Hematology Education

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

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London, United Kingdom, June 9-12, 2011

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Preface

On behalf of the EHA Board and the Scientific Program Committee we are pleased to present the Education Book of the 16th Congress of EHA. The Education Sessions, on which the content of this book is based, cover a large spectrum of basic, translational and clinical research in hematology, presented and chaired by internationally recognized experts.

We would like to thank the authors and speakers for their valuable contributions as well as the chairs and external advisors, who served as reviewers for the Education Book. Without their efforts, the quality of the book could not have been so high.

In addition to enjoying the presentations, we hope you will find the peer-reviewed manuscripts in this edition a useful source of information and reference.

Ivo Touw
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The molecular basis of T-cell acute lymphoblastic leukemia

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2011;5:1-8

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic neoplasm resulting from the malignant transformation of T-cell progenitor cells. T-ALL accounts for 10–15% of pediatric and 25% of adult ALL cases[1] and is characteristically more prevalent in males than females (male to female ratio 3:1). Clinically, T-ALL patients typically show diffuse infiltration of the bone marrow by T-cell lymphoblast, hyperleukocytosis, mediastinal enlargement, pleural effusions, and often central nervous system involvement. The introduction of intensified chemotherapy has gradually improved the outcome of T-ALL patients so that over 75% of children and 40–50% of adults with this disease achieve long term and durable remissions.[2] However, the outcome of T-ALL patients with primary resistant or relapsed disease remains very poor.[3,4] Therefore, current research efforts are focused on a better understanding of the molecular pathogenesis of T-ALL in order to search for molecular drug targets and tumor-specific therapies that may facilitate the development of more effective and less toxic anti-leukemic drugs.[5]

T-cell transformation is a multistep process, in which multiple genetic alterations cooperate to disrupt the mechanisms that control cell growth, proliferation, survival, and differentiation during normal T-cell development. In this context, *NOTCH1* is the most prominent T-ALL specific oncogene, to the extent that most T-ALLs can be primarily defined as tumors driven by constitutive activation of NOTCH signaling.[6] However, loss of the tumor suppressor genes *p16/INK4A* and *p14/ARF* in chromosome band 9p21, present in more than 70% of all T-ALL cases, is the most prevalent genetic abnormality in this disease.[1,7] Thus, constitutive activation of *NOTCH* signaling and loss of *p16/INK4A* and *p14/ARF* constitute the core of the oncogenic program responsible for transformation of T-cell progenitors.

In addition T-ALLs are characterized by the translocation and aberrant expression of transcription factor oncogenes. These chromosomal alterations arise from errors in the recombination process responsible for the rearrangement of T-cell receptor (TCR) genes during normal thymocyte development, and typically place T-ALL transcription factor oncogenes under the control of T-cell specific enhancers in the vicinity of the *TCRB* (7q34) or *TCRA-TCRD* (14q11) loci. These translocations are present in approximately 33% of T