(21):4284-92.
29. Baek JH, D'Agnillo F, Vallelian F, et al. Hemoglobin-driven pathophysiology is an in vivo consequence of the red blood cell storage lesion that can be attenuated in guinea pigs by haptoglobin therapy. J Clin Invest. 2012;122(4):1444-58.
30. Solomon SB, Wang D, Sun J, et al. Mortality increases after massive exchange transfusion with older stored blood in canines with experimental pneumonia. Blood. 2012 Dec 18. [Epub ahead of print]
31. Lacroix J. The Age of Blood Evaluation (ABLE) Study - International Standard Randomized Controlled Trial Number Register (ISRCTN) 2008. Available from: http://www.controlled-trials.com/isrctn/pf/44878718.
32. Koch C. The Red Cell Storage Duration and Outcomes in Cardiac Surgery study. 2009. Available from: http://clinicaltrials.gov/.
33. Assmann SF. The Red Cell Storage Duration Study (RECESS) 2010. Available from: http://clinicaltrials.gov/.
34. Heddle N, Cook R, Arnold D, Crowther MA, et al. The effect of blood storage duration on in-hospital mortality: a randomized controlled pilot feasibility trial. Transfusion. 2012;52:1203-12.
35. Aubron C, Syres G, Nichol A et al. A pilot feasibility trial of allocation of freshest available RBC cells versus standard care in critically ill patients. Transfusion. 2012;52:1196-202.
36. Red Cell Storage Duration and Outcomes in Cardiac Surgery. http://clinicaltrials.gov/ct2/results?term=NCT00458783&Search=Search
37. Fergusson DA, Hébert P, Hogan D, et al. Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial. JAMA. 2012;308(14):1443-51.

HIV-associated lymphomas in the HAART era

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A B S T R A C T

The incidence of lymphoma is increased in patients with HIV infection, partly related to their immunosuppression. The advent of highly-active anti-retroviral therapy (HAART) resulted in a significant improvement in the outcome of patients diagnosed with HIV and lymphoma. This led to a generalized tendency to treat these patients with the same chemotherapy protocols used in the general population. Nevertheless, this is undoubtedly a subset of patients with some specific particularities which might result in an increased risk of toxicity during treatment, and this should be taken into account when managing them. Thus, the selection of the appropriate HAART combination and the use of prophylactic antibiotics are crucial to ensure a good outcome. The prognosis of patients with HIV and lymphoma is comparable to that of the non-infected population, as long as patients with HIV infection receive the same regimens as HIV-negative patients, and provided they receive adequate support therapy, in terms of prophylactic antibiotics and anti-retroviral treatment.

Learning goals

At the conclusion of this activity, participants should be able to:
- describe how the introduction of HAART has impacted on the incidence and outcome of lymphoma in patients with HIV infection;
- enumerate arguments in favor and against the concomitant use of HAART during chemotherapy;
- describe the management of patients with HIV and lymphoma and discuss how their outcome compares with that of patients with the same types of lymphoma in the general population.

Introduction

The incidences of both non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma (HL) are significantly increased in patients with HIV infection in comparison with those in the general population. NHL in HIV patients, frequently grouped under the term AIDS-related lymphoma (ARL), is an AIDS-defining malignancy (ADM), whereas HL is a non-AIDS defining malignancy (NADM). The poor prognosis of patients with AIDS in the pre-highly active anti-retroviral therapy (HAART) era led to a nihilistic symptomatic/palliative approach in the management of patients with HIV and lymphoma. The advent of HAART resulted in a significant improvement in the outcome of patients diagnosed with HIV and lymphoma and a growing tendency to treat them following the same protocols used in the non-infected population. Nevertheless, some controversial issues in the management of patients with HIV and lymphoma remain, which will be reviewed in this session.

Etiopathology and classification

The development of lymphoma in patients with HIV infection is, undoubtedly, related to their immunosuppression and low CD4 counts, as demonstrated by the almost complete disappearance of primary central nervous system lymphoma (PCNSL) (characterized by extremely low CD4 counts at diagnosis) in the HAART era.[1] However, the relationship is not a simple lineal one. The conflicting reports on the incidence of HL in the HAART era illustrate this, with some articles reporting that the incidence has not decreased in the HAART era,[1,2] and others reporting a higher incidence in recent years.[3] In fact, some studies suggest that the incidence of HL is higher in patients with moderate CD4 counts than in those with severe immunosuppression, so that the risk of developing HL might actually increase when patients start HAART.[4] The fact that the risk of being diagnosed with lymphoma does not disappear in patients on HAART with adequate viral suppression and CD4 counts further demonstrates that a low CD4 count and a high viral load are not essential for the development of lymphoma in these patients. Although the CD4 count recovers in patients responding to HAART, it is clear that their immune system is not fully functional. Furthermore, HIV results in chronic antigen stimulation, and all of these circumstances contribute to the increased risk of lymphoma. It is important to note, at this point, that patients with HIV infection can develop many different types of lymphoma. The last update of the Centers for

Disease Control and Prevention (CDC) definitions for AIDS in 2008 confirmed the 24 AIDS-defining conditions previously included in 1999,[5] amongst them Burkitt's lymphoma (BL), immunoblastic lymphoma (or equivalent) and primary central nervous system lymphoma (PCNSL), which are frequently grouped under the term 'AIDS-related lymphoma' (ARL). In contrast, the World Health Organization (WHO) 2008 classification acknowledges the diversity of subtypes of lymphoma that can be diagnosed in individuals with HIV infection (Table 1).[6] This follows the spirit of the Revised European American Lymphoma Classification (REAL) classification, continued in the WHO, recognizing that each subtype of lymphoma is an individual and specific entity, with its specific pathogenesis, clinical features, treatment and prognosis. In this regard, the pathogenic mechanisms leading to the development of lymphoma in patients with HIV infection vary in different types of lymphoma, the degree of immunosuppression/chronic stimulation required, the involvement of other viruses such as Epstein-Barr virus (EBV) or human herpes virus-8 (HHV-8), and the presence of genetic abnormalities, differing from one subtype to the other.[7,8]

Concomitant administration of HAART during chemotherapy

It was clear in the pre-HAART era that the concomitant administration of anti-retroviral (ARV) therapy during chemotherapy resulted in a significant increase in the toxicity of chemotherapy with a very poor tolerance.[9,10] This is not surprising considering that almost the only available ARV at that time was zidovudine (AZT), which is characterized by its myelotoxicity. This led in many centers to the avoidance of ARV in patients receiving chemotherapy, even when newer less toxic combinations were developed. In contrast, the recommendation on the use of HAART concomitantly with chemotherapy is based on the evidence that the outcome of patients with HIV and lymphomas has significantly improved in the HAART era.[11] Moreover, several studies have demonstrated that the prognosis of patients with HIV and lymphoma is better amongst those receiving HAART during treatment[12,13] and, even more, in those responding to HAART.[14,15] In addition, the interruption of HAART in patients on therapy prior to the diagnosis of lymphoma might lead to the development of viral resistance.[16] There are two reasons for the increased toxicity of chemotherapy in combination with ARV. On the one hand, many ARV have overlapping toxicities with some of the chemotherapy drugs most frequently used in patients with lymphoma. On the other hand, some ARV, especially protease inhibitors (PI), alter the metabolism of cytotoxic drugs via inhibition of CYP3A4. In this sense, Bower *et al*. showed that PI-based HAART results in a significantly increased myelotoxicty when given in combination with chemotherapy, in comparison with other HAART regimens.[17] This supports the importance of an appropriate choice of ARVs in patients receiving chemotherapy to ensure the benefit obtained by continuing HAART without its potential downsides.

Infusional *versus* conventional regimens

The poor results obtained in the pre-HAART era with conventional regimens led some investigators to explore the use of infusional regimens to treat patients with HIV and lymphoma;[18] cyclophosphamide, doxorubicin and etoposide (CDE)[19] and dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin (DA-EPOCH)[20] were the most commonly used. These studies often include patients with 'ARL'. Therefore, patients are treated with the same regimens regardless of their histological subtype, i.e. diffuse large B-cell lymphoma (DLBCL) or BL, in spite of the fact that there is some suggestion that patients with BL might benefit less than patients with DLBCL from some of these regimens.[21] In contrast, with the advent of HAART, there was a movement in many centers towards managing HIV patients with lymphoma using the same chemotherapy schedules used in the general population, including the intensive chemotherapy regimens generally administered to patients with BL. Several studies have demonstrated that the outcome of patients with HIV and DLBCL,[22] BL,[23] and HL[24] are comparable to those obtained in the non-infected population when patients receive the same protocols. In spite of this, some centers still advocate the use of infusional regimens. The National Cancer Institute presented excellent results of the DA-EPOCH regimen in combination

Table 1. WHO 2008 classification.

Lymphomas also occurring in immuno-competent patients
Burkitt's and Burkitt-like lymphomas
Diffuse large B-cell lymphomas Centroblastic Immunoblastic (including PCNSL)
Extra-nodal MALT lymphoma
Peripheral T-NHL
Classic Hodgkin's lymphoma
Lymphomas occurring more specifically in HIV-positive patients
Primary effusion lymphoma
Plasmablastic lymphoma of the oral cavity
Lymphomas also occurring in other immune-deficiency states
Polymorphic B-cell lymphoma (PTLD-like)

PCNSL: primary central nervous system lymphoma; MALT lymphoma: mucosa associated lymphoid tissue lymphoma; PTLD: post-transplant lymphoproliferative disorder.

Table 2. Complete response/complete response uncertain and survival in patients treated with ABVD according to their serological status.

End point	HIV-negative	HIV-positive	*P*
CR/CRu	79%	74%	0.34
5-year EFS	66%	59%	0.5
5-year DFS	85%	87%	0.5
5-year OS	88%	81%	0.15

ABVD: doxorubicin-bleomycin-vinblastine-dacarbazine; CR/CRu: complete response/complete response uncertain; EFS: event-free survival; DFS: disease-free survival; OS: overall survival.

with rituximab for patients with BL (with or without HIV infection) a few years ago,[25] and is currently running a phase II study of this regimen (NCT01092182). A pooled analysis of two consecutive trials compared the outcome of patients with HIV and lymphoma treated with either R-CHOP or with R-EPOCH and concluded that R-EPOCH resulted in a significant better complete remission (CR) rate, event-free survival (EFS) and overall survival (OS).[26] Thus, the data supporting the superiority of infusional over conventional regimens in HIV-positive patients come from phase II studies, with no randomized trials comparing conventional *versus* infusional regimens in the HIV-positive population. In view of this, the results of the randomized study CALGB 50203 comparing R-CHOP with DA-EPOCH-R in HIV-negative patients with DLBCL might shed some light on this issue.

Use of rituximab in patients with HIV infection

The excellent results achieved with the addition of rituximab to CHOP in patients with DLBCL in the general population[27] led the AIDS-Malignancies Consortium (AMC) to run one of the few randomized studies in patients with HIV and lymphoma, comparing R-CHOP with CHOP.[28] It is important to note that in this study by Kaplan and colleagues, 20% of the patients had histological subtypes other than DLBCL (including BL), and that the rituximab arm included a short 'maintenance' phase with three monthly additional doses of rituximab. The results of this study are well known and can be summarized by saying that the addition of rituximab did not result in a significant advantage, which was partly attributed to an increased toxicity in patients receiving rituximab, especially in those with very low CD4 counts. This study raised obvious concerns on the safety of rituximab in severely immune-depressed patients. More recently, another AMC randomized phase II study compared the concurrent administration of rituximab and EPOCH with the sequential administration of rituximab, at the end of EPOCH chemotherapy.[29] Although not the primary end point of the study, the toxicity profile was comparable in both arms with no evidence that the concurrent administration of rituximab and chemotherapy substantially increases its toxicity in comparison with the sequential administration. The results of this study, as well of those of other phase II studies,[15,30] support the safety of the administration of rituximab in patients with HIV infection, with the caveat that patients with very low CD4 counts (defined in different studies as <50 or <100) might present increased toxicity. Whether rituximab should be omitted in these patients or whether it can be given supported with prophylactic antibiotics is a matter of debate.

Management of patients with HL

As mentioned above, the incidence of HL, one of the most frequent NADMs, has been reported to be increasing in the HAART era,[3] and its management has also evolved. Similarly, as in other types of lymphomas, there was some reluctance to treat patients with HIV and HL with the standard chemotherapy regimens used in the general population before the introduction of HAART. Again, this changed in the HAART era with many investigators moving towards the use of standard regimens for HL, such as doxorubicin, bleomycin, vinblastine and dacarbazine (ABVD). The largest series of 62 patients with HIV infection and HL treated with ABVD reported a CR rate of 87% with a 5-year EFS and OS of 71% and 75%, respectively,[14] results similar to those seen in non-infected patients. The lack of any difference in patient outcome, with or without HIV infection, with HL treated with ABVD was recently demonstrated in a multicenter study (Table 2).[24] An important difference in the management of HL in HIV patients in comparison with HL in non-infected patients is that risk-adapted strategies are less frequently followed. This is partly due to the smaller proportion of patients with early favorable disease in the HIV population on the one hand, and, on the other hand, to the perceived increased risk of toxicity in this group of patients. Nevertheless, a recent study has demonstrated the feasibility of following a risk-adapted strategy in patients with HIV and HL,[31] in line with the philosophy of offering patients with HIV infection the same curative protocols used in the general population.

Management of relapsed/refractory disease

In spite of the excellent results achieved in the management of patients with HIV and lymphoma, there will be, as in the non-HIV population, a percentage of patients with either refractory or recurrent disease. The standard management of refractory/relapsed HL and DLBCL (amongst other types of lymphomas) in fit patients, based on the results of several randomized studies,[32-34] consists of the administration of salvage chemotherapy with the aim at obtaining a response, and consolidating it with high-dose therapy and autologous stem cell rescue (HDT/ASCR). At the beginning of this century, several small pilot studies demonstrated the feasibility of HDT/ASCR in HIV patients (Table 3),[35-38] with adequate neutrophil and

Table 3. High-dose therapy with autologous stem cell rescue in HIV patients with relapsed/refractory lymphoma.

Series	N.	On HAART	Neutrophil count>0.5 (days, median)	Platelet count>20 (days, median)	OS	EFS
Re* (2003)[35]	10	9	10	13	Median 18 mo	Median 11 mo
Gabarre (2004)[36]	14	14	12	11	NR	NR
Serrano (2005)[37]	11	10	16	20	81% (at 15 mo)	65% (at 32 mo)
Krishnan** (2005)[38]	20	9	11	NR	85%	85%

*HAART: highly active antiretroviral therapy; OS: overall survival; EFS: event-free survival; mo: months; *median follow up: 8 months; **median follow up: 32 months.*

platelet recovery, and outcomes comparable to those expected in uninfected patients. The Lymphoma Working Party (LWP) of the European Group for Blood and Marrow Transplantation (EBMT) performed a case-matched comparative analysis of the outcomes of patients receiving HDT/ASCR for relapsed/refractory lymphoma and demonstrated the lack of significant differences according to their serological status,[39] supporting the management of patients with HIV and lymphoma with the same strategies used in non-HIV patients, also in the refractory/relapse setting. Regarding the salvage regimens used prior to HDT/ASCR, no series have been published on the second-line treatment in HIV patients with relapsed/refractory lymphoma and, thus, the scarce available data are extracted from series on first-line treatment or studies of HDT/ASCR. The salvage protocols used are varied and include the same range of different regimes used in the general population, with small numbers of patients treated with the same regimen, precluding any conclusion as to the superiority of one protocol over the others.[40]

Conclusions

The introduction of HAART represented a huge advance in the outcome of patients with HIV diagnosed with lymphoma and allowed them to benefit from the same chemotherapy regimens employed in the general population. This massive step, moving from a palliative approach in the pre-HAART era to a curative one in recent times, has eliminated the differences in the prognosis of patients with lymphoma according to their serological status, provided HIV-patients receive the appropriate regimens for the histological subtype and stage of the disease, and the adequate prophylactic antibiotics and HAART regimen.

References

1. International Collaboration on HIV and Cancer. Highly active antiretroviral therapy and incidence of cancer in human immunodeficiency virus-infected adults. J Natl Cancer Inst. 2000;92(22):1823-30.
2. Bedimo R, Chen RY, Accortt NA, Raper JL, Linn C, Allison JJ, et al. Trends in AIDS-defining and non-AIDS-defining malignancies among HIV-infected patients: 1989-2002. Clin Infect Dis. 2004;39(9):1380-4.
3. Herida M, Mary-Krause M, Kaphan R, Cadranel J, Poizot-Martin I, Rabaud C, et al. Incidence of non-AIDS-defining cancers before and during the highly active antiretroviral therapy era in a cohort of human immunodeficiency virus-infected patients. J Clin Oncol. 2003;21(18):3447-53.
4. Lanoy E, Rosenberg PS, Fily F, Lascaux AS, Martinez V, Partisani M, et al. HIV-associated Hodgkin lymphoma during the first months on combination antiretroviral therapy. Blood.2011;118(1):44-9.
5. Schneider E, Whitmore S, Glynn KM, Dominguez K, Mitsch A, McKenna MT. Revised surveillance case definitions for HIV infection among adults, adolescents, and children aged <18 months and for HIV infection and AIDS among children aged 18 months to <13 years—United States, 2008. MMWR Recomm Rep. 2008;57(RR-10):1-12.
6. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri S, Stein H, et al. WHO Classification of tumors of haematopoietic and lymphoid tissues. Lyon: IARC, 2008.
7. Carbone A, Gloghini A, Capello D, Gaidano G. Genetic pathways and histogenetic models of AIDS-related lymphomas. Eur J Cancer. 2001;37(10):1270-5.
8. Carbone A, Gloghini A. AIDS-related lymphomas: from pathogenesis to pathology. Br J Haematol. 2005;130(5):662-70.
9. Tirelli U, Errante D, Oksenhendler E, Spina M, Vaccher E, Serraino D, et al. Prospective study with combined low-dose chemotherapy and zidovudine in 37 patients with poor-prognosis AIDS-related non-Hodgkin's lymphoma. French-Italian Cooperative Study Group. Ann Oncol. 1992;3(10):843-7.
10. Levine AM. Hodgkin's disease in the setting of human immunodeficiency virus infection. J Natl Cancer Inst Monogr. 1998(23):37-42.
11. Diamond C, Taylor TH, Aboumrad T, Anton-Culver H. Changes in acquired immunodeficiency syndrome-related non-Hodgkin lymphoma in the era of highly active antiretroviral therapy: incidence, presentation, treatment, and survival. Cancer. 2006;106(1):128-35.
12. Vaccher E, Spina M, di Gennaro G, Talamini R, Nasti G, Schioppa O, et al. Concomitant cyclophosphamide, doxorubicin, vincristine, and prednisone chemotherapy plus highly active antiretroviral therapy in patients with human immunodeficiency virus-related, non-Hodgkin lymphoma. Cancer. 2001;91(1):155-63.
13. Hentrich M, Maretta L, Chow KU, Bogner JR, Schurmann D, Neuhoff P, et al. Highly active antiretroviral therapy (HAART) improves survival in HIV-associated Hodgkin's disease: results of a multicenter study. Ann Oncol. 2006;17(6):914-9.
14. Xicoy B, Ribera JM, Miralles P, Berenguer J, Rubio R, Mahillo B, et al. Results of treatment with doxorubicin, bleomycin, vinblastine and dacarbazine and highly active antiretroviral therapy in advanced stage, human immunodeficiency virus-related Hodgkin's lymphoma. Haematologica. 2007;92(2):191-8.
15. Ribera JM, Oriol A, Morgades M, Gonzalez-Barca E, Miralles P, Lopez-Guillermo A, et al. Safety and efficacy of cyclophosphamide, adriamycin, vincristine, prednisone and rituximab in patients with human immunodeficiency virus-associated diffuse large B-cell lymphoma: results of a phase II trial. Br J Haematol. 2008;140(4):411-9.
16. Balduin M, Sierra S, Daumer MP, Rockstroh JK, Oette M, Fatkenheuer G, et al. Evolution of HIV resistance during treatment interruption in experienced patients and after restarting a new therapy. J Clin Virol. 2005;34(4):277-87.
17. Bower M, McCall-Peat N, Ryan N, Davies L, Young AM, Gupta S, et al. Protease inhibitors potentiate chemotherapy-induced neutropenia. Blood. 2004;104(9):2943-6.
18. Sparano JA, Wiernik PH, Strack M, Leaf A, Becker N, Valentine ES. Infusional cyclophosphamide, doxorubicin, and etoposide in human immunodeficiency virus- and human T-cell leukemia virus type I-related non-Hodgkin's lymphoma: a highly active regimen. Blood. 1993;81(10):2810-5.
19. Sparano JA, Lee S, Chen MG, Nazeer T, Einzig A, Ambinder RF, et al. Phase II trial of infusional cyclophosphamide, doxorubicin, and etoposide in patients with HIV-associated non-Hodgkin's lymphoma: an Eastern Cooperative Oncology Group Trial (E1494). J Clin Oncol. 2004;22(8):1491-500.
20. Little RF, Pittaluga S, Grant N, Steinberg SM, Kavlick MF, Mitsuya H, et al. Highly effective treatment of acquired immunodeficiency syndrome-related lymphoma with dose-adjusted EPOCH: impact of antiretroviral therapy suspension and tumor biology. Blood. 2003;101(12):4653-9.
21. Spina M, Jaeger U, Sparano JA, Talamini R, Simonelli C, Michieli M, et al. Rituximab plus infusional cyclophosphamide, doxorubicin, and etoposide in HIV-associated non-Hodgkin lymphoma: pooled results from 3 phase 2 trials. Blood. 2005;105(5):1891-7.
22. Navarro JT, Lloveras N, Ribera JM, Oriol A, Mate JL, Feliu E. The prognosis of HIV-infected patients with diffuse large B-cell lymphoma treated with chemotherapy and highly active antiretroviral therapy is similar to that of HIV-negative patients receiving chemotherapy. Haematologica. 2005;90(5):704-6.
23. Oriol A, Ribera JM, Bergua J, Gimenez Mesa E, Grande C, Esteve J, et al. High-dose chemotherapy and immunotherapy in adult Burkitt lymphoma: comparison of results in human immunodeficiency virus-infected and noninfected patients. Cancer. 2008;113(1):117-25.
24. Montoto S, Shaw K, Okosun J, Gandhi S, Fields P, Wilson A, et al. HIV status does not influence outcome in patients with classical Hodgkin lymphoma treated with chemotherapy using doxorubicin, bleomycin, vinblastine, and dacarbazine in the highly active antiretroviral therapy era. J Clin Oncol. 2012;30(33):4111-6.
25. Dunleavy K, Little RF, Pittaluga S, Grant N, Shovlin M, Steinberg SM, et al. A prospective study of dose-adjusted (DA) EPOCH with rituximab in adults with newly diagnosed Burkitt lymphoma: a regimen with high efficacy and low tox-

icity. Article appears in: 10th International Conference on Malignant Lymphoma. Lugano, Switzerland, 2008.
26. Barta SK, Lee JY, Kaplan LD, Noy A, Sparano JA. Pooled analysis of AIDS malignancy consortium trials evaluating rituximab plus CHOP or infusional EPOCH chemotherapy in HIV-associated non-Hodgkin lymphoma. Cancer. 2012;118(16):3977-83.
27. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002;346(4):235-42.
28. Kaplan LD, Lee JY, Ambinder RF, Sparano JA, Cesarman E, Chadburn A, et al. Rituximab does not improve clinical outcome in a randomized phase 3 trial of CHOP with or without rituximab in patients with HIV-associated non-Hodgkin lymphoma: AIDS-Malignancies Consortium Trial 010. Blood. 2005;106(5):1538-43.
29. Sparano JA, Lee JY, Kaplan LD, Levine AM, Ramos JC, Ambinder RF, et al. Rituximab plus concurrent infusional EPOCH chemotherapy is highly effective in HIV-associated B-cell non-Hodgkin lymphoma. Blood. 2010;115(15):3008-16.
30. Boue F, Gabarre J, Gisselbrecht C, Reynes J, Cheret A, Bonnet F, et al. Phase II trial of CHOP plus rituximab in patients with HIV-associated non-Hodgkin's lymphoma. J Clin Oncol. 2006;24(25):4123-8.
31. Hentrich M, Berger M, Wyen C, Siehl J, Rockstroh JK, Muller M, et al. Stage-adapted treatment of HIV-associated Hodgkin lymphoma: results of a prospective multicenter study. J Clin Oncol. 2012;30(33):4117-23.
32. Linch DC, Winfield D, Goldstone AH, Moir D, Hancock B, McMillan A, et al. Dose intensification with autologous bone-marrow transplantation in relapsed and resistant Hodgkin's disease: results of a BNLI randomised trial. Lancet. 1993;341(8852):1051-4.
33. Schmitz N, Pfistner B, Sextro M, Sieber M, Carella AM, Haenel M, et al. Aggressive conventional chemotherapy compared with high-dose chemotherapy with autologous haemopoietic stem-cell transplantation for relapsed chemosensitive Hodgkin's disease: a randomised trial. Lancet. 2002;359(9323):2065-71.
34. Philip T, Guglielmi C, Hagenbeek A, Somers R, Van der Lelie H, Bron D, et al. Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. N Engl J Med. 1995;333(23):1540-5.
35. Re A, Cattaneo C, Michieli M, Casari S, Spina M, Rupolo M, et al. High-dose therapy and autologous peripheral-blood stem-cell transplantation as salvage treatment for HIV-associated lymphoma in patients receiving highly active antiretroviral therapy. J Clin Oncol. 2003;21(23):4423-7.
36. Gabarre J, Marcelin AG, Azar N, Choquet S, Levy V, Levy Y, et al. High-dose therapy plus autologous hematopoietic stem cell transplantation for human immunodeficiency virus (HIV)-related lymphoma: results and impact on HIV disease. Haematologica. 2004;89(9):1100-8.
37. Serrano D, Carrion R, Balsalobre P, Miralles P, Berenguer J, Buno I, et al. HIV-associated lymphoma successfully treated with peripheral blood stem cell transplantation. Exp Hematol. 2005;33(4):487-94.
38. Krishnan A, Molina A, Zaia J, Smith D, Vasquez D, Kogut N, et al. Durable remissions with autologous stem cell transplantation for high-risk HIV-associated lymphomas. Blood. 2005;105(2):874-8.
39. Diez-Martin JL, Balsalobre P, Re A, Michieli M, Ribera JM, Canals C, et al. Comparable survival between HIV+ and HIV- non-Hodgkin and Hodgkin lymphoma patients undergoing autologous peripheral blood stem cell transplantation. Blood. 2009;113(23):6011-4.
40. Re A, Michieli M, Casari S, Allione B, Cattaneo C, Rupolo M, et al. High-dose therapy and autologous peripheral blood stem cell transplantation as salvage treatment for AIDS-related lymphoma: long-term results of the Italian Cooperative Group on AIDS and Tumors (GICAT) study with analysis of prognostic factors. Blood. 2009;114(7):1306-13.

HCV-associated lymphomas

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A B S T R A C T

The association between hepatitis C virus (HCV) infection and B-cell non-Hodgkin's lymphomas (NHL) has been demonstrated by epidemiological studies, in particular in highly endemic geographical areas. Marginal zone lymphoma and diffuse large B-cell lymphoma are the histotypes most frequently associated with HCV infection. Many mechanisms have been proposed for explaining HCV-induced lymphoproliferation; antigenic stimulation by HCV seems to be fundamental in establishing B-cell expansion as observed in mixed cryoglobulinemia and in NHL. Moreover, HCV-infected cells display a mutator phenotype with increased mutation frequency of some genes, such as immunoglobulin heavy chain. Recently, antiviral treatment has been proved to be effective in the treatment of HCV-positive indolent lymphomas, in particular splenic marginal zone lymphoma. Across different studies, overall response rate was approximately 75% and responses were linked to the eradication of the HCV-RNA. More recently, a subset of apparently *de novo* diffuse large B-cell lymphoma emerged as a separate entity associated with HCV infection. In this setting, antigenic trigger seems no longer necessary to support the lymphoproliferation and antiviral treatment is not sufficient to control the disease. On the other hand, the impact of liver toxicity after immunochemotherapy in patients with HCV-positive diffuse large B-cell lymphoma is a relevant clinical issue that has not yet been completely clarified.

Learning goals

At the conclusion of this activity, participants should be able to:
- understand the mechanisms of HCV-related lymphoproliferation;
- describe the epidemiological evidences of association of HCV with NHL;
- describe the anti-lymphoma activity of antiviral treatment in HCV-associated indolent B-cell NHL;
- understand biological and clinical features of HCV-associated DLBCL.

Introduction

Hepatitis C virus (HCV) infection is a worldwide problem, with up to 200 million people infected worldwide. There are important regional differences in the prevalence of HCV infection: the lowest rates are reported in Northern Europe while in Italy, Japan, Egypt and southern parts of United States, prevalence estimates exceed 2%.[1]

HCV infection is the cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) and has been associated to a spectrum of extra-hepatic manifestations. Due to the lymphotropism of HCV[2] and consequent lymphatic infection, some lymphoproliferative disorders have been linked to the virus: mixed cryoglobulinemia (MC),[3] monoclonal gammopathies[4] and B-cell non-Hodgkin's lymphomas (B-NHL).[5]

In the last 20 years, not only biological and epidemiological studies but also therapeutic observations provided solid evidence for the association between HCV and B-cell NHL. In particular, HCV has been associated with B-cell low grade indolent NHL, especially of marginal zone origin, as well as with aggressive lymphomas, mainly diffuse large B-cell lymphoma (DLBCL). More recently, interventional studies demonstrated that in HCV-positive patients affected by indolent NHL, eradication of HCV with antiviral treatment (AT) could directly induce lymphoma regression, providing a strong argument in favor of a causative link between HCV and lymphoproliferation.[6]

Mixed cryoglobulinemia

Mixed cryoglobulinemia (MC) is the most well defined lymphoproliferative disorder associated with HCV infection. It is a clinically benign pre-lymphomatous disease characterized by a very high prevalence (nearly 90%-100%)[3] of HCV infection, and by bone marrow and liver B-cell clones resembling a picture of low grade NHL. In fact, monoclonal or oligoclonal B-cell expansion are frequently detected in the intrahepatic infiltrates and in the bone marrow of HCV-infected patients. Immunophenotipic profile of the cells in the intraportal lymphoid nodules shows that they are mostly B cells expressing IgM and, in some cases, CD5 antigen.[7] Molecular studies of V-D-J pattern of the B-cell component in HCV-positive patients with MC demonstrated that more than one clone with an oligoclonal

pattern sustains the lymphoproliferation.[8]

The main clinical features of MC are palpable purpura, arthralgia, weakness, organ involvement (liver, kidney), peripheral neuropathy and vasculitis. The vasculitic lesions are the consequence of vascular deposition of circulating immune complexes and complement.[9]

In HCV-infected patients with MC, the risk for developing a NHL is greatly increased with respect to the general population (about 35 times according to a multicenter Italian study):[10] approximately 10% of patients with MC evolve to a frank NHL. AT with pegylated interferon (PEG-IFN) + ribavirin (RBV) has been shown to reverse bone marrow B-cell expansion in patients with HCV-MC, leading to clinical and virological responses in more than half of cases.[11]

HCV and lymphoproliferation

Mechanisms of lymphoproliferation in HCV-infected subjects are not unique; many models have been proposed and they may be not mutually exclusive (Figure 1).

The role of HCV infection in lymphomagenesis may be related to the chronic antigenic stimulation of B-cell response,[12] similar to the well characterized induction of gastric mucosa-associated lymphoid tissues (MALT) lymphoma development by *Helicobacter pylori* chronic infection.[13] In a similar way, chronic HCV infection may possibly sustain a multi-step evolution from MC to overt low-grade NHL and eventually to high-grade NHL.[12,13] During this process, independence from antigenic stimulation can develop due to additional genetic aberrations. Regarding the antigenic trigger, the monoclonal component of MC is often an IgM with a rheumatoid factor activity (anti-IgG cross-reactive binding) that mirrors the expansion of a B-cell monoclonal population[14] not only in bone marrow but also in hepatic follicles.[7]

It has also been hypothesized that the HCV antigens such as NS3 may be involved in the induction of MC and lymphoma.[15] In addition, envelope protein such as E2 protein can play a role in lymphomagenesis; it interacts with the tetraspanin CD81, present also on the B-cell surface, lowering the threshold and leading to a polyclonal B-cell activation.[16] In a case of HCV-associated NHL, it has been

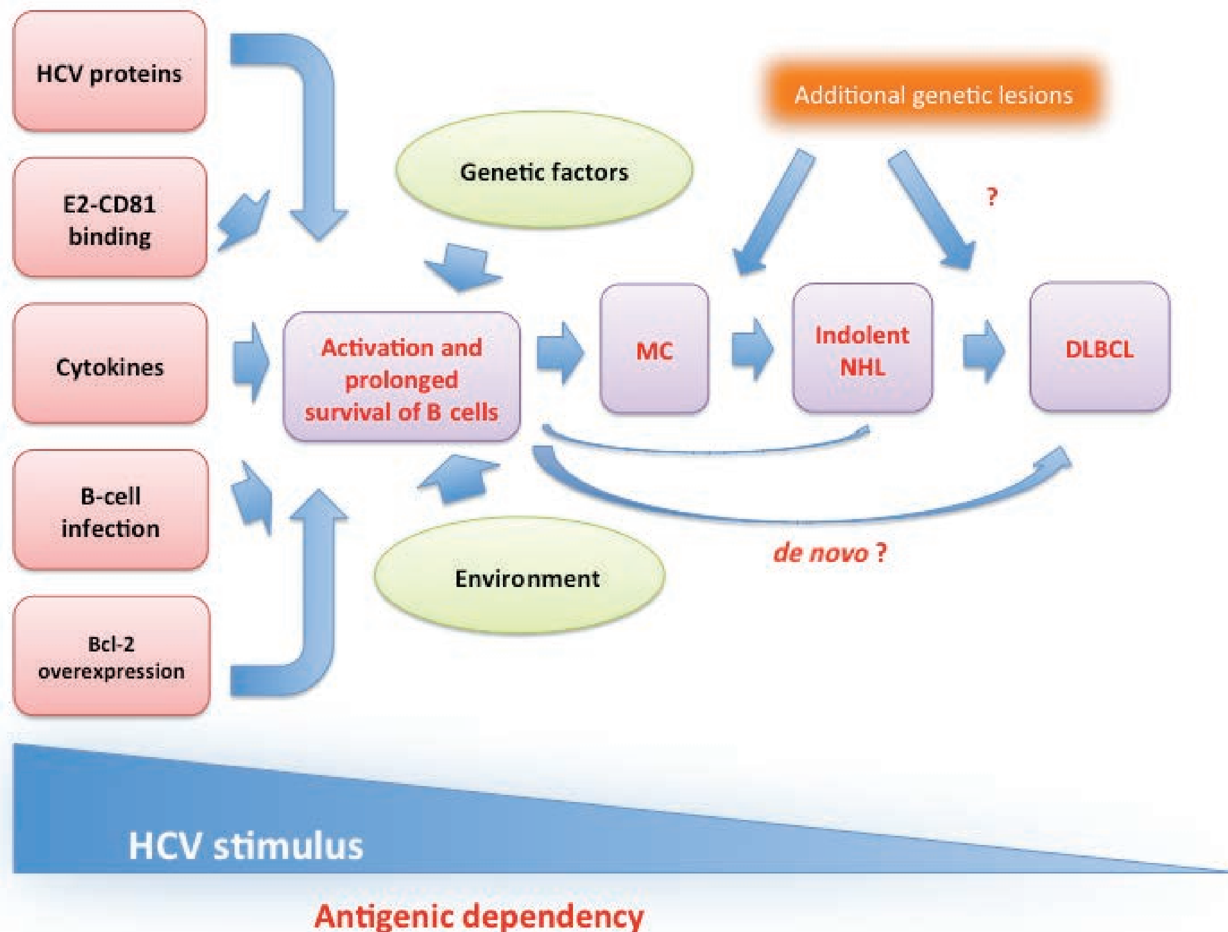

Figure 1. Mechanisms of HCV-induced lymphoproliferation.

elegantly demonstrated that the specific immunoglobulin binds the E2 protein as a human anti-E2 antibody.[17] In addition, E2 protein appears to mimic human Ig[18] and induces the production of IgM monoclonal factor (rheumatoid factor) in MC patients.[19]

A role in development of HCV-related lymphoproliferative disorders could be played also by chromosomal alterations: for instance, MC with or without lymphoma is characterized by translocation t(14;18) with the overexpression of the antiapoptotic bcl-2 gene leading to prolonged B-cell survival.[19] It is also worthy of note that an increased amount of aneuploidy has also been reported in patients with chronic hepatitis and in patients with NHL compared to healthy subjects.[20]

Another mechanism of HCV-related lymphomagenesis is associated with lymphotropism of HCV, as demonstrated by the viral replication in lymphatic tissue.[2] In addition, regarding molecular signals in HCV-related lymphomagenesis, it has been demonstrated[21] that HCV-infected cells display a mutator phenotype with increased mutation frequency of immunoglobulin heavy chain, *BCL-6*, p53, and β-catenin genes.

It was also postulated that cytokines and chemokines are involved in the mechanisms of HCV-induced lymphoproliferation: IFNγ, TNFα,[22] CXCL13[23] and BAFF[24] in MC, as well as osteopontin[25] in B-NHL.

Epidemiology

Several epidemiological studies have been conducted since the 1990s to investigate the link between HCV and NHL. These studies, sometimes based on relatively small numbers of cases, suggested a significant increased risk of B-cell NHL in HCV-positive patients, especially in countries with high prevalence of HCV infection such as Italy,[26] Egypt[27] and Japan[28] while studies from areas with low HCV prevalence, such as Northern Europe and the US, failed to show any evident association.[29]

Meta-analyses of studies evaluating prevalence of HCV infection in B-cell NHL confirmed the association between HCV and NHL.[30-33] In 2003, a meta-analysis[31] evaluated 48 studies (5542 patients) with a mean HCV infection prevalence of 13%. In 10 case-control studies examined, HCV prevalence in B-cell NHL was 17% compared with 1.5% in healthy subjects (Odds ratio (OR) 10.8). In a 2006 up-dated meta-analysis[33] of 15 case-control studies, relative risk of lymphoma among HCV-positive subjects was 2-2.5. Overall, the fraction of NHL attributable to HCV infection varies greatly according to geographical area, reaching 10% in highly endemic countries. Interestingly, in a large Danish-Swedish population-based case-control study on 2819 lymphoma patients and 1856 controls of second-generation Danish-Swedish origin, a positive association between HCV and risk of NHL has been demonstrated in a population with a low HCV prevalence.[34]

A major limitation is that the numbers of cases analyzed in these cohorts were too small to establish a clear correlation between HCV and specific histotypes of NHL. In the Epilymph[35] study, the subtypes associated with HCV infection resulted DLBCL, marginal zone lymphoma (MZL) and lymphoplasmacytic lymphoma (LPL). In addition, the International Lymphoma Epidemiology Consortium (InterLymph), based in Europe, North America, and Australia, performed a pooled case-control study including in the analysis data of 7 previous surveys: among 4784 cases of NHL and 6269 controls, HCV infection was detected in 3.6% of NHL cases and in 2.7% of controls. In subtype-specific analyses, HCV prevalence was associated with DLBCL, MZL and LPL.[36] On the other hand, results from a population-based study from the US are somewhat unexpected: from the US Surveillance, Epidemiology, and End Results (SEER)-Medicare database, 61,464 cases were selected and HCV was associated not only with increased risk of DLBCL (OR 1.5) and MZL (OR 2.2) but also with Burkitt's lymphoma (OR 5.2) and follicular lymphoma (OR 1.88).[37]

HCV and indolent lymphomas

Several clinical-pathological studies investigated the association of HCV-infection with specific indolent NHL subtypes. Within indolent NHL subtypes reported in the World Health Organization (WHO) classification,[38] the association with HCV infection has been best characterized in MZLs. It is well known that many infectious agents have been involved in the pathogenesis of specific types of MZLs: *Helicobacter pylori* for gastric MALT lymphoma,[13] *Borrelia burgdorferi* for MALT lymphoma of the skin,[39] *Chlamydophila psittaci* for MALT lymphoma of the orbit, *Campylobacter jejuni* for immunoproliferative small intestine disease. In these clinical scenarios, eradication of the antigen after antimicrobial therapy may lead to a regression of the underlying lymphoma.

According to this scenario, also chronic stimulation by HCV may play a role in development of a subgroup of MZL cases; however, the role of HCV in marginal zone lymphomagenesis can reflect geographical differences considering the relatively high seroprevalence in some series of MZL[40] and the rarity of HCV-positive cases in others.[41]

Splenic marginal zone lymphoma (SMZL) is an indolent lymphoma that accounts for less than 2% of all NHL.[42] In a large Italian series, HCV serology was positive in 19% and cryoglobulins were detected in 10%. In 2005, French authors described a form of splenic lymphoma associated with MC and HCV infection:[43] all 18 patients had MC, symptomatic in 13. In addition, in a series of 133 SMZL (26 HCV-positive) 12% showed stereotyped B-cell receptors, suggesting that the pathogenesis of SMZL may involve also HCV-related and unrelated epitopes or an antigenic trigger common to other indolent lymphomas.

Recently, unsupervised hierarchical clustering of miRNA expression profiles demonstrated a distinct signature of SMZL compared with the normal splenic marginal zone. Supervised analysis revealed differentially expressed miRNAs and miR-26b, a miRNA with tumor suppressive properties, was significantly down-regulated in HCV-positive patients with SMZL.

Primary nodal marginal zone lymphoma is listed in the 2008 edition of WHO lymphoma classification as a distinct clinical-pathological subtype characterized by exclusive primary lymph node localization in the absence of extranodal site of involvement. This rare form of indolent NHL has been linked to HCV infection with preferential

use of specific VH segments.[44] In a relatively large Italian series, HCV serology was positive in 24% and HCV-RNA was detectable in half the patients studied.[45]

Gastric and non-gastric extranodal MZL of MALT represent 8% of all NHL. They are typically indolent diseases of middle and advanced age; disseminated disease is present in nearly one-third of cases.[46] Interestingly, an increased prevalence of HCV infection has been reported in unselected series of patients with gastric lymphoma.[47] In an Italian multicenter study, HCV serology was available in 35% of non-gastric MALT lymphoma. Interestingly, three specific MALT lymphoma sites showed an elevated prevalence of HCV infection: salivary glands, skin and orbit.[48]

The association of HCV infection and salivary glands lymphoma has been clearly demonstrated.[49] In 33 cases of primary salivary MALT lymphoma, 7 had HCV infection and of these 11 patients with cryoglobulinemia, 5 were HCV-positive and 6 were affected by Sjögren's syndrome. Interestingly, a study on B-cell lymphoma in patients with Sjögren's syndrome and HCV infection reported an elevated occurrence of parotid involvement and a high proportion of MALT lymphomas with primary extranodal involvement (exocrine glands, liver, and stomach).[50] A series of 12 HCV-positive subjects presenting with subcutaneous nodules resembling 'lipomas' with a typical histology of extranodal MZL of MALT have been recently reported.[51] From a clinical point of view, the clinical benign appearance of these 'lipoma-like' lesions and their indolent clinical behavior may delay correct diagnosis. HCV-RNA was detectable in 10 of the 10 patients tested; cryoglobulins were found in 4 patients and 2 presented cryoglobulinemic purpura. Molecular analysis of immunoglobulin heavy chain gene rearrangements documented the presence of somatic mutations in 14 of 17 (82%) clonal rearrangements; in some cases different V-D-J rearrangements were found at diagnosis and at relapse.

Beside MZLs, also LPL/Waldenström's macroglobulinemia (WM) has been associated to HCV infection.[52] However, this association is not completely defined. For example, a survey from the US did not find any HCV-positive case among 100 untreated patients affected by WM.[53] Comparing Waldenström's macroglobulinemia and SMZL, it seems that SMZL, despite some common features, displayed a clearly higher association with HCV infection than WM.[54]

B-cell chronic lymphoproliferative disorders are defined as the miscellaneous category of leukemic lymproliferative disorders distinct from chronic lymphocytic leukemia (Royal Marsden Hospital scoring system ≤3[55]). Association of these entities with HCV is not clear. A monocentric Bayesian study[56] showed a high prevalence of HCV infection while another series reported a low rate of HCV-positivity (5%) in CD5/CD10-negative B-cell chronic lymphoproliferative disorders.[57] Further investigations are needed to clarify this issue, given the heterogeneity and the small numbers of studies focusing on these entities. Interestingly, monoclonal B-cell lymphocytosis (MBL), a pre-clinical condition characterized by an expansion of clonal B cells in the absence of frank lymphocytosis, was identified in nearly 30% of HCV-positive subjects with a significantly higher frequency than in the general population.

Antiviral treatment of HCV-positive indolent lymphomas

The aim of treatment for patients with HCV-related chronic hepatitis is to prevent disease complications through HCV eradication, defined as sustained virological response (SVR), i.e. undetectable HCV-RNA by a sensitive polymerase chain reaction (PCR)-based assay 24 weeks after discontinuation of therapy. The current standard of care is combination of PEG-IFN and weight-based RBV for 48 weeks for genotype 1 and 4, and for 24 weeks for genotype 2 and 3. Patients with genotype 2 or 3 obtain a SVR in 75%-90% of cases, while patients with genotype 1 and 4 achieve SVR in nearly 50%.[58] Recently, the introduction of the HCV NS3/4A protease inhibitors boceprevir[59] and teleprevir,[60] the first two drugs belonging to a new generation of direct-acting antiviral agents, has been demonstrated to improve virological responses in genotype 1 patients. Other novel potent protease inhibitors showing promising activity are currently under development, as well as other new upcoming classes of direct-acting antiviral agents like polymerase inhibitors. Their potential combination seems to herald the possibility of obtaining highly efficacious IFN-free regimens for HCV chronic hepatitis in the near future.[61,62]

Strong additional evidence supporting the etiological role of HCV in lymphomagenesis is the reported regression of indolent NHL after eradication of HCV infection with AT. In 2005, a systematic review concerning the efficacy of AT in lymphoproliferative disorders was published[63] covering 16 studies reporting the employment of AT as primary anti-lymphoma treatment in 65 HCV-infected patients diagnosed with lymphoproliferative disorders. Complete response (CR) was reported in three-quarters of cases. However, some reports were based on relatively few patients and included also subjects with MC with presence of B-cell monoclonality.[64] Data regarding 124 cases of HCV-associated indolent NHL treated with AT for lymphoma control are summarized in Table 1.[65-80] Nearly half of these reports are case reports regarding 1-2 patients.

In 2002, Hermine *et al.* reported the outcome of 9 patients with splenic lymphoma with villous lymphocytes and HCV infection treated with IFN. Complete response and HCV-RNA clearance were obtained in 7 of 9 patients. Two patients who did not respond were subsequently treated with IFN plus RBV and obtained HCV-RNA negativity as well as lymphoma regression. This anti-lymphoma activity was absent in HCV-negative patients with SMZL. A subsequent report from the same group expanded these results in 18 patients with chronic HCV infection, MC and splenic lymphoma with villous lymphocytes.[43] All patients were treated with IFN (plus RBV in 10) and 14 patients obtained a CR after clearance of HCV-RNA. Viral genotype did not seem to correlate with the response. Another study reported first-line AT with IFN and RBV in 8 HCV-positive patients with different subtypes of MZL: 60% obtained a response and this was correlated to virological response in most cases.[73]

An Italian multicenter study reported results of AT in 13 HCV-positive indolent B-cell NHL, including non-marginal zone cases.[74] Among 12 assessable patients, 7 achieved a CR, 2 partial responses (PR); lymphoma regression was highly significantly associated to clearance

or decrease in serum HCV viral load. Virological response was more frequent in HCV genotype 2; however, lymphoma response did not correlate with the HCV genotype. One of the greatest achievements of this study was the demonstration of the efficacy of AT in a wide range of HCV-positive low-grade NHL subtypes other than splenic MZL, as CRs were actually observed without significant differences in all indolent NHL histologies. More recently, Mazzaro *et al*.[76] reported a comparison of PEG- IFN and standard IFN (plus RBV) as first-line treatment in 18 patients with HCV-positive low-grade B-cell NHL. CR and SVR rates were higher in the group treated with PEG-IFN (6 of 10 patients, 60%) with respect to the group treated with standard IFN (3 of 8 patients, 37%). Achievement of lymphoma response was significantly related to the clearance of HCV-RNA.

Data regarding molecular eradication with AT in HCV-associated indolent NHL are conflicting. The French[74] and Italian[66] experiences reported that no molecular response was obtained along with clinical remission. On the other hand, Italian and US authors reported an impact of AT on disappearance of immunoglobulin heavy-chain gene rearrangement and t(14;18) translocation in HCV infected patients.[19,64,81] This observation along with other similar reports suggest that AT can eliminate clonal B-cell proliferation and possibly prevent the subsequent development

Table 1. Series of patients with indolent B-cell lymphoma associated with HCV infection treated with antiviral treatment as anti-lymphoma approach.

	Year	N. of pts	Type of NHL	N. of pts with MC	Type of anti-viral treatment	NHL response	HCV response
Mazzaro *et al.*[65]	1996	6	Immunocytoma	6	α-IFN-2b	3 CR	3
Bauduer[66]	1996	1	EMZL (oral cavity)	-	α-IFN	1 PR	1
Caramaschi *et al.*[67]	1999	1	EMZL (salivary glands)	-	α-IFN	1 CR	NA
Moccia *et al.*[68]	1999	3	SMZL	-	α-IFN	2 CR	NA
Patriarca *et al.*[69]	2001	1	LPL	-	α-IFN	1 CR	1
Hermine *et al.*[6]	2002	9	SLVL	6	α-IFN	7 CR	7
Casato *et al.*[70]	2002	1	Leukemic MZL	1	α-IFN	1 CR	Decreased HCV-RNA
Pitini *et al.*[71]	2004	2	SMZL	-	α-IFN	2 CR	2
Tursi *et al.*[72]	2004	16	EMZL (stomach)	-	α-IFN-2b + RBV	16 CR	11/16
Kelaidi *et al.*[73]	2004	8	SMZL (n=4) Disseminated MZL (n=1) Leukemic MZL (n=1) EMZL (n=2) (1 duodenus; 1 ileus)	8	α-IFN-2b + RBV	5 CR	5 SVR, 2 PR
Vallisa *et al.*[74]	2005	13	SMZL (n=4) NMZL (n=2) EMZL (n=2) FL (n=1) LPL (n=4)	5	Peg-IFN + RBV	7 CR, 2 PR	7 SVR, 1 PR
Svoboda *et al.*[75]	2005	1	EMZL (salivary gland, liver)	-	Peg-IFN + RBV	CR	1
Saadoun *et al.*[43]	2005	18	SLVL	18	α-IFN (+ RBV in 10)	14 CR, 4 PR	14 CR, 4 PR
Paulli *et al*[51]	2009	2	EMZL (subcutaneous tissue)	2	Peg-IFN + RBV	1 CR, 1 PR	2 CR
Mazzaro *et al.*[76]	2009	18	1 SLVL 1 FL 16 LPL	13	α-IFN + RBV (n=8) Peg-IFN + RBV (n=10)	9 CR, 4 PR	9 SVR
Oda *et al.*[77]	2010	1	B-NHL (liver)	-	Peg-IFN + RBV	CR	SVR
Saadoun *et al.*[78]	2010	13	8 MZL 4 LPL SLL 1	13	Peg-IFN + RBV (+ R in 7)	11 CR	
Pellicelli *et al.*[79]	2011	9	3 EMZL 3 SMZL 1 NMZL 2 FL	5	Peg-IFN + RBV	5 CR 2 PR	7 SVR
Mauro *et al.*[80]	2012	1	LPL	1	Peg-IFN + RBV	CR	SVR

SMZL: splenic marginal zone lymphoma; NMZL: nodal marginal zone lymphoma; SLVL: splenic lymphoma with villous lymphocytes; EMZL: extranodal marginal zone lymphoma of MALT; FL: follicular lymphoma; LPL: lymphoplasmacytic lymphoma; SLL: small lymphocytic lymphoma; NHL: non-Hodgkin's lymphoma; NOS: not otherwise specified; pts: patients; IFN: interferon; RBV: ribavirin; R: rituximab; CR: complete response; PR: partial response; SVR: sustained virological response.

of lymphoma. Regarding this, Kawamura *et al.* reported 501 consecutive patients carrying HCV infection who had never received IFN and 2708 consecutive patients who received IFN therapy. They demonstrated that sustained virological response induced by AT protects against the development of NHL in these patients.[82] In the non-treated group, the cumulative rate of NHL development was 2.6% at 15 years; the cumulative rate of NHL development in IFN-group with SVR was 0% at 15 years while the cumulative rate in patients with persistent infection was 2.6% at 15 years.

In the future, several lines can be pursued with the aim of further improving these results. First, investigations should examine whether the combination of PEG-IFN + RBV + rituximab (PIRR scheme) tested in symptomatic MC[78,83] is able to obtain a better long-term control of disease also in indolent B-cell NHL. Interestingly, while in an Italian study[83] no patient with NHL were included, in a French study,[78] 7 patients with lymphoma were treated with the PIRR scheme and all obtained CR for lymphoma.

Second, investigations should also examine if new antiviral combinations with new anti-HCV agents (i.e. PEG-IFN and RBV plus boceprevir or teleprevir) that obtain higher rates of SVR in genotypes 1 carriers,[59,60] could increase the possibility of lymphoma response also in patients with more resistant genotypes. Finally, will future IFN-free regimens with direct antiviral agents only allow access to AT also for HCV-positive NHL patients with contraindications to IFN use, e.g. because of advanced age, cytopenias and/or comorbidities. In addition, considering the anti-proliferative properties of IFN, lymphoma regression with IFN-free regimens could definitely demonstrate that lymphoma regression is strictly linked to the HCV eradication.

HCV and aggressive lymphomas

Despite the classical association of HCV with indolent NHL, aggressive NHL, in particular DLBCL, are emerging as diseases linked to HCV infection. An Italian case-control study reported an even higher association of HCV infection with DLBCL (OR 3.5) with respect to indolent NHL (OR 2.3), suggesting that approximately 1 in 20 cases of DLBCL in Italy may be attributable to HCV.[84] On the other hand, an experimental model in which transgenic mice expressing the whole HCV genome in CD19-positive B cells display a high incidence of B-NHL with the features of typical DLBCL.[85] Reports have also shown a higher seroprevalance of genotype 1 with a short duration of infection in HCV-positive DLBCL as compared to patients with indolent NHL, who showed a higher prevalence of genotype 2.[79]

HCV-positive DLBCL patients display specific presentation with respect to HCV-negative DLBCL.[86,87] In particular, residual signs of low-grade lymphoma and extranodal disease such as spleen are more frequently detected in HCV-positive cases in comparison with HCV. Data regarding clinical features of HCV-associated DLBCL series reported in literature are summarized in Table 2.[86-89]

As far as outcome is concerned, some clinical features included in common prognostic scores for DLBCL such as older age, advanced stage and number of extranodal sites may be confounding because they are differently distributed in this setting. Moreover, many laboratory parameters of common prognostic value (i.e. LDH, blood cell counts) are influenced not only by lymphoma but also by HCV infection. As a consequence, little is known about precise prognostication of HCV-positive DLBCL.

Unlike indolent B-NHL, AT seems not to play a central role in the first-line approach for HCV-positive DLBCL, because lymphoma cells are most likely to be independent from chronic antigenic stimulation due to the acquisition of additional oncogenic lesions. For this reason, although anecdotal cases of aggressive NHL such as DLBCL[90] and mantle cell lymphoma[91] treated with AT and obtaining remission have been reported, HCV-positive DLBCL patients have to be treated with anthracycline-based chemotherapy coupled with rituximab.

In the pre-rituximab era, in a French study[86] focusing on 23 HCV-positive DLBCL patients, 52% developed hepatic toxicity during chemotherapy. This was significantly higher than matched HCV-negative patients. HCV positivity also showed a negative impact on overall survival.

In an Italian study,[87] among 132 patients with HCV-positive DLBCL, only 4% had to discontinue treatment due to severe hepatotoxicity, while 11% required dose reduction or prolongation of treatment intervals.

Based on the parallel with the phenomenon of HBV reactivation and the risk of acute exacerbation of hepatitis, there have been many concerns about the use of rituximab. In MC syndrome with advanced liver disease, rituximab appeared to be safe and effective. Moreover, the depletion of CD20+ B cells was followed by cirrhosis syndrome

Table 2. Series of patients with diffuse large B-cell lymphoma associated with HCV infection.

	Year	N. of pts	M/F N. (%)	Transformed NHL N. (%)	Primary extranodal N. (%) N.	Spleen involvement	OS	PFS
Besson *et al.*[86]	2006	26	17/9 (27/73)	7 (32)	NA	12 (46)	2-y OS 56%	2-y 53%
Visco *et al.*[87]	2006	156	74/82 (47/53)	14 (8)	67 (43)	53 (34)	5-y OS 75%	5-y PFS 51%
Arcaini *et al.*[88]	2010	99	46/53 (46/53)*	NA	14 (14)*	34 (34)	5-y OS 68%	Median PFS 1.9 y
Ennishi e *et al.*[89]	2010	131	79/52 (60/40)	5 (4)	NA	24 (18)	3-y OS 75%	3-y PFS 69%

**L Arcaini, personal data, 2008. M: males; F: females; OS: overall survival; PFS: progression-free survival; y: years..*

improvement despite the possibility of transient increases in HCV-RNA.[92] On the other hand, it is arguable that immunodeficiency linked to hematologic malignancies and combination of rituximab with immunosuppressive and chemotherapy drugs may result in a more aggressive evolution of liver damage.[93]

Regarding the use of rituximab in HCV-positive NHL, in an Italian series[87] rituximab was coupled with standard chemotherapy in 35 patients. None of the rituximab-treated patients developed moderate-severe hepatic toxicity. In another Italian report of 160 HCV-positive NHL (101 were DLBCL and 28 had been treated with rituximab)[94] among 93 patients with normal ALT at presentation, 16 patients developed WHO grade 3-4 liver toxicity, and among 67 patients with abnormal ALT, 8 patients had a 3.5 times elevation during treatment. Among 28 patients treated with rituximab and chemotherapy, 5 patients (18%) developed liver toxicity. Severe hepatic toxicity occurred more frequently in genotype 1 carriers.

A Japanese survey analyzed 553 patients with DLBCL (131 HCV-positive) treated with R-CHOP.[89] HCV infection was not a significant risk factor for prognosis. Of 131 HCV-positive patients, 36 (27%) had severe hepatic toxicity (grade 3-4), compared with 13 of 422 (3%) patients who were negative for HCV infection. Multivariate analysis revealed that HCV infection was a significant risk factor for severe hepatic toxicity. However, this excess of hepatic toxicity seems to only slightly affect the outcome, as the authors found only a trend toward a worst overall survival in HCV-positive patients, without any difference in response rate or progression-free survival between the two cohorts.

The interplay between HCV-RNA levels and transaminases in HCV-positive NHL during chemotherapy with or without rituximab has only been analyzed in some reports and with conflicting conclusions.[94-102] For instance, Ennishi and co-workers found that in 34 patients treated with R-CHOP, HCV-RNA load significantly increased during immunochemotherapy.[95] While relatively small case series suggested that HCV-RNA peak may precede or coincide with the occurrence of a transaminases flare (possibly at the time of CD20 lymphocyte recovery),[98,100] in the majority of studies, ALT levels seem to have no impact on HCV-RNA quantification and, therefore, this does not appear to be useful to predict liver toxicity.[94,97,99,102,103]

Many researchers have explored the option of integrating AT in the context of immunochemotherapy programs in HCV-positive DLBCL. Although some rare cases of concurrent delivery of AT and immunochemotherapy with the aim of preventing or treating hepatitis flares have been reported,[95] treatment with AT is usually not feasible because of hematologic toxicity, as shown by a pilot study by Musto in 4 patients with DLBCL,[104] while a sequential approach (immunochemotherapy followed by AT) seem effective and well tolerated also in aggressive forms.[105] These preliminary experiences are encouraging and have to be confirmed in larger prospective series.

Conclusions

The association between HCV infection and B-cell NHL has been demonstrated by epidemiological studies, in particular in highly endemic geographical areas. MZL and DLBCL are the histotypes most frequently associated with HCV infection. Many mechanisms have been proposed to explain the wide spectrum of HCV-induced lymphoproliferation that ranges from MC to indolent and aggressive NHL.

AT has been shown to be effective in the treatment of HCV-positive indolent lymphomas while it is not sufficient to treat HCV-associated DLBCL. It is likely that future improvements in AT and advances in prognostication and monitoring of hepatic toxicity may directly result in an increase in cure rates of HCV-associated NHL.

References

1. Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis. 2005;5:558-67.
2. Zignego AL, Macchia D, Monti M, Thiers V, Mazzetti M, Foschi M, et al. Infection of peripheral mononuclear blood cells by hepatitis C virus. J Hepatol. 1992;15:382-6.
3. Agnello V, Chung RT, Kaplan LM. A role for hepatitis C virus infection in type II cryoglobulinemia. N Engl J Med. 1992;327:1490-5.
4. Andreone P, Gramenzi A, Cursaro C, Bernardi M, Zignego AL. Monoclonal gammopathy in patients with chronic hepatitis C virus infection. Blood. 1996;88:1122.
5. Ferri C, Caracciolo F, Zignego AL, La Civita L, Monti M, Longombardo G, et al. Hepatitis C virus infection in patients with non-Hodgkin's lymphoma. Br J Haematol. 1994;88:392-4.
6. Hermine O, Lefrere F, Bronowicki JP, Mariette X, Jondeau K, Eclache-Saudreau V, et al. Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. N Engl J Med. 2002;347:89-94.
7. Sansonno D, De Vita S, Iacobelli AR, Cornacchiulo V, Boiocchi M, Dammacco F. Clonal analysis of intrahepatic B cells from HCV-infected patients with and without mixed cryoglobulinemia. J Immunol. 1998;160:3594-601.
8. De Re V, De Vita S, Marzotto A, Rupolo M, Gloghini A, Pivetta B, et al. Sequence analysis of the immunoglobulin antigen receptor of hepatitis C virus-associated non-Hodgkin lymphomas suggests that the malignant cells are derived from the rheumatoid factor-producing cells that occur mainly in type II cryoglobulinemia. Blood. 2000;96:3578-84.
9. Tedeschi A, Barate C, Minola E, Morra E. Cryoglobulinemia. Blood Rev. 2007;21:183-200.
10. Monti G, Pioltelli P, Saccardo F, Campanini M, Candela M, Cavallero G, et al. Incidence and characteristics of non-Hodgkin lymphomas in a multicenter case file of patients with hepatitis C virus-related symptomatic mixed cryoglobulinemias. Arch Intern Med. 2005;165:101-5.
11. Mazzaro C, Monti G, Saccardo F, Zignego AL, Ferri C, De Vita S, et al. Efficacy and safety of peginterferon alfa-2b plus ribavirin for HCV-positive mixed cryoglobulinemia: a multicentre open-label study. Clin Exp Rheumatol. 2011;29:933-41.
12. Marcucci F, Mele A. Hepatitis viruses and non-Hodgkin lymphoma: epidemiology, mechanisms of tumorigenesis, and therapeutic opportunities. Blood.117:1792-8.
13. Suarez F, Lortholary O, Hermine O, Lecuit M. Infection-associated lymphomas derived from marginal zone B cells: a model of antigen-driven lymphoproliferation. Blood. 2006;107:3034-44.
14. Agnello V, Zhang QX, Abel G, Knight GB. The association of hepatitis C virus infection with monoclonal rheumatoid factors bearing the WA cross-idiotype: implications for the etiopathogenesis and therapy of mixed cryoglobulinemia. Clin Exp Rheumatol. 1995;13(13 Suppl):S101-4.
15. De Re V, Sansonno D, Simula MP, Caggiari L, Gasparotto D, Fabris M, et al. HCV-NS3 and IgG-Fc crossreactive IgM in patients with type II mixed cryoglobulinemia and B-cell clonal proliferations. Leukemia. 2006;20:1145-54.
16. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, et al. Binding of hepatitis C virus to CD81. Science. 1998;282:938-41.
17. Quinn ER, Chan CH, Hadlock KG, Foung SK, Flint M, Levy S. The B-cell receptor of a hepatitis C virus (HCV)-associated non-Hodgkin lymphoma binds the viral E2 envelope protein,

implicating HCV in lymphomagenesis. Blood. 2001;98:3745-9.
18. Hu YW, Rocheleau L, Larke B, Chui L, Lee B, Ma M, et al. Immunoglobulin mimicry by Hepatitis C Virus envelope protein E2. Virology. 2005;332:538-49.
19. Zignego AL, Ferri C, Giannelli F, Giannini C, Caini P, Monti M, et al. Prevalence of bcl-2 rearrangement in patients with hepatitis C virus-related mixed cryoglobulinemia with or without B-cell lymphomas. Ann Intern Med. 2002;137:571-80.
20. Goldberg-Bittman L, Kitay-Cohen Y, Hadari R, Yukla M, Fejgin MD, Amiel A. Random aneuploidy in chronic hepatitis C patients. Cancer Genet Cytogenet. 2008;180:20-3.
21. Machida K, Cheng KT, Sung VM, Shimodaira S, Lindsay KL, Levine AM, et al. Hepatitis C virus induces a mutator phenotype: enhanced mutations of immunoglobulin and protooncogenes. Proc Natl Acad Sci USA. 2004;101:4262-7.
22. Saadoun D, Bieche I, Maisonobe T, Asselah T, Laurendeau I, Piette JC, et al. Involvement of chemokines and type 1 cytokines in the pathogenesis of hepatitis C virus-associated mixed cryoglobulinemia vasculitis neuropathy. Arthritis Rheum. 2005;52:2917-25.
23. Sansonno D, Tucci FA, Troiani L, Lauletta G, Montrone M, Conteduca V, et al. Increased serum levels of the chemokine CXCL13 and up-regulation of its gene expression are distinctive features of HCV-related cryoglobulinemia and correlate with active cutaneous vasculitis. Blood. 2008;112:1620-7.
24. Fabris M, Quartuccio L, Sacco S, De Marchi G, Pozzato G, Mazzaro C, et al. B-Lymphocyte stimulator (BLyS) up-regulation in mixed cryoglobulinaemia syndrome and hepatitis-C virus infection. Rheumatology. 2007;46:37-43.
25. Libra M, Indelicato M, De Re V, Zignego AL, Chiocchetti A, Malaponte G, et al. Elevated Serum Levels of Osteopontin in HCV-Associated Lymphoproliferative Disorders. Cancer Biol Ther. 2005;4:1192-4.
26. Cocco P, Piras G, Monne M, Uras A, Gabbas A, Ennas MG, et al. Risk of malignant lymphoma following viral hepatitis infection. Int J Hematol. 2008;87:474-83.
27. Goldman L, Ezzat S, Mokhtar N, Abdel-Hamid A, Fowler N, Gouda I, et al. Viral and non-viral risk factors for non-Hodgkin's lymphoma in Egypt: heterogeneity by histological and immunological subtypes. Cancer Causes Control. 2009;20(6):981-7.
28. Mizorogi F, Hiramoto J, Nozato A, Takekuma Y, Nagayama K, Tanaka T, et al. Hepatitis C virus infection in patients with B-cell non-Hodgkin's lymphoma. Intern Med. 2000;39:112-7.
29. Collier JD, Zanke B, Moore M, Kessler G, Krajden M, Shepherd F, et al. No association between hepatitis C and B-cell lymphoma. Hepatology. 1999;29:1259-61.
30. Negri E, Little D, Boiocchi M, La Vecchia C, Franceschi S. B-cell non-Hodgkin's lymphoma and hepatitis C virus infection: a systematic review. Int J Cancer. 2004;111:1-8.
31. Gisbert JP, Garcia-Buey L, Pajares JM, Moreno-Otero R. Prevalence of hepatitis C virus infection in B-cell non-Hodgkin's lymphoma: systematic review and meta-analysis. Gastroenterology. 2003;125:1723-32.
32. Matsuo K, Kusano A, Sugumar A, Nakamura S, Tajima K, Mueller NE. Effect of hepatitis C virus infection on the risk of non-Hodgkin's lymphoma: a meta-analysis of epidemiological studies. Cancer Sci. 2004;95:745-52.
33. Dal Maso L, Franceschi S. Hepatitis C virus and risk of lymphoma and other lymphoid neoplasms: a meta-analysis of epidemiologic studies. Cancer Epidemiol Biomarkers Prev. 2006;15:2078-85.
34. Schollkopf C, Smedby KE, Hjalgrim H, Rostgaard K, Panum I, Vinner L, et al. Hepatitis C infection and risk of malignant lymphoma. Int J Cancer. 2008;122:1885-90.
35. Nieters A, Kallinowski B, Brennan P, Ott M, Maynadie M, Benavente Y, et al. Hepatitis C and risk of lymphoma: results of the European multicenter case-control study EPILYMPH. Gastroenterology. 2006;131:1879-86.
36. de Sanjose S, Benavente Y, Vajdic CM, Engels EA, Morton LM, Bracci PM, et al. Hepatitis C and non-Hodgkin lymphoma among 4784 cases and 6269 controls from the International Lymphoma Epidemiology Consortium. Clin Gastroenterol Hepatol. 2008;6:451-8.
37. Anderson LA, Pfeiffer R, Warren JL, Landgren O, Gadalla S, Berndt SI, et al. Hematopoietic malignancies associated with viral and alcoholic hepatitis. Cancer Epidemiol Biomarkers Prev. 2008;17:3069-75.
38. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: IARC Press; 2008.
39. Roggero E, Zucca E, Mainetti C, Bertoni F, Valsangiacomo C, Pedrinis E, et al. Eradication of Borrelia burgdorferi infection in primary marginal zone B-cell lymphoma of the skin. Hum Pathol. 2000;31:263-8.
40. Arcaini L, Paulli M, Boveri E, Vallisa D, Bernuzzi P, Orlandi E, et al. Splenic and nodal marginal zone lymphomas are indolent disorders at high hepatitis C virus seroprevalence with distinct presenting features but similar morphologic and phenotypic profiles. Cancer. 2004;100:107-15.
41. Thieblemont C, Felman P, Berger F, Dumontet C, Arnaud P, Hequet O, et al. Treatment of splenic marginal zone B-cell lymphoma: an analysis of 81 patients. Clin Lymphoma. 2002;3:41-7.
42. Matutes E, Oscier D, Montalban C, Berger F, Callet-Bauchu E, Dogan A, et al. Splenic marginal zone lymphoma proposals for a revision of diagnostic, staging and therapeutic criteria. Leukemia. 2008;22:487-95.
43. Saadoun D, Suarez F, Lefrere F, Valensi F, Mariette X, Aouba A, et al. Splenic lymphoma with villous lymphocytes, associated with type II cryoglobulinemia and HCV infection: a new entity? Blood. 2005;105:74-6.
44. Marasca R, Vaccari P, Luppi M, Zucchini P, Castelli I, Barozzi P, et al. Immunoglobulin gene mutations and frequent use of VH1-69 and VH4-34 segments in hepatitis C virus-positive and hepatitis C virus-negative nodal marginal zone B-cell lymphoma. Am J Pathol. 2001;159:253-61.
45. Arcaini L, Paulli M, Burcheri S, Rossi A, Spina M, Passamonti F, et al. Primary nodal marginal zone B-cell lymphoma: clinical features and prognostic assessment of a rare disease. Br J Haematol. 2007;136:301-4.
46. Thieblemont C, Berger F, Dumontet C, Moullet I, Bouafia F, Felman P, et al. Mucosa-associated lymphoid tissue lymphoma is a disseminated disease in one third of 158 patients analyzed. Blood. 2000;95:802-6.
47. Luppi M, Longo G, Ferrari MG, Ferrara L, Marasca R, Barozzi P, et al. Additional neoplasms and HCV infection in low-grade lymphoma of MALT type. Br J Haematol. 1996;94:373-5.
48. Arcaini L, Burcheri S, Rossi A, Paulli M, Bruno R, Passamonti F, et al. Prevalence of HCV infection in nongastric marginal zone B-cell lymphoma of MALT. Ann Oncol. 2007;18:346-50.
49. Ambrosetti A, Zanotti R, Pattaro C, Lenzi L, Chilosi M, Caramaschi P, et al. Most cases of primary salivary mucosa-associated lymphoid tissue lymphoma are associated either with Sjoegren syndrome or hepatitis C virus infection. Br J Haematol. 2004;126:43-9.
50. Ramos-Casals M, la Civita L, de Vita S, Solans R, Luppi M, Medina F, et al. Characterization of B cell lymphoma in patients with Sjogren's syndrome and hepatitis C virus infection. Arthritis Rheum. 2007;57:161-70.
51. Paulli M, Arcaini L, Lucioni M, Boveri E, Capello D, Passamonti F, et al. Subcutaneous 'lipoma-like' B-cell lymphoma associated with HCV infection: a new presentation of primary extranodal marginal zone B-cell lymphoma of MALT. Ann Oncol.21:1189-95.
52. Silvestri F, Barillari G, Fanin R, Salmaso F, Pipan C, Falasca E, et al. Impact of hepatitis C virus infection on clinical features, quality of life and survival of patients with lymphoplasmacytoid lymphoma/immunocytoma. Ann Oncol. 1998;9:499-504.
53. Leleu X, O'Connor K, Ho AW, Santos DD, Manning R, Xu L, et al. Hepatitis C viral infection is not associated with Waldenstrom's macroglobulinemia. Am J Hematol. 2007;82:83-4.
54. Arcaini L, Varettoni M, Boveri E, Orlandi E, Rattotti S, Zibellini S, et al. Distinctive clinical and histological features of Waldenstrom's macroglobulinemia and splenic marginal zone lymphoma. Clin Lymphoma Myeloma Leuk. 2011;11:103-5.
55. Matutes E, Owusu-Ankomah K, Morilla R, Garcia Marco J, Houlihan A, Que TH, et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia. 1994;8:1640-5.
56. Arcaini L, Pascutto C, Passamonti F, Bruno R, Merli M, Rizzi S, et al. Bayesian models identify specific lymphoproliferative disorders associated with hepatitis C virus infection. Int J Cancer. 2009;124:2246-9.
57. Goldaniga M, Ferrario A, Cortelazzo S, Guffanti A, Pavone E, Ambrosetti A, et al. A multicenter retrospective clinical study of CD5/CD10-negative chronic B cell leukemias. Am J Hematol. 2008;83:349-54.
58. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med.

2002;347:975-82.
59. Poordad F, McCone J Jr, Bacon BR, Bruno S, Manns MP, Sulkowski MS, et al. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med.364:1195-206.
60. Hezode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, et al. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med. 2009;360:1839-50.
61. Poordad F, Lawitz E, Kowdley KV, Cohen DE, Podsadecki T, Siggelkow S, et al. Exploratory study of oral combination antiviral therapy for hepatitis C. N Engl J Med. 2013;368:45-53.
62. Gane EJ, Stedman CA, Hyland RH, Ding X, Svarovskaia E, Symonds WT, et al. Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. N Engl J Med. 2013;368:34-44.
63. Gisbert JP, Garcia-Buey L, Pajares JM, Moreno-Otero R. Systematic review: regression of lymphoproliferative disorders after treatment for hepatitis C infection. Aliment Pharmacol Ther. 2005;21:653-62.
64. Zuckerman E, Zuckerman T, Sahar D, Streichman S, Attias D, Sabo E, et al. The effect of antiviral therapy on t(14;18) translocation and immunoglobulin gene rearrangement in patients with chronic hepatitis C virus infection. Blood. 2001;97:1555-9.
65. Mazzaro C, Franzin F, Tulissi P, Pussini E, Crovatto M, Carniello GS, et al. Regression of monoclonal B-cell expansion in patients affected by mixed cryoglobulinemia responsive to alpha-interferon therapy. Cancer. 1996;77:2604-13.
66. Bauduer F. MALT non-Hodgkin's lymphoma associated with hepatitis C virus infection treated by interferon alpha. Am J Hematol. 1996;53:209.
67. Caramaschi P, Biasi D, Carletto A, Ambrosetti A, Mocella S, Randon M, et al. [MALT lymphomas of the salivary glands. Review of the literature apropos of a case in a patient with hepatitis C virus infection]. Recenti Prog Med. 1999;90:585-91.
68. Moccia F, Tognoni E, Boccaccio P. The relationship between splenic marginal zone B-cell lymphoma and chronic liver disease associated with hepatitis C virus infection. Ann Ital Med Int. 1999;14:288-93.
69. Patriarca F, Silvestri F, Fanin R, Zaja F, Sperotto A, Baccarani M. Long-lasting complete remission of hepatitis C virus (HCV) infection and HCV-associated immunocytoma with alpha-interferon treatment. Br J Haematol. 2001;112:370-2.
70. Casato M, Mecucci C, Agnello V, Fiorilli M, Knight GB, Matteucci C, et al. Regression of lymphoproliferative disorder after treatment for hepatitis C virus infection in a patient with partial trisomy 3, Bcl-2 overexpression, and type II cryoglobulinemia. Blood. 2002;99:2259-61.
71. Pitini V, Arrigo C, Righi M, Scaffidi M, Sturniolo G. Systematic screening for HCV infection should be performed in patients with splenic marginal zone lymphoma. Br J Haematol. 2004;124:252-3.
72. Tursi A, Brandimarte G, Torello M. Disappearance of gastric mucosa-associated lymphoid tissue in hepatitis C virus-positive patients after anti-hepatitis C virus therapy. J Clin Gastroenterol. 2004;38:360-3.
73. Kelaidi C, Rollot F, Park S, Tulliez M, Christoforov B, Calmus Y, et al. Response to antiviral treatment in hepatitis C virus-associated marginal zone lymphomas. Leukemia. 2004;18:1711-6.
74. Vallisa D, Bernuzzi P, Arcaini L, Sacchi S, Callea V, Marasca R, et al. Role of anti-hepatitis C virus (HCV) treatment in HCV-related, low-grade, B-cell, non-Hodgkin's lymphoma: a multicenter Italian experience. J Clin Oncol. 2005;23:468-73.
75. Svoboda J, Andreadis C, Downs LH, Miller WT Jr, Tsai DE, Schuster SJ. Regression of advanced non-splenic marginal zone lymphoma after treatment of hepatitis C virus infection. Leuk Lymphoma. 2005;46:1365-8.
76. Mazzaro C, De Re V, Spina M, Dal Maso L, Festini G, Comar C, et al. Pegylated-interferon plus ribavirin for HCV-positive indolent non-Hodgkin lymphomas. Br J Haematol. 2009;145:255-7.
77. Oda Y, Kou T, Watanabe M, Sakuma Y, Taguchi N, Kato Y, et al. Regression of B-cell lymphoma of the liver with hepatitis C virus infection after treatment with pegylated interferon-alpha and ribavirin. Dig Dis Sci. 2010;55(6):1791-3.
78. Saadoun D, Resche Rigon M, Sene D, Terrier B, Karras A, Perard L, et al. Rituximab plus Peg-interferon-alpha/ribavirin compared with Peg-interferon-alpha/ribavirin in hepatitis C-related mixed cryoglobulinemia. Blood. 2010;116:326-34.
79. Pellicelli AM, Marignani M, Zoli V, Romano M, Morrone A, Nosotti L, et al. Hepatitis C virus-related B cell subtypes in non Hodgkin's lymphoma. World Journal of Hepatology. 2011;3:278-84.
80. Mauro E, Pedata M, Ermacora A, Mazzaro C. An additional line of therapy with pegylated interferon and ribavirin after rituximab in a patient with hepatitis C virus-related mixed cryoglobulinaemia and indolent non-Hodgkin's lymphoma previously treated with interferon. Blood Transfus. 2012;10:101-3.
81. Giannelli F, Moscarella S, Giannini C, Caini P, Monti M, Gragnani L, et al. Effect of antiviral treatment in patients with chronic HCV infection and t(14;18) translocation. Blood. 2003;102:1196-201.
82. Kawamura Y, Ikeda K, Arase Y, Yatsuji H, Sezaki H, Hosaka T, et al. Viral elimination reduces incidence of malignant lymphoma in patients with hepatitis C. Am J Med. 2007;120:1034-41.
83. Dammacco F, Tucci FA, Lauletta G, Gatti P, De Re V, Conteduca V, et al. Pegylated interferon-alpha, ribavirin, and rituximab combined therapy of hepatitis C virus-related mixed cryoglobulinemia: a long-term study. Blood. 2010;116:343-53.
84. Mele A, Pulsoni A, Bianco E, Musto P, Szklo A, Sanpaolo MG, et al. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. Blood. 2003;102:996-9.
85. Kasama Y, Sekiguchi S, Saito M, Tanaka K, Satoh M, Kuwahara K, et al. Persistent expression of the full genome of hepatitis C virus in B cells induces spontaneous development of B-cell lymphomas in vivo. Blood.116:4926-33.
86. Besson C, Canioni D, Lepage E, Pol S, Morel P, Lederlin P, et al. Characteristics and outcome of diffuse large B-cell lymphoma in hepatitis C virus-positive patients in LNH 93 and LNH 98 Groupe d'Etude des Lymphomes de l'Adulte programs. J Clin Oncol. 2006;24:953-60.
87. Visco C, Arcaini L, Brusamolino E, Burcheri S, Ambrosetti A, Merli M, et al. Distinctive natural history in hepatitis C virus positive diffuse large B-cell lymphoma: analysis of 156 patients from northern Italy. Ann Oncol. 2006;17:1434-40.
88. Arcaini L, Merli M, Passamonti F, Bruno R, Brusamolino E, Sacchi P, et al. Impact of treatment-related liver toxicity on the outcome of HCV-positive non-Hodgkin's lymphomas. Am J Hematol. 2010;85:46-50.
89. Ennishi D, Maeda Y, Niitsu N, Kojima M, Izutsu K, Takizawa J, et al. Hepatic toxicity and prognosis in hepatitis C virus-infected patients with diffuse large B-cell lymphoma treated with rituximab-containing chemotherapy regimens: a Japanese multicenter analysis. Blood. 2010;116:5119-25.
90. Iannitto E, Ammatuna E, Tripodo C, Marino C, Calvaruso G, Florena AM, et al. Long-lasting remission of primary hepatic lymphoma and hepatitis C virus infection achieved by the alpha-interferon treatment. Hematol J. 2004;5:530-3.
91. Levine AM, Shimodaira S, Lai MM. Treatment of HCV-related mantle-cell lymphoma with ribavirin and pegylated interferon Alfa. N Engl J Med. 2003;349:2078-9.
92. Petrarca A, Rigacci L, Caini P, Colagrande S, Romagnoli P, Vizzutti F, et al. Safety and efficacy of rituximab in patients with hepatitis C virus-related mixed cryoglobulinemia and severe liver disease. Blood. 2010;116:335-42.
93. Zignego AL, Giannini C, Gragnani L, Piluso A, Fognani E. Hepatitis C virus infection in the immunocompromised host: a complex scenario with variable clinical impact. J Transl Med. 2012;10:158.
94. Arcaini L, Merli M, Passamonti F, Bruno R, Brusamolino E, Sacchi P, et al. Impact of treatment-related liver toxicity on the outcome of HCV-positive non-Hodgkin's lymphomas. Am J Hematol. 2010;85:46-50.
95. Ennishi D, Maeda Y, Niitsu N, Kojima M, Izutsu K, Takizawa J, et al. Hepatic toxicity and prognosis in hepatitis C virus-infected patients with diffuse large B-cell lymphoma treated with rituximab-containing chemotherapy regimens: a Japanese multicenter analysis. Blood. 2010;116:5119-25.
96. Ennishi D, Terui Y, Yokoyama M, Mishima Y, Takahashi S, Takeuchi K, et al. Monitoring serum hepatitis C virus (HCV) RNA in patients with HCV-infected CD20-positive B-cell lymphoma undergoing rituximab combination chemotherapy. Am J Hematol. 2008;83:59-62.
97. Marignani M, Mangone M, Cox MC, Angeletti S, Veggia B, Ferrari A, et al. HCV-positive status and hepatitis flares in patients with B-cell non-Hodgkin's lymphoma treated with rituximab-containing regimens. Dig Liver Dis. 2011;43:139-42.
98. Pitini V, Sturniolo G, Arrigo C, Leonardi S, Pino S, Altavilla G. HCV genotype 2 as a risk factor for reactivation in patients with B-cell lymphoma undergoing rituximab combination chemotherapy. Br J Haematol. 2010;150:116-8.
99. Nooka A, Shenoy PJ, Sinha R, Lonial S, Flowers CR. Hepatitis C reactivation in patients who have diffuse large B-

cell lymphoma treated with rituximab: a case report and review of literature. Clin Lymphoma Myeloma Leuk. 2011;11:379-84.
100. Hsieh CY, Huang HH, Lin CY, Chung LW, Liao YM, Bai LY, et al. Rituximab-induced hepatitis C virus reactivation after spontaneous remission in diffuse large B-cell lymphoma. J Clin Oncol. 2008;26:2584-6.
101. Ennishi D, Yokoyama M, Terui Y, Takeuchi K, Ikeda K, Tanimoto M, et al. Does rituximab really induce hepatitis C virus reactivation? J Clin Oncol. 2008;26:4695-6.
102. Tsutsumi Y, Ichiki K, Shiratori S, Kawamura T, Tanaka J, Asaka M, et al. Changes in hepatitis C virus antibody titer and viral RNA load in non-Hodgkin's lymphoma patients after rituximab chemotherapy. Int J Lab Hematol. 2009;31:468-70.
103. Aksoy S, Abali H, Kilickap S, Erman M, Kars A. Accelerated hepatitis C virus replication with rituximab treatment in a non-Hodgkin's lymphoma patient. Clin Lab Haematol. 2006;28:211-4.
104. Musto P, Dell'Olio M, La Sala A, Mantuano S, Cascavilla N. Diffuse B-Large Cell Lymphomas (DBLCL) with Hepatitis-C Virus (HCV) Infection: Clinical Outcome and Preliminary Results of a Pilot Study Combining R-CHOP with Antiviral Therapy. ASH Annual Meeting Abstracts. 2005;106:2447.
105. La Mura V, De Renzo A, Perna F, D'Agostino D, Masarone M, Romano M, et al. Antiviral therapy after complete response to chemotherapy could be efficacious in HCV-positive non-Hodgkin's lymphoma. J Hepatol. 2008;49:557-63.

Epstein-Barr virus-driven lymphomas: an update

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A B S T R A C T

Epstein-Barr virus (EBV) is a ubiquitous human γ-herpes virus that has established an elegant strategy to persist as a life-long asymptomatic infection in memory B lymphocytes. Despite its ubiquity, EBV has potent transforming properties that may arise from its strategy to permanently infect individuals and that may lead to the development of a variety of lymphomas of B- or NK-/T- cell origin. In the last decade, our understanding of the latency programs activated by the virus in different EBV-driven lymphomas and the function of EBV-encoded proteins has considerably improved. The emerging picture clearly indicates that EBV latency proteins are able to hijack or deregulate critical cellular pathways to promote the proliferation and survival of infected cells, while impairing anti-viral immune responses. Similar effects may be also induced by EBV-encoded micro-RNAs, which may have a relevant pathogenic role, particularly in lymphomas showing a restricted expression of viral proteins. Moreover, recent data have challenged the view that only the latency phase of EBV infection is relevant for EBV-driven lymphomagenesis, suggesting that lytic EBV replication may also contribute to the development of EBV-associated lymphoproliferations. The recent advances in the clarification of the mechanisms underlying EBV-induced cell transformation and immune evasion help to design novel treatment approaches for EBV-related lymphomas.

Learning goals

At the conclusion of this activity, participants should be able to:
- provide an update on the continuously expanding spectrum of EBV-associated lymphomas in terms of classification, epidemiology, extent of EBV-association, and pattern of EBV latent proteins expressed;
- highlight recent data indicating that EBV lytic reactivation may also contribute to the EBV-mediated lymphomagenesis, particularly in the early phases;
- review available evidence supporting a pathogenic role of EBV-encoded miRNA in the development of EBV-associated lymphomas, particularly for the histotypes in which only a restricted set of viral proteins is expressed;
- highlight how the ever-increasing understanding of the complex relationship between EBV and its host may translate into new therapeutic approaches.

Introduction

The Epstein-Barr virus (EBV) was first identified 40 years ago in cultured Burkitt's lymphoma (BL) cells when no human lymphoid cell had ever been maintained in culture.[1] EBV has a worldwide distribution being able to establish a lifelong infection in more than 90% of individuals. Primary infection is usually asymptomatic, and when it is delayed until adolescence or adulthood, a benign lymphoproliferative disease, known as infectious mononucleosis (IM), may occur. B lymphocytes are the main target of EBV infection *in vivo*, although epithelial cells, and T or NK cells may also carry EBV. Infection of B lymphocytes is usually non-productive or latent, whereas intermittent reactivation and virus replication at epithelial surfaces allow the spreading of EBV to new hosts. Healthy EBV-seropositive adults usually carry 1-50 EBV-infected B lymphocytes per million cells in the peripheral blood.[2] These latently infected B cells show features of resting memory B lymphocytes[2,3] and are considered the reservoir of EBV latency. After infection of naïve B cells, EBV normally establishes different programs of latency that are sequentially expressed according to the type, differentiation, and activation status of infected cells, finally driving them to the memory B-cell compartment. The first program is expressed shortly after infection and includes the full set of EBV-encoded latency proteins, including six EBV nuclear antigens (EBNAs) and three latent membrane proteins (LMP-1, LMP-2A, LMP-2B). This broad latency pattern (Latency III) promotes the activation and growth of B lymphocytes which can be initiated *in vitro* into continuously proliferating lymphoblastoid cell lines (LCLs). Similarly to normal B lymphocytes activated by interaction with the cognate antigen, also EBV-carrying activated B lymphoblasts migrate to the germinal centers

of lymphoid follicles where the viral transcription program changes.[4] In these cells, only EBNA-1 and the LMPs (Latency II) are expressed, a 'rescue' program that provides signals allowing infected lymphoblasts to survive and differentiate into memory B cells.[5] Cells leaving the germinal center as resting memory B lymphocytes silence the expression of all EBV latency proteins (Latency 0) or may express EBNA-1 upon cell division (Latency I), a strict requirement for replication of the viral genome and its maintenance within the infected cell. This elegant strategy allows EBV-carrying cells to escape immune surveillance while establishing lifelong persistence in the infected host. In all forms of latency, EBV expresses the EBERs, small non-polyadenylated, non-coding double-strand RNAs, which may also contribute to EBV-driven B-cell immortalization.[6] When the EBV-infected memory B cell differentiates into antibody-secreting plasma cells, EBV activates an additional program, the lytic replication, which favors the spreading of the virus both within and outside the host.[5] From this scenario, it appears that the virus is able to exploit several steps of normal B-cell physiology to establish (activation and differentiation) and maintain (long-term memory) a persistent latent infection and then be released (terminal differentiation). Although well equipped to promote the growth of B lymphocytes, EBV may drive the proliferation of these cells only transiently in immunocompetent hosts. This may also explain why only a limited proportion of EBV-seropositive individuals develop EBV-associated lymphomas, even in the setting of immune deficiency. In rare cases, therefore, a derangement of the normal differentiation pathway may prevent infected B cells from entering into a resting state, thus allowing EBV to fully manifest its transforming potential.

The relevance of the different EBV latency programs is strongly supported by studies of EBV-associated lymphomas, which mainly include tumors of B-cell origin, consistently with the preferential tropism of the virus (Table 1). In fact, the same latency programs that EBV has evolved to successfully establish persistence in the infected host are expressed in different EBV-associated tumors (Table 2).[7] These observations also led to the hypothesis that distinct stages of the EBV life cycle may be pathogenically linked to the development of specific types of lymphoma expressing the equivalent latency program.[5] The various forms of latency observed in EBV-positive lymphomas are strongly influenced by the degree of EBV-specific immune responses that normally control very efficiently EBV-infected cells. In particular, expression of the full repertoire of growth-transformation-associated antigens can be tolerated only in conditions of profound immune suppression, whereas more restricted patterns of EBV latency are usually found in lymphomas of immunocompetent patients.

Lymphomas in immunosuppressed patients

Post-transplant lymphoproliferative disorders

Post-transplant lymphoproliferative disorders (PTLDs) are a heterogeneous group of disorders arising in iatrogenically immunosuppressed recipients of organ or hematopoietic stem-cell transplantation. EBV is mainly associated with early-onset PTLDs that are frequently, although not invariably, poly- or oligo-clonal, whereas most late-onset PTLDs are true monoclonal lymphoid malignancies associated with EBV in only a fraction of cases. Infusion of a T-cell depleted donor stem-cell product, a high degree of HLA mismatch between donor and recipient, the use of anti-thymocyte globulin with reduced-intensity transplant conditioning, the cumulative load of immunosuppressive drugs and the EBV seronegativity of the recipient at the time of transplantation constitute the main risk factors for PTLD development.[8,9] PTLD is the most immunogenic EBV-driven lymphoma, characterized by the expression of all latency proteins, including the EBNA family proteins, most of which are immunodominant in eliciting $CD8^+$ T-cell responses. Nevertheless, impairment of EBV-specific T-cell responses alone is not sufficient to explain PTLD development, which requires additional factors able to maintain EBV-carrying B cells in a continuous proliferative state, preventing their exit from the cell cycle and evolution to the harmless resting memory B lymphocytes.[5]

HIV-associated lymphoproliferative disorders

HIV-associated lymphoproliferative disorders are clinico-pathologically heterogeneous tumors and include the same lymphomas that may occur in the general population and those seen much more often in the setting of HIV infection. The most common HIV-associated lymphomas are BL and diffuse large B-cell lymphoma (DLBCL) (also involving the central nervous system, CNS). Lymphomas occurring specifically in HIV-infected patients include two rare entities: primary effusion lymphoma and plasmablastic lymphoma. Globally, EBV is identified in the neoplastic cells of approximately 40% of HIV-associated lymphomas, but the detection of EBV and the pattern of viral latency vary considerably with the site of presentation and histological subtype (Table 1). While the broad latency III pattern is consistently found in DLBCL, particularly in immunoblastic and CNS lymphomas, more restricted and still poorly defined forms of latency are detected in primary effusion lymphoma and plasmablastic lymphoma (Table 1).

Burkitt's lymphoma

Although BL is rather monomorphous in terms of morphological, immunophenotypic and genetic features, the World Health Organization (WHO) classification identifies three clinical BL subtypes: the *endemic* form, associated with EBV in the majority of cases and occurring in tropical Africa and New Guinea where malaria is holoendemic; the *sporadic* variant, affecting children and young adults from the rest of the world with no climatic or geographical link, and rarely EBV-associated; and the *immunodeficiency-associated* form, mainly occurring in HIV-infected patients with more than 200 CD4 T cells per μL, and carrying EBV in 30%-50% of the cases. The strict pathogenic association between EBV and endemic BL is supported by the constant presence of EBV genome in lymphoma cells,[10] by data indicating that EBV infection precedes lymphomagenesis[11] and by the relationship between high antibody titers to EBV VCA and increased risk for developing this lymphoma.[12] Nevertheless, the mechanisms by which EBV contributes to the pathogene-

sis of endemic BL are still unclear. In these tumors, in fact, EBV usually shows the most restricted pattern of latency, which is limited to EBERs and the EBNA-1 protein (Latency I), stimulating a debate as to how EBNA-1 or, indeed, the non-coding EBER RNAs (present in all forms of latency) might contribute to BL pathogenesis. The operational classification of EBV latency patterns has been recently challenged by the demonstration that other EBV latent proteins can be expressed by tumor cells in a fraction of BL. In particular, a broadened expression of EBV antigens, including EBNA-3 to -6 and BHRF1 proteins, has been demonstrated in BLs carrying an EBNA-2 gene-deleted EBV genome.[13] Available data indicate that loss of the c-MYC antagonist EBNA-2 may provide a survival advantage to lymphoma cells,[14] suggesting that EBV may act as an anti-apoptotic rather than a growth-promoting agent in BL, by selecting restricted transcriptional programs compatible with the inherently high c-MYC expression. In fact, forced expression of the full Latency III program in BL cells is incompatible with high c-MYC expression and with maintenance of the malignant BL cell phenotype.[15,16] Malaria has long been linked to the development of endemic BL, mainly based on the geographical correlations between the prevalence of malaria and the reported incidence of endemic BL.[17] Notably, two recent case-control studies demonstrated a markedly high risk of BL in individuals with the highest antibody titers against both EBV and *Plasmodium falciparum*, also suggesting a synergistic effect of the two infections.[18,19] Moreover, impaired EBV-specific T-cell responses have been observed in children from malaria endemic regions at the same age in which BL incidence peaks.[20] This malaria-induced immune aberration seems specific for the responses to EBV, leaving unaltered the cellular immunity to other herpes viruses, and mainly consists in a decrease in the number of central memory T cells specific for EBV.[20] A direct link between malaria and EBV-infected cells is represented by the cysteine-rich interdomain-1a of the *P. falciparum* erythrocyte membrane protein that was shown to trigger EBV reactivation.[22] Moreover, through activation of Toll-like receptor-9, *P. falciparum* may induce in B lymphocytes the expression of activation-induced cysteine deaminase (AID), a DNA modifying enzyme that may contribute to the induction of c-MYC-activating chromosomal translocations characteristic of

Table 1. EBV-driven lymphoproliferative disorders.

EBV-associated B-cell lymphoproliferative disorders
Burkitt's lymphoma
Hodgkin's lymphoma
PTLD
HIV-related lymphoproliferative disorders
Lymphomatoid gramulomatosis
Pyothorax-associated lymphoma
EBV- positive DLBCL of the elderly
EBV-associated T/NK-cell lymphoproliferative disorders
Peripheral T-cell lymphoma
AILT
Extranodal nasal T-/NK-cell lymphoma
Hepatosplenic T-cell lymphoma
Non-hepatosplenic γδ T-cell lymphomas
Enteropathy-type T-cell lymphoma

Table 2. EBV-associated lymphoproliferative disorders and the corresponding forms of viral latency.

Disease	% of EBV-related cases	Viral proteins expressed	Latency type
Infectious mononucleosis	>99	EBNA-1, -2, -3, -4, -5, -6, LMP-1, LMP-2A, -2B	III, II, I
Burkitt's lymphoma			
Endemic	>95	EBNA-1, EBERs, BARF0	I
Sporadic	20-80[a]		
AIDS-related	30-50		
Post-transplant lymphoproliferative disorders	>90	EBNA-1, -2, -3, -4, -5, -6, LMP-1, LMP-2A, EBERs, BARF0	III, II, I
AIDs-related DLBCL			
Immunoblastic	70-100	All EBNAs, LMPs, EBERs, BARF0	III
Non-immunoblastic	10-30	All EBNAs, LMPs, EBERs, BARF0	III
CNS lymphomas	>95	All EBNAs, LMPs, EBERs, BARF0	III
Plasmablastic lymphoma	60-75	EBERs only??	0?
Primary effusion lymphoma	70-90	EBNA-1, EBERs	I
Primary effusion lymphoma			
HIV-unrelated	70-90	EBNA-1, EBERs	I
HIV-associated	70-90		
Hodgkin's lymphoma			
HIV-unrelated	20-90[b]	EBNA-1, LMP-1, LMP-2A, EBERs, BARF0	II
HIV-associated	≈100		
EBV+ DLBCL of the elderly	≈100	EBNA-1, EBNA-2 (30%), LMP-1, LMP-2A, -2B, EBERs, BARF0	II/III
Extranodal, T-/NK-cell lymphoma, nasal type	≈100	EBNA-1, LMP-1, LMP-2A, EBERs, BARF0	II

[a]*The prevalence of EBV associated cases varies widely in different areas of the world, with 20% in North America and Europe and higher percentages in some regions of South America, North Africa and Asia.* [b]*In Western countries the prevalence of EBV-association is approximately 40%, whereas in developing countries the global proportion of EBV-associated HL is higher (up to 90%), particularly for those cases occurring in early childhood.*[7]

BL.[23] With regard to HIV-associated BLs, the observation that these lymphomas arise in patients with a relatively high CD4 T-cell count indicates that immunosuppression *per se* is not sufficient to explain the development of these cases. Similarly to chronic *P. falciparum* infection and endemic BL, also in HIV-associated BL a chronic antigenic stimulation of B lymphocytes may be of pathogenic relevance, as also suggested by the increased serum levels of B-cell activation markers detected prior to the onset of these lymphomas.[24,25] Chronic activation induced by HIV and EBV infection may concur in the induction in B lymphocytes of DNA-modifying enzymes, such as AID[26] and RAG1/2[27] which may favor the generation of chromosomal translocation of lymphomagenic relevance.

Diffuse large B-cell lymphoma of the elderly

EBV-positive diffuse large B-cell lymphoma (DLBCL) of the elderly was first recognized in the Japanese population[28] and it has been recently included as a provisional entity among the DLBCL subtypes within the WHO classification. It is defined as blastic proliferation of an EBV+ B-cell clone in patients over 50 years of age with no known cause of immunodeficiency or prior lymphoma.[29] Nevertheless, lymphomas with the same histopathological features and EBV association may also occur in younger individuals and in children.[30,31] EBV-positive DLBCL of the elderly accounts for approximately 10% of all DLBCL among Asian patients, whereas the limited data available indicate that it is quite rare in Western countries.[30,32] On the basis of the obvious morphological similarities with immunodeficiency-associated lymphoproliferative disorders, it has been proposed that the development of EBV-positive DLBCL of the elderly may be favored by the immune senescence inherent to aging. In addition to a decrease in naïve T cells, the decreased function of EBV-specific T lymphocytes characterizing the elderly[33] may allow an uncontrolled proliferation of EBV-infected B cells, with a consequent increased risk for developing a lymphoma. The usually broad pattern of EBV latency observed in these lymphomas is consistent with a subclinical impairment of immune responses and this may include EBERs, EBNA-1, and LMP-1 (Latency II) and also EBNA-2 (latency III) in up to 32% of cases.[28,30,34] The prognosis of this subset of DLBCL is generally worse than the age-matched cases without EBV infection.[28,32]

Hodgkin's lymphoma

Hodgkin's lymphoma (HL) is a distinct disorder characterized by a relatively low proportion (<10%) of the distinguishing malignant cells, the so-called Hodgkin-Reed-Sternberg cells (HRS), which are scattered in an abundant admixture of inflammatory and accessory cells. The WHO classification identifies classic and non-classic HL based on different morphological, phenotypic, and molecular features.[29] Classic HL includes the nodular-sclerosis, mixed-cellularity, lymphocyte-rich, and lymphocyte-depleted histological subtypes, whereas nodular lymphocyte-predominant HL represents the non-classic form. EBV is more commonly associated with classic HL, especially the mixed-cellularity subtypes. The non-classic nodular lymphocyte-predominant HL cases are very rarely associated with EBV. In Western countries, EBV is infrequently found in HL of young adults, although an association with delayed exposure to EBV and a history of IM is recognized.[7] In contrast, HL of the elderly is more frequently associated with EBV, as a possible consequence of the declined immune functions related to aging. In developing countries, the global proportion of EBV-associated HL is markedly higher, particularly for the cases occurring in early childhood.[35] The pathogenic role of EBV is supported by the demonstration of high titers of EBV-specific antibodies at diagnosis and, more compellingly, before onset of the disease. The virus is harbored in monoclonal form in all HRS cells, which express EBERs, EBNA-1, LMP-1, and LMP-2. Available evidence indicates that HRS cells are derived from pre-apoptotic germinal center B lymphocytes carrying crippling immunoglobulin gene mutations being, therefore, cells committed to die by apoptosis because of missing functional B-cell receptors (BCRs). EBV infection can rescue these cells from apoptosis, mainly thanks to the ability of LMP-1 and LMP-2 to mimic CD40- and BCR-mediated signaling.[35]

EBV-associated NK-cell and T-cell lymphomas

Extranodal nasal NK-/T-cell lymphoma (ENKL) is an angiocentric and angiodestructive tumor associated with prominent necrosis which predominantly involves the nasal cavity and has a geographical predilection for East Asia and Central America.[36] Most of the cases are EBV-associated as shown by the presence of clonal virus genomes in all tumor cells.[37,38] While the non-coding EBERs are abundantly expressed, a heterogeneous expression of LMP-1 and LMP-2 was found, indicating a prevalent latency II program.[38,39] The reported difficulty in detecting LMP-2 mRNA and protein in ENKL biopsies has recently found a possible explanation in the discovery of a novel LMP-2 transcript encoding for a truncated protein, which may have a role in the pathogenesis if these lymphomas.[40] Recently, expression of LMP-1, detected in more than 70% of ENKL, was correlated with activation of NF-κB and Akt and with a mainly localized disease with favorable clinical outcome.[41] A small proportion of tumor cells were found to enter the lytic cycle of the virus, suggesting a possible pathogenic involvement of a local EBV reactivation.[41] Aggressive NK cell leukemia is a closely related entity presenting at a younger age than ENKL and often showing a fulminant clinical course. Genetic and immunophenotypic profile and association with EBV are similar to those of ENKL.

Other histotypes of T/NK lymphomas

Other T-/NK-cell lymphoproliferative disorders that have been rarely reported to be EBV associated include angioimmunoblastic T-cell lymphoma,[42] a subset of peripheral T-cell lymphomas, unspecified,[43] enteropathy-type T-cell lymphoma,[44,45] γδ T-cell lymphomas (hepatosplenic and non-hepatosplenic),[46] T-cell lymphoproliferative disorders after chronic EBV infection,[47] and EBV-associated cutaneous T-cell lymphoproliferative disorders (especially in Asia).[48]

Pathogenic role of EBV latency proteins

The generation of recombinant forms of EBV lacking individual latent genes allowed the identification of the genes that are essential for B-cell immortalization *in vitro*. These studies have provided convincing evidence that EBNA-2 and LMP-1 are essential requirements for initiating the growth program in B cells, whereas a crucial role is played by EBNA-1, EBNA-3, -5, and -6.[49] Immortalization is achieved through the activity of viral proteins that derange cellular pathways controlling growth and/or survival. These viral proteins usually act co-operatively and may induce different biological effects in different cellular backgrounds. The main biological effects of latent EBV proteins more frequently expressed in EBV-driven lymphomas and contributing to lymphomagenesis are briefly summarized below. For a more detailed description see Middeldorp *et al*.[50] and Saha and Robertson.[51]

EBNA-1

EBNA-1 is a DNA-binding phosphoprotein essential for replication and stable persistence of viral episomes within EBV-infected cells and is the only EBV-encoded protein consistently expressed in all EBV-driven lymphomas.[50] Although transgenic expression of EBNA-1 in mouse B cells appears to lead to B-cell lymphoma only inconsistently, more convincing data point to an oncogenic role of this protein. In particular, EBNA-1 was shown to induce genomic instability and a DNA damage response through increased production of reactive oxygen species.[52] Moreover, EBNA-1 can induce the expression of the recombinases RAG-1 and RAG-2, suggesting that this protein might facilitate genomic recombination events and could thereby contribute to the c-myc/Ig locus translocation that is crucial for BL development.[27,53] EBNA-1 also promotes B-cell survival as shown by the pro-apoptotic effects induced in BL cells by EBNA-1 silencing and by inhibition of p53-dependent apoptosis after ectopic expression in an EBV-negative background.[54] In addition, EBNA-1 may indirectly destabilize p53 by blocking the interaction between p53 and USP7, the p53 ubiquitin protease.[55] The anti-apoptotic properties of EBNA-1 probably contribute to the persistence of EBV-infected B cells *in vivo*. This ability is particularly relevant in the EBV biology if it is considered that the majority of B lymphocytes that physiologically progress through the various stages of B-cell differentiation die by apoptosis. Rescuing from apoptosis B cells that are committed to die may allow EBV to contribute to lymphomagenesis, as shown in the case of HRS cells of HL, which may survive in the presence of EBV infection despite the fact they carry non-functional rearrangements of their immunoglobulin genes.[35] Moreover, the anti-apoptotic function of EBNA-1 may sustain the survival of transformed cells long after they have accumulated genetic changes that render them independent of normal cellular controls. This hypothesis is supported by the observation that inhibition of EBNA-1 functions with a dominant negative EBNA-1 derivative decreased survival in several EBV-carrying tumor cell lines.[54]

EBNA-2

The EBNA-2 protein is localized in the nucleus and is one of the first viral proteins expressed in EBV infected B-lymphocytes.[56] In co-operation with EBNA-5, EBNA-2 induces the G_0 to G_1 transition of resting B cells and is a key regulator of several virus-encoded genes (i.e. LMP-1 and LMP-2).[56] In addition, EBNA-2 modulates the transcriptional activity of several B-cell activation markers including CD21, CD23, hes-1, runx3, and the proto-oncogene c-MYC.[56] The function of EBNA-2 as transcriptional transactivator mainly depends on its ability to bind RBP-Jκ, thus mimicking a constitutively activated Notch receptor, which is frequently activated in several lymphomas. Considering that EBNA-2 is immunogenic and, consequently, that its expression is allowed only in immunocompromised patients, EBNA-2 is probably not required for the maintenance of the transformed phenotype in lymphomas of immunocompetent patients.

LMP-1

LMP-1 is expressed in most EBV-associated lymphoproliferations and is essential for EBV-mediated B-cell transformation.[49] LMP-1 resembles a constitutively active cellular receptor whose ligand-independent signaling activity is attributable to spontaneous homo-oligomerization of LMP-1 molecules within the membrane.[57] By the recruitment of tumor necrosis factor receptor-associated factors molecules, LMP-1 mimics molecular functions of the CD40 receptor in B-cell activation and proliferation. However, compared to CD40, LMP-1 assembles a unique and more efficient signaling complex.[58,59] LMP-1 activates multiple signaling pathways including mitogen-activated protein kinase, c-jun N-terminal kinase, phosphatidylinositol 3-kinase/Akt, IRF, WNT, and NF-κB.[49] Constitutive expression of LMP-1 in the B-cell compartment of transgenic mice induces B-cell lymphomas.[60,61] LMP-1 alone can induce many of the phenotypic and functional changes associated with EBV infection, including increased homotypic adhesion and upregulation of adhesion molecules (LFA-1, ICAM-1, LFA-3), B-cell activation markers (CD23, CD30, CD40, CD71), and anti-apoptotic genes (Bcl-2, Bcl-xL, Mcl-1, A20).[49,50,62] Expression of LMP-1 in B lymphocytes up-regulates IL-10 production which stimulates the growth of these cells and may inhibit local immune responses.[63] LMP-1 was also shown to transactivate the hTERT promoter and enhance telomerase activity in B cells, thereby contributing to the establishment of latency and transformation.[64] On the basis of these pleiotropic properties, LMP-1 is considered the major EBV-encoded oncoprotein.

LMP-2

LMP-2 is a hydrophobic membrane protein that exists as two different forms, LMP-2A and LMP-2B, resulting from alternative spliced mRNAs transcribed from the region spanning the terminal repeats of the EBV genome.[65] Although the LMP-2 protein is not essential for B-cell transformation *in vitro*, the expression of this viral gene in EBV-carrying memory B cells from healthy individuals suggests that it plays an important role in mediating virus persistence.[65] Expression of LMP-2A in transgenic mice was shown to provide survival signals allowing immature B cells to progress through developmental checkpoints that would normally result in cell death.[66] This has been related to the ability of LMP-2A to activate the PI3-K/Akt pathway that physiologically provides a

survival signal in response to BCR triggering.[67] LMP-2 was also shown to block BCR signaling through its binding to the cellular tyrosine kinases Lyn and Syk, thus preventing the possible induction of EBV lytic cycle promoted by BCR triggering.[65] Results from gene expression profiling demonstrated that LMP-2A expression is associated with a marked downregulation of several transcription factors crucial for B-lymphocyte development, such as E2A, early cell factor, and Pax-5.[68] Strikingly, malignant HRS cells of HL show a similar pattern of global downregulation of B cell-specific genes,[69] pointing to a role for LMP-2A in the induction of the transcriptional alterations that characterize these cells and allow them to survive without a functional BCR.

Role of EBV lytic replication in lymphomagenesis

The characteristics of EBV-driven lymphomas are a predominantly latent pattern of viral gene expression and that several EBV latency proteins are strictly required for B-cell immortalization *in vitro*.[70-73] These findings have suggested that only the latency phase of EBV infection is relevant for EBV-driven lymphomagenesis. Nevertheless, recent data have challenged this scenario suggesting that lytic EBV replication may also contribute to the development of EBV-associated lymphoproliferations (Figure 1). In fact, small numbers of lytically infected cells are frequently detected in tumor tissues of EBV-driven lymphomas.[74,75] Besides favoring the local and systemic spread of the virus, lytic infection may increase the pool of latently infected cells, which is associated with a higher risk of a clonal expansion of EBV-carrying B lymphocytes in the immune compromised host. Consistent with a lymphomagenic role of lytic infection, prophylactic treatment of transplant patients with antiviral drugs able to inhibit EBV lytic replication was shown to reduce the occurrence of EBV-associated PTLD.[76,77] More direct insights came from the observation that LCLs generated with EBV strains defective for lytic replication are markedly less effective in the induction of EBV-positive lymphoproliferations in SCID mice.[78] The impaired *in vivo* growth showed by LCLs with lytic-defective viruses could be overcome by restoration of lytic gene expression, further supporting a pathogenic role for these viral products in EBV-driven lymphomagenesis.[78] Notably, early-passage LCLs obtained with lytic-defective EBV produce markedly lower amounts of the B-cell growth-promoting factors IL-6, cIL-10, and vIL-10,[78] suggesting that local induction or reactivation of EBV lytic cycle may contribute to the growth of latently infected cells by promoting the release of paracrine B-cell growth factors. This phenomenon may be at least in part ascribed to the activity of BZLF1, the main EBV lytic transactivator.[78,79] BZLF1 was also shown to transactivate the expression of IL-13,[80] another B-cell growth-promoting cytokine frequently detected in HRS cells of HL and in NK cells of patients with chronic active EBV infection.[81,82] IL-13 may also promote the induction of a microenvironment sustaining the growth and survival of EBV-infected tumor cells. In fact, IL-13 downstream proteins, such as ICAM-1, macrophage-derived chemokine, thymus and activated related chemokine, and eotaxin, may attract macrophages, Th2 cells and fibroblasts and favor their clustering with tumor cells, as it may occur in HL.[83,84] However, the role of BZLF1 in the IL-13 induction in EBV-associated HL remains to be clarified, since BZLF1 is only infrequently expressed by HRS cells.[85] Lytically EBV-infected B lymphocytes were also shown to secrete factors, such as VEGF and IL-8, which may promote angiogenesis.[86] In addition to cIL-10 and vIL-10, B cells undergoing EBV lytic replication also pro-

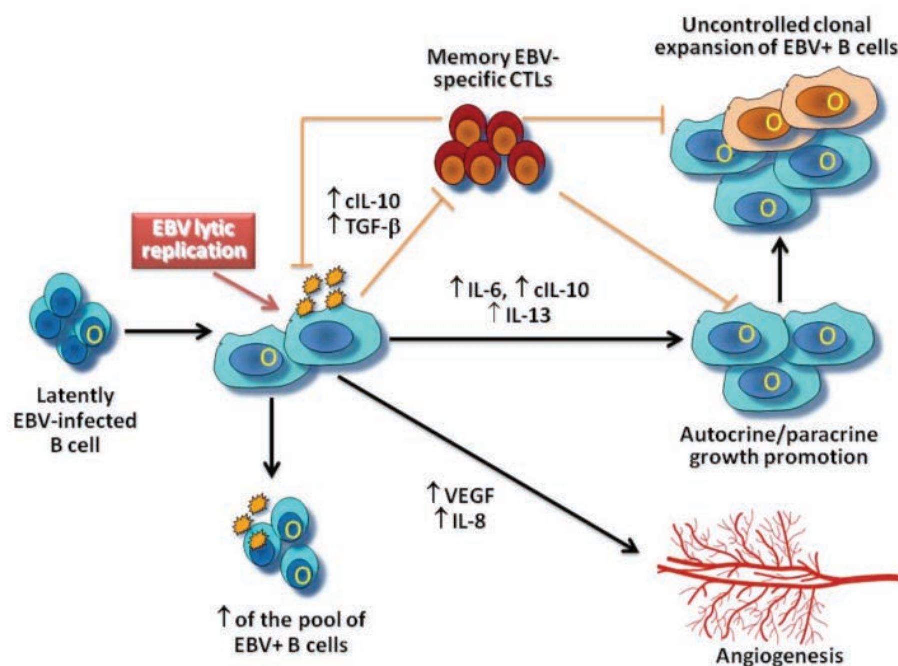

Figure 1. Contribution of EBV lytic replication to the early phases of B-cell lymphomagenesis.

duce high levels of TGF-β, which further contributes to the generation of an immunosuppressive microenvironment.[87] The pathogenic involvement of EBV lytic replication has been convincingly demonstrated in a new humanized NOD/LtSz-*scid*/*IL2Rγ*^{null} mouse model in which both human CD34^{+} hematopoietic stem cells and human thymus/liver tissue are engrafted.[88] In fact, animals infected with fully competent virus developed lymphomas more frequently than those infected with BZLF1-deleted, lytic cycle-defective EBV.[88] Considering that this animal model allows effective immune responses to be generated against EBV-infected B cells, these findings are consistent with a crucial lymphomagenic role of lytic EBV infection also in the context of an active immune response.[88]

Viral and cellular miRNA: new players in the field of EBV-driven lymphomas?

The study of the complex interactions between viral and cellular proteins has long characterized the efforts made to understand the mechanisms underlying viral oncogenesis. Nevertheless, a new class of molecules, the microRNAs (miRNAs), has recently emerged as a further critical mediator of virus-host interactions, starting from the discovery of virally-encoded miRNAs in EBV[89] and other herpes viruses. miRNAs are small (≈22 nucleotides), non-coding single-stranded RNAs that regulate gene expression by binding to complementary sequences within mRNAs. They are first transcribed by RNA polymerase II as primary transcripts (pri-miRNA) and are then processed into mature miRNAs by the type-III RNase endonucleases Drosha and Dicer.[90] Mature miRNAs can be incorporated into the RNA-induced silencing complex, where they can interact with several target mRNAs and inhibit their expression through mRNA destabilization followed by degradation and/or impairment of protein translation. The use of molecular and bio-informatic approaches allowed 44 mature EBV miRNAs derived from 25 precursors to be identified.[89,91,92] Two groups of EBV miRNAs can be distinguished based on their locations in the EBV genome: BHRF1 miRNAs located within introns of the *BHRF1* gene (encoding for a bcl-2 homolog) and produced from the long EBNA transcript and BART miRNAs, located in introns contained within the BART transcripts.[89,91] Studies carried out with viral miRNA deletion mutants indicate that EBV miRNAs contribute to EBV-driven B-cell immortalization *in vitro*, although they are not essential.[93-95] Despite the fact that the EBV B95.8 laboratory strain bears a deletion within the BART region and is able to encode only 5 of the 22 BART miRNAs, this virus is still able to immortalize B cells.[93] Nevertheless, mutational inactivation of the BHRF1 miRNAs inhibits the outgrowth of LCLs, slows down the G_1 to S phase progression of these cells and enhances their apoptotic rates.[93-95] Therefore, BHRF1 miRNAs may provide an advantage during the early stages of primary B-cell infection when the viral life cycle is critical for the establishment of long-term latency. Expression of the BHRF1 miRNAs *in vivo* is, however, restricted to type III latency, which is mainly observed in immunocompromised hosts, while BART miRNAs are variably expressed at all latency stages.[91,96] Notably, EBV-driven lymphomas characterized by the more restricted forms of latency, such as BL (latency I) and HL (latency II), were shown to express the broad spectrum of BART miRNAs detected in latency III tumors, whereas BHRF1 miRNAs were not expressed or only barely detectable.[96] These intriguing results point to a pathogenic involvement of BART miRNA deregulation in EBV-associated lymphomas. In particular, expression of latency III-associated BART miRNAs may successfully deregulate viral and cellular functions that are relevant for B-cell transformation in the face of a competent immunity that does not allow the expression of transforming but immunogenic viral proteins. In this respect, the identification of cellular mRNAs targeted by BART miRNAs will be crucial for a deeper understanding of the still obscure mechanisms underlying EBV-mediated transformation in immunocompetent individuals. Available evidence indicates that BART miRNAs have only a minor role in regulating viral genes, most likely functioning in down-regulating transcripts from the host cell.[97] In particular, BART miRNAs were shown to down-regulate PUMA and Bim, two BH3-only proteins of the Bcl-2 family with pro-apoptotic functions, thus promoting enhanced cell survival.[98,99] Moreover, BART miRNAs was also shown to suppress expression of the stress-induced NK cell ligand MICB in B cells, to escape recognition and consequent elimination by NK cells.[100] Notably, miRNAs with similar function but engaging different target sites within the MICB transcript have also been identified in cytomegalovirus and Kaposi sarcoma human herpesvirus, indicating that several herpesviruses have independently evolved mechanisms able to successfully counteract this critical mediator of innate immunity.

EBV not only expresses its own set of viral miRNAs, but also has the ability to strongly affect the cellular miRNA profile, inducing a downregulation of the overall level of cellular miRNAs in primary B lymphocytes.[101] However, the level of a subset of cellular miRNAs, including miR-155, is markedly increased by EBV infection of B cells, an effect apparently due to activation of pri-miR-155 transcription.[102,103] This is an intriguing finding, considering that other transforming herpesviruses encode viral orthologs of cellular miR155 that down-regulate a similar set of cellular mRNAs. Notably, miR-155 is an oncogenic miRNA critical for B-cell maturation and immunoglobulin production in response to antigen.[104] In particular, blocking miR-155 function was found to induce cell cycle arrest and apoptosis of EBV-immortalized LCLs.[105] Moreover, ectopic miR-155 expression in mice B cells has been shown to induce pre-B-cell proliferation followed by high-grade lymphoma/leukemia, effects mediated by downregulation of the key transcriptional repressor and proto-oncogene Bcl-6.[106] The study of cellular miRNA expression during EBV-mediated primary B-cell growth transformation also demonstrated that the virus up-regulates mir-21, another putative pro-growth onco-miR, and represses putative tumor suppressor onco-miRs, such as let-7 and miR-29 family members.[107] In B cells, the EBV-encoded LMP-1 oncoprotein up-regulates mir-146a, which acts as an inhibitor of interferon response pathway, thereby helping suppress immune-mediated surveillance of EBV-infected cells through interferon signaling.[108] EBV LMP-1 also up-regulates miR-34a, whose expression is required for the proliferation of EBV-transformed B cells.[107] This pro-growth function of miR-34a

contrasts with its canonical role as tumor suppressor and strengthens the need to study miRNA functions in different cell types.

New therapeutic perspectives

The progressive increase in our knowledge of the multiple mechanisms exploited by EBV for the development of lymphoid malignancies is providing an increasing number of new therapeutic targets. The immunogenic latent EBV proteins can be targeted by specific cytotoxic T lymphocytes that can be easily generated and infused in patients with EBV-driven lymphoproliferations.[109,110] Although relevant clinical benefits were especially registered in the management of PTLD, the outcome of patients with lymphomas expressing more restricted patterns of EBV latency, such as in HL, is not satisfactory and the treatment protocols still require specific improvements.[109,110] Identification of the cellular signalling pathways hijacked by EBV offers the opportunity to design small molecules or pharmacological inhibitors potentially able to selectively block critical interactions with EBV oncoproteins. Further studies on the altered expression of viral or cellular miRNAs that influence specific pathways involved in lymphoma development and progression may help in the identification and validation of new therapeutic options for EBV-associated lymphoproliferations. This perspective is particularly encouraging since miRNAs can regulate multiple targets of the same or different signaling pathways, thereby minimizing the development of resistance or compensatory mechanisms. Notably, studies carried out in pre-clinical animal models have validated several miRNAs as suitable pharmacological targets, demonstrating similar, enhanced or additive effects to standard treatments. There is also an increasing interest in developing strategies able to reactivate EBV lytic gene expression in latently infected tumor cells for the treatment of overt EBV-associated lymphomas. In fact, lytic infection may promote the death of EBV-positive lymphoma cells *in vivo*, an effect that may be particularly effective and therapeutically relevant since it favors immune recognition of viral antigens that further enhances the killing of tumor cells. Several chemotherapeutic drugs are known to trigger EBV replication, and combination of antivirals with lytic cycle inducers is emerging as a highly promising strategy for the treatment of EBV-driven lymphomas.[111] Successful exploitation of these and other virus-related characteristics will lead to improved control of EBV-associated lymphomas.

References

1. Epstein MA, Achong BG, Barry YM. Virus particles in cultured lymphoblasts from Burkitt's Lymphoma. Lancet. 1964;1(7335):702-3.
2. Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA. EBV persistence in memory B cells in vivo. Immunity. 1998;9(3):395-404.
3. Miyashita EM, Yang B, Babcock GJ, Thorley-Lawson DA. Identification of the site of Epstein-Barr virus persistence in vivo as a resting B cell. J Virol. 1997;71(7):4882-91.
4. Babcock GJ, Hochberg D, Thorley-Lawson AD. The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. Immunity. 2000;13(4):497-506.
5. Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med. 2004;350(13):1328-37.
6. Yajima M, Kanda T, Takada K. Critical role of Epstein-Barr Virus (EBV)-encoded RNA in efficient EBV-induced B-lymphocyte growth transformation. J Virol. 2005;79(7):4298-307.
7. Farrell K, Jarrett RF. The molecular pathogenesis of Hodgkin lymphoma. Histopathology. 2011;58(1):15-25.
8. Landgren O, Gilbert ES, Rizzo JD, Socie G, Banks PM, Sobocinski KA, et al. Risk factors for lymphoproliferative disorders after allogeneic hematopoietic cell transplantation. Blood. 2009;113(20):4992-5001.
9. Gross TG, Savoldo B, Punnett A. Posttransplant lymphoproliferative diseases. Pediatr Clin North Am. 2010;57(2):481-503.
10. Niedobitek G, Agathanggelou A, Rowe M, Jones EL, Jones DB, Turyaguma P, et al. Heterogeneous expression of Epstein-Barr virus latent proteins in endemic Burkitt's lymphoma. Blood. 1995;86(2):659-65.
11. Neri A, Barriga F, Inghirami G, Knowles DM, Neequaye J, Magrath IT, et al. Epstein-Barr virus infection precedes clonal expansion in Burkitt's and acquired immunodeficiency syndrome-associated lymphoma. Blood. 1991;77(5):1092-5.
12. Geser A, de Thé G, Lenoir G, Day NE, Williams EH. Final case reporting from the Ugandan prospective study of the relationship between EBV and Burkitt's lymphoma. Int J Cancer. 1982;29(4):397-400.
13. Kelly G, Bell A, Rickinson A. Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. Nat Med. 2002;8(10):1098-104.
14. Kelly GL, Milner AE, Tierney RJ, Croom-Carter DS, Altmann M, Hammerschmidt W, et al. Epstein-Barr virus nuclear antigen 2 (EBNA2) gene deletion is consistently linked with EBNA3A, -3B, and -3C expression in Burkitt's lymphoma cells and with increased resistance to apoptosis. J Virol. 2005;79(16):10709-17.
15. Polack A, Hortnagel K, Pajic A, Christoph B, Baier B, Falk M, et al. c-myc activation renders proliferation of Epstein-Barr virus (EBV)-transformed cells independent of EBV nuclear antigen 2 and latent membrane protein 1. Proc Natl Acad Sci USA. 1996;93(19):10411-6.
16. Pajic A, Staege MS, Dudziak D, Schuhmacher M, Spitkovsky D, Eissner G, et al. Antagonistic effects of c-myc and Epstein-Barr virus latent genes on the phenotype of human B cells. Int J Cancer. 2001;93(6):810-6.
17. Rainey JJ, Mwanda WO, Wairiumu P, Moormann AM, Wilson ML, Rochford R. Spatial distribution of Burkitt's lymphoma in Kenya and association with malaria risk. Trop Med Int Health. 2007;12(8):936-43.
18. Carpenter LM, Newton R, Casabonne D, Ziegler J, Mbulaiteye S, Mbidde E, et al. Antibodies against malaria and Epstein-Barr virus in childhood Burkitt lymphoma: a case-control study in Uganda. Int J Cancer. 2008;122(6):1319-23.
19. Mutalima N, Molyneux E, Jaffe H, Kamiza S, Borgstein E, Mkandawire N, et al. Associations between Burkitt lymphoma among children in Malawi and infection with HIV, EBV and malaria: results from a case-control study. PLoS One. 2008;3(6):e2505.
20. Moormann AM, Chelimo K, Sumba OP, Lutzke ML, Ploutz-Snyder R, Newton D, et al. Exposure to holoendemic malaria results in elevated Epstein-Barr virus loads in children. J Infect Dis. 2005;191(8):1233-8.
21. Chattopadhyay PK, Chelimo K, Embury PB, Mulama DH, Sumba PO, Gostick E, et al. Holoendemic Malaria Exposure Is Associated with Altered Epstein-Barr Virus-Specific CD8+ T-Cell Differentiation. J Virol. 2013;87(3):1779-88.
22. Chêne A, Donati D, Guerreiro-Cacais AO, Levitsky V, Chen Q, Falk KI, et al. A molecular link between malaria and Epstein-Barr virus reactivation. PLoS Pathog. 2007;3(6):e80.
23. Potup P, Kumsiri R, Kano S, Kalambaheti T, Looareesuwan S, Troye-Blomberg M, et al. Blood stage Plasmodium falciparum antigens induce immunoglobulin class switching in human enriched B cell culture. Southeast Asian J Trop Med Public Health. 2009;40(4):651-64.
24. Grulich AE, Wan X, Law MG, Milliken ST, Lewis CR, Garsia RJ, et al. B-cell stimulation and prolonged immune deficiency are risk factors for non-Hodgkin's lymphoma in people with AIDS. AIDS. 2000;14(2):133-40.
25. Breen EC, Fatahi S, Epeldegui M, Boscardin WJ, Detels R, Martinez-Maza O. Elevated serum soluble CD30 precedes the development of AIDS-associated non-Hodgkin's B cell lymphoma. Tumour Biol. 2006;27(4):187-94.
26. Epeldegui M, Breen EC, Hung YP, Boscardin WJ, Detels R, Martinez-Maza O. Elevated expression of activation induced

cytidine deaminase in peripheral blood mononuclear cells precedes AIDS-NHL diagnosis. AIDS. 2007;21(17):2265-70.
27. Srinivas SK, Sixbey JW. Epstein-Barr virus induction of recombinase-activating genes RAG1 and RAG2. J Virol. 1995;69(12):8155-8.
28. Oyama T, Ichimura K, Suzuki R, Suzumiya J, Ohshima K, Yatabe Y, et al. Senile EBV+ B-cell lymphoproliferative disorders: a clinicopathologic study of 22 patients. Am J Surg Pathol. 2003;27(1):16-26.
29. Swerdlow S, Campo E, Harris N, Jaffe E, Pileri SA, Stein H, et al. World Health Organization classification of tumors, pathology and genetics of tumours of Hematopoietic and Lymphoid Tissues. International Agency of Research on Cancer (IARC). 2008.
30. Kuze T, Nakamura N, Hashimoto Y, Sasaki Y, Abe M. The characteristics of Epstein-Barr virus (EBV)-positive diffuse large B-cell lymphoma: comparison between EBV(+) and EBV(-) cases in Japanese population. Jpn J Cancer Res. 2000;91(12):1233-40.
31. Cohen M, De Matteo E, Narbaitz M, Carreno FA, Preciado MV, Chabay PA. Epstein-Barr virus presence in pediatric diffuse large B-cell lymphoma reveals a particular association and latency patterns: Analysis of viral role in tumor microenvironment. Int J Cancer. 2013;132(7):1572-80..
32. Park S, Lee J, Ko YH, Han A, Jun HJ, Lee SC, et al. The impact of Epstein-Barr virus status on clinical outcome in diffuse large B-cell lymphoma. Blood. 2007;110(3):972-8.
33. Ouyang Q, Wagner WM, Walter S, Muller CA, Wikby A, Aubert G, et al. An age-related increase in the number of CD8+ T cells carrying receptors for an immunodominant Epstein-Barr virus (EBV) epitope is counteracted by a decreased frequency of their antigen-specific responsiveness. Mech Ageing Dev. 2003;124(4):477-85.
34. Shimoyama Y, Asano N, Kojima M, Morishima S, Yamamoto K, Oyama T, et al. Age-related EBV-associated B-cell lymphoproliferative disorders: diagnostic approach to a newly recognized clinicopathological entity. Pathol Int. 2009;59(12):835-43.
35. Kuppers R, Engert A, Hansmann ML. Hodgkin lymphoma. J Clin Invest. 2012;122(10):3439-47.
36. Chan J, Wong KF, Jaffe ES, Ralfkiaer E. Aggressive NK-cell leukemia. World Health Organization classification of tumors Pathology and Genetics, Tumours of Haematopoietic and Lymphoid Tissue IARC Press. 2001.
37. Harabuchi Y, Yamanaka N, Kataura A, Imai S, Kinoshita T, Mizuno F, et al. Epstein-Barr virus in nasal T-cell lymphomas in patients with lethal midline granuloma. Lancet. 1990; 335(8682):128-30.
38. Minarovits J, Hu LF, Imai S, Harabuchi Y, Kataura A, Minarovits-Kormuta S, et al. Clonality, expression and methylation patterns of the Epstein-Barr virus genomes in lethal midline granulomas classified as peripheral angiocentric T cell lymphomas. J Gen Virol. 1994;75(Pt 1):77-84.
39. Chiang AK, Tao Q, Srivastava G, Ho FC. Nasal NK- and T-cell lymphomas share the same type of Epstein-Barr virus latency as nasopharyngeal carcinoma and Hodgkin's disease. Int J Cancer. 1996;68(3):285-90.
40. Fox CP, Haigh TA, Taylor GS, Long HM, Lee SP, Shannon-Lowe C, et al. A novel latent membrane 2 transcript expressed in Epstein-Barr virus-positive NK- and T-cell lymphoproliferative disease encodes a target for cellular immunotherapy. Blood. 2010;116(19):3695-704.
41. Kanemitsu N, Isobe Y, Masuda A, Momose S, Higashi M, Tamaru J, et al. Expression of Epstein-Barr virus-encoded proteins in extranodal NK/T-cell Lymphoma, nasal type (ENKL): differences in biologic and clinical behaviors of LMP1-positive and -negative ENKL. Clin Cancer Res. 2012;18(8):2164-72.
42. Rudiger T, Weisenburger DD, Anderson JR, Armitage JO, Diebold J, MacLennan KA, et al. Peripheral T-cell lymphoma (excluding anaplastic large-cell lymphoma): results from the Non-Hodgkin's Lymphoma Classification Project. Ann Oncol. 2002;13(1):140-9.
43. Dupuis J, Boye K, Martin N, Copie-Bergman C, Plonquet A, Fabiani B, et al. Expression of CXCL13 by neoplastic cells in angioimmunoblastic T-cell lymphoma (AITL): a new diagnostic marker providing evidence that AITL derives from follicular helper T cells. Am J Surg Pathol. 2006;30(4):490-4.
44. Quintanilla-Martinez L, Lome-Maldonado C, Ott G, Gschwendtner A, Gredler E, Angeles-Angeles A, et al. Primary intestinal non-Hodgkin's lymphoma and Epstein-Barr virus: high frequency of EBV-infection in T-cell lymphomas of Mexican origin. Leuk Lymphoma. 1998;30(1-2):111-21.
45. de Bruin PC, Jiwa NM, Oudejans JJ, Radaszkiewicz T, Meijer CJ. Epstein-Barr virus in primary gastrointestinal T cell lymphomas. Association with gluten-sensitive enteropathy, pathological features, and immunophenotype. Am J Pathol. 1995;146(4):861-7.
46. Ohshima K, Haraoka S, Harada N, Kamimura T, Suzumiya J, Kanda M, et al. Hepatosplenic gammadelta T-cell lymphoma: relation to Epstein-Barr virus and activated cytotoxic molecules. Histopathology. 2000;36(2):127-35.
47. Quintanilla-Martinez L, Kumar S, Fend F, Reyes E, Teruya-Feldstein J, Kingma DW, et al. Fulminant EBV(+) T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. Blood. 2000;96(2):443-51.
48. Kim YC, Yang WI, Lee MG, Kim SN, Cho KH, Lee SJ, et al. Epstein-Barr virus in CD30 anaplastic large cell lymphoma involving the skin and lymphomatoid papulosis in South Korea. Int J Dermatol. 2006;45(11):1312-6.
49. Raab-Traub N. EBV-induced oncogenesis. In: Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Arvin A, Campadelli-Fiume G, Mocarski E, et al. (eds.)Cambridge: Cambridge University Press; 2007.
50. Middeldorp JM, Brink AA, van den Brule AJ, Meijer CJ. Pathogenic roles for Epstein-Barr virus (EBV) gene products in EBV-associated proliferative disorders. Crit Rev Oncol Hematol. 2003;45(1):1-36.
51. Saha A, Robertson ES. Epstein-Barr virus-associated B-cell lymphomas: pathogenesis and clinical outcomes. Clin Cancer Res. 2011;17(10):3056-63.
52. Gruhne B, Sompallae R, Marescotti D, Kamranvar SA, Gastaldello S, Masucci MG. The Epstein-Barr virus nuclear antigen-1 promotes genomic instability via induction of reactive oxygen species. Proc Natl Acad Sci USA. 2009;106(7):2313-8.
53. Kuhn-Hallek I, Sage DR, Stein L, Groelle H, Fingeroth JD. Expression of recombination activating genes (RAG-1 and RAG-2) in Epstein-Barr virus-bearing B cells. Blood. 1995;85(5):1289-99.
54. Kennedy G, Komano J, Sugden B. Epstein-Barr virus provides a survival factor to Burkitt's lymphomas. Proc Natl Acad Sci USA. 2003;100(24):14269-74.
55. Saridakis V, Sheng Y, Sarkari F, Holowaty MN, Shire K, Nguyen T, et al. Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1 implications for EBV-mediated immortalization. Mol Cell. 2005;18(1):25-36.
56. Zimber-Strobl U, Strobl LJ. EBNA2 and Notch signalling in Epstein-Barr virus mediated immortalization of B lymphocytes. Semin Cancer Biol. 2001;11(6):423-34.
57. Gires O, Zimber-Strobl U, Gonnella R, Ueffing M, Marschall G, Zeidler R, et al. Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule. EMBO J. 1997;16(20):6131-40.
58. Kilger E, Kieser A, Baumann M, Hammerschmidt W. Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. EMBO J. 1998;17(6):1700-9.
59. Kaykas A, Worringer K, Sugden B. CD40 and LMP-1 both signal from lipid rafts but LMP-1 assembles a distinct, more efficient signaling complex. EMBO J. 2001;20(11):2641-54.
60. Kulwichit W, Edwards RH, Davenport EM, Baskar JF, Godfrey V, Raab-Traub N. Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. Proc Natl Acad Sci USA. 1998;95(20):11963-8.
61. Stunz LL, Busch LK, Munroe ME, Sigmund CD, Tygrett LT, Waldschmidt TJ, et al. Expression of the cytoplasmic tail of LMP1 in mice induces hyperactivation of B lymphocytes and disordered lymphoid architecture. Immunity. 2004;21(2):255-66.
62. D'Souza BN, Edelstein LC, Pegman PM, Smith SM, Loughran ST, Clarke A, et al. Nuclear factor kappa B-dependent activation of the antiapoptotic bfl-1 gene by the Epstein-Barr virus latent membrane protein 1 and activated CD40 receptor. J Virol. 2004;78(4):1800-16.
63. Nakagomi H, Dolcetti R, Bejarano MT, Pisa P, Kiessling R, Masucci MG. The Epstein-Barr virus latent membrane protein-1 (LMP1) induces interleukin-10 production in Burkitt lymphoma lines. Int J Cancer. 1994;57(2):240-4.
64. Terrin L, Dal Col J, Rampazzo E, Zancai P, Pedrotti M, Ammirabile G, et al. Latent membrane protein 1 of Epstein-Barr virus activates the hTERT promoter and enhances telomerase activity in B lymphocytes. J Virol. 2008;82(20):10175-87.
65. Longnecker R. Epstein-Barr virus latency: LMP2, a regulator or means for Epstein-Barr virus persistence? Adv Cancer Res. 2000;79):175-200.
66. Caldwell RG, Brown RC, Longnecker R. Epstein-Barr virus LMP2A-induced B-cell survival in two unique classes of EmuLMP2A transgenic mice. J Virol. 2000;74(3):1101-13.
67. Swart R, Ruf IK, Sample J, Longnecker R. Latent membrane protein 2A-mediated effects on the phosphatidylinositol 3-

Kinase/Akt pathway. J Virol. 2000;74(22):10838-45.
68. Portis T, Dyck P, Longnecker R. Epstein-Barr Virus (EBV) LMP2A induces alterations in gene transcription similar to those observed in Reed-Sternberg cells of Hodgkin lymphoma. Blood. 2003;102(12):4166-78.
69. Schwering I, Brauninger A, Klein U, Jungnickel B, Tinguely M, Diehl V, et al. Loss of the B-lineage-specific gene expression program in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. Blood. 2003;101(4):1505-12.
70. Skare J, Farley J, Strominger JL, Fresen KO, Cho MS, zur Hausen H. Transformation by Epstein-Barr virus requires DNA sequences in the region of BamHI fragments Y and H. J Virol. 1985;55(2):286-97.
71. Kaye KM, Izumi KM, Kieff E. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc Natl Acad Sci USA. 1993;90(19):9150-4.
72. Tomkinson B, Robertson E, Kieff E. Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. J Virol. 1993;67(4):2014-25.
73. Humme S, Reisbach G, Feederle R, Delecluse HJ, Bousset K, Hammerschmidt W, et al. The EBV nuclear antigen 1 (EBNA1) enhances B cell immortalization several thousandfold. Proc Natl Acad Sci USA. 2003;100(19):10989-94.
74. Montone KT, Hodinka RL, Salhany KE, Lavi E, Rostami A, Tomaszewski JE. Identification of Epstein-Barr virus lytic activity in post-transplantation lymphoproliferative disease. Mod Pathol. 1996;9(6):621-30.
75. Xue SA, Labrecque LG, Lu QL, Ong SK, Lampert IA, Kazembe P, et al. Promiscuous expression of Epstein-Barr virus genes in Burkitt's lymphoma from the central African country Malawi. Int J Cancer. 2002;99(5):635-43.
76. Hanto DW, Frizzera G, Gajl-Peczalska KJ, Sakamoto K, Purtilo DT, Balfour HH Jr, et al. Epstein-Barr virus-induced B-cell lymphoma after renal transplantation: acyclovir therapy and transition from polyclonal to monoclonal B-cell proliferation. N Engl J Med. 1982;306(15):913-8.
77. Darenkov IA, Marcarelli MA, Basadonna GP, Friedman AL, Lorber KM, Howe JG, et al. Reduced incidence of Epstein-Barr virus-associated posttransplant lymphoproliferative disorder using preemptive antiviral therapy. Transplantation. 1997;64(6):848-52.
78. Hong GK, Gulley ML, Feng WH, Delecluse HJ, Holley-Guthrie E, Kenney SC. Epstein-Barr virus lytic infection contributes to lymphoproliferative disease in a SCID mouse model. J Virol. 2005;79(22):13993-4003.
79. Mahot S, Sergeant A, Drouet E, Gruffat H. A novel function for the Epstein-Barr virus transcription factor EB1/Zta: induction of transcription of the hIL-10 gene. J Gen Virol. 2003;84(Pt 4):965-74.
80. Tsai SC, Lin SJ, Chen PW, Luo WY, Yeh TH, Wang HW, et al. EBV Zta protein induces the expression of interleukin-13, promoting the proliferation of EBV-infected B cells and lymphoblastoid cell lines. Blood. 2009;114(1):109-18.
81. Kapp U, Yeh WC, Patterson B, Elia AJ, Kagi D, Ho A, et al. Interleukin 13 is secreted by and stimulates the growth of Hodgkin and Reed-Sternberg cells. J Exp Med. 1999;189(12):1939-46.
82. Kimura H, Hoshino Y, Hara S, Sugaya N, Kawada J, Shibata Y, et al. Differences between T cell-type and natural killer cell-type chronic active Epstein-Barr virus infection. J Infect Dis. 2005;191(4):531-9.
83. van den Berg A, Visser L, Poppema S. High expression of the CC chemokine TARC in Reed-Sternberg cells. A possible explanation for the characteristic T-cell infiltratein Hodgkin's lymphoma. Am J Pathol. 1999;154(6):1685-91.
84. Aldinucci D, Lorenzon D, Olivo K, Rapana B, Gattei V. Interactions between tissue fibroblasts in lymph nodes and Hodgkin/Reed-Sternberg cells. Leuk Lymphoma. 2004;45(9):1731-9.
85. Pallesen G, Sandvej K, Hamilton-Dutoit SJ, Rowe M, Young LS. Activation of Epstein-Barr virus replication in Hodgkin and Reed-Sternberg cells. Blood. 1991;78(5):1162-5.
86. Hong GK, Kumar P, Wang L, Damania B, Gulley ML, Delecluse HJ, et al. Epstein-Barr virus lytic infection is required for efficient production of the angiogenesis factor vascular endothelial growth factor in lymphoblastoid cell lines. J Virol. 2005;79(22):13984-92.
87. Cayrol C, Flemington EK. Identification of cellular target genes of the Epstein-Barr virus transactivator Zta: activation of transforming growth factor beta igh3 (TGF-beta igh3) and TGF-beta 1. J Virol. 1995;69(7):4206-12.
88. Ma SD, Hegde S, Young KH, Sullivan R, Rajesh D, Zhou Y, et al. A new model of Epstein-Barr virus infection reveals an important role for early lytic viral protein expression in the development of lymphomas. J Virol. 2011;85(1):165-77.
89. Pfeffer S, Zavolan M, Grasser FA, Chien M, Russo JJ, Ju J, et al. Identification of virus-encoded microRNAs. Science. 2004;304(5671):734-6.
90. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97.
91. Cai X, Schafer A, Lu S, Bilello JP, Desrosiers RC, Edwards R, et al. Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. PLoS Pathog. 2006;2(3):e23.
92. Chen SJ, Chen GH, Chen YH, Liu CY, Chang KP, Chang YS, et al. Characterization of Epstein-Barr virus miRNAome in nasopharyngeal carcinoma by deep sequencing. PLoS One. 2010;5(9.
93. Seto E, Moosmann A, Gromminger S, Walz N, Grundhoff A, Hammerschmidt W. Micro RNAs of Epstein-Barr virus promote cell cycle progression and prevent apoptosis of primary human B cells. PLoS Pathog. 2010;6(8):e1001063.
94. Feederle R, Haar J, Bernhardt K, Linnstaedt SD, Bannert H, Lips H, et al. The members of an Epstein-Barr virus microRNA cluster cooperate to transform B lymphocytes. J Virol. 2011;85(19): 9801-10.
95. Feederle R, Linnstaedt SD, Bannert H, Lips H, Bencun M, Cullen BR, et al. A viral microRNA cluster strongly potentiates the transforming properties of a human herpesvirus. PLoS Pathog. 2011;7(2):e1001294.
96. Qiu J, Cosmopoulos K, Pegtel M, Hopmans E, Murray P, Middeldorp J, et al. A novel persistence associated EBV miRNA expression profile is disrupted in neoplasia. PLoS Pathog. 2011;7(8):e1002193.
97. Marquitz AR, Raab-Traub N. The role of miRNAs and EBV BARTs in NPC. Semin Cancer Biol. 2012;22(2):166-72.
98. Choy EY, Siu KL, Kok KH, Lung RW, Tsang CM, To KF, et al. An Epstein-Barr virus-encoded microRNA targets PUMA to promote host cell survival. J Exp Med. 2008;205(11):2551-60.
99. Marquitz AR, Mathur A, Nam CS, Raab-Traub N. The Epstein-Barr Virus BART microRNAs target the pro-apoptotic protein Bim. Virology. 2011;412(2):392-400.
100. Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe. 2009;5(4):376-85.
101. Godshalk SE, Bhaduri-McIntosh S, Slack FJ. Epstein-Barr virus-mediated dysregulation of human microRNA expression. Cell Cycle. 2008;7(22):3595-600.
102. Gatto G, Rossi A, Rossi D, Kroening S, Bonatti S, Mallardo M. Epstein-Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-kappaB pathway. Nucleic Acids Res. 2008;36(20):6608-19.
103. Yin Q, McBride J, Fewell C, Lacey M, Wang X, Lin Z, et al. MicroRNA-155 is an Epstein-Barr virus-induced gene that modulates Epstein-Barr virus-regulated gene expression pathways. J Virol. 2008;82(11):5295-306.
104. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet. 2009;10(10):704-14.
105. Linnstaedt SD, Gottwein E, Skalsky RL, Luftig MA, Cullen BR. Virally induced cellular microRNA miR-155 plays a key role in B-cell immortalization by Epstein-Barr virus. J Virol. 2010;84(22):11670-8.
106. Sandhu SK, Volinia S, Costinean S, Galasso M, Neinast R, Santhanam R, et al. miR-155 targets histone deacetylase 4 (HDAC4) and impairs transcriptional activity of B-cell lymphoma 6 (BCL6) in the Emu-miR-155 transgenic mouse model. Proc Natl Acad Sci USA. 2012;109(49):20047-52.
107. Forte E, Salinas RE, Chang C, Zhou T, Linnstaedt SD, Gottwein E, et al. The Epstein-Barr virus (EBV)-induced tumor suppressor microRNA MiR-34a is growth promoting in EBV-infected B cells. J Virol. 2012;86(12):6889-98.
108. Cameron JE, Yin Q, Fewell C, Lacey M, McBride J, Wang X, et al. Epstein-Barr virus latent membrane protein 1 induces cellular MicroRNA miR-146a, a modulator of lymphocyte signaling pathways. J Virol. 2008;82(4):1946-58.
109. Merlo A, Turrini R, Dolcetti R, Martorelli D, Muraro E, Comoli P, et al. The interplay between Epstein-Barr virus and the immune system: a rationale for adoptive cell therapy of EBV-related disorders. Haematologica. 2010;95(10):1769-77.
110. Bollard CM, Rooney CM, Heslop HE. T-cell therapy in the treatment of post-transplant lymphoproliferative disease. Nat Rev Clin Oncol. 2012;9(9):510-9.
111. Wildeman MA, Novalic Z, Verkuijlen SA, Juwana H, Huitema AD, Tan IB, et al. Cytolytic virus activation therapy for epstein-barr virus-driven tumors. Clin Cancer Res. 2012;18(18):5061-70.

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Bristol Myers Squibb (Speakers Bureau, Advisory Board); Novartis (Researcher, Consultant, Speakers Bureau, Advisory Board)

Santini V
Affiliations to disclose: Yes
Celgene (Speakers Bureau); Janssen Cilag (Speakers Bureau); Novartis (Speakers Bureau)

Sanz G
Affiliations to disclose: Yes
Amgen (Honoraria, Advisory Board, Research Funding); Boehringer-Ingelheim (Advisory Board); Celgene (Honoraria, Advisory Board, Research Funding); Janssen-Cilag (Honoraria, Advisory Board); Merck Sharp & Dohme (Advisory Board); Novartis (Honoraria, Advisory Board, Research Funding)

Sanz M
Affiliations to disclose: No Affiliations

Schmiegelow K
Affiliations to disclose: Yes
Only for Children Pharmaceutical (Consultancy)

Schrappe M
Affiliations to disclose: Yes
Novartis (Research Support); Medac (Research Support); Eusapharma (Research Support); Sigmatau Rare Diseases (Research Support)

Sierra J
Affiliations to disclose: Yes
Novartis (Researcher; Advisory board); Celgene (Researcher); Roche (Researcher); Amgen (Researcher; Advisory board)

Simonsson B
Affiliations to disclose: Yes
Bristol Myers Squibb (Consultant); Novartis (Researcher, Consultant)

Socié G
Affiliations to disclose: Yes
Fresenius HemoCare BV (Speaker); Therakos (symposia); Alexion (Consultant)

Soulier J
Affiliations to disclose: No Affiliations

Stanulla M
Affiliations to disclose: No Affiliations

Stasi R
Affiliations to disclose: Yes
Symphogen (Advisory Board); Glaxosmithkline (Speakers Bureau, Advisory Board); Amgen (Speakers Bureau, Advisory Board); Suppremol (Consultant)

Stavenuiter F
Affiliations to disclose: No Affiliations

Steele A
Affiliations to disclose: No Affiliations

Stevenson F
Affiliations to disclose: No Affiliations

Sureda A
Affiliations to disclose: Yes
Takeda (Advisory board, Speaker Bureau); Novartis (Advisory Board); Sanofi (Advisory Board)

Tallman M
Affiliations to disclose: No Affiliations

Thein S-L
Affiliations to disclose: Yes
Shire Pharmaceuticals (research support, consultant); Sangart (Advisory board); Novartis (Speaker's Bureau); TauRx Therapeutics Ltd (DSMB)

Valent P
Affiliations to disclose: Yes
Novartis (Researcher); Domantis (Researcher); Celgene (Researcher); Amgen (Researcher); Genzyme (Researcher)

Valk P
Affiliations to disclose: Yes
Skyline diagnostics (ownership interests)

van Bruggen R
Affiliations to disclose: Yes
Sanquin (Research)

van de Watering L
Affiliations to disclose: Yes
Sanquin Blood Supply (employment)

van Leeuwen F
Affiliations to disclose: No Affiliations

Viprakasit V
Affiliations to disclose: Yes
Novartis Co. (Advisory Board, Speaker's Bureau And Consultant); Shire (Reseach Grant); GPO-Thailand (Research Support)

Voorberg J
Affiliations to disclose: No Affiliations

Vora A
Affiliations to disclose: No Affiliations

Walker B
Affiliations to disclose: Yes
Onyx (Honoraria); Celgene (Honoraria)

Wallis J
Affiliations to disclose: Yes
Ortho (Clinical Diagnostics, Advisory Board And Occasional Speaker)

Welch J
Affiliations to disclose: Yes
Eisai (Research Support); Sanofi (Speaker's Bureau)

Welte K
Affiliations to disclose: No Affiliations

Whitlock J
Affiliations to disclose: Yes
Mundipharma (DSMC Chair, No Honoria Or Compensation); Micromet/Amgen (Advisory Board, No Honoria Or Compensation); Pfizer (Advisory Board, No Honoria Or Compensation); Glaxo-Smith-Kline (Advisory Board, No Honoria Or Compensation); Ariad Pharmaceuticals (Advisory Board, No Honoria Or Compensation); Jazz Pharmaceuticals (Advisory Board, No Honoria Or Compensation)

Wilson W
Affiliations to disclose: No Affiliations

Young N
Affiliations to disclose: No Affiliations

Yuruk K
Affiliations to disclose: Unknown

Zolla L
Affiliations to disclose: No Affiliations

Zwaan M
Affiliations to disclose: Yes
Bristol Myers Squibb (research support); GlaxoSmithKline (consultant); Celgene (Consultant); Merck, Scarpe & Dohme (advisory board); Pfizer (consultant)

Zwick C
Affiliations to disclose: No Affiliations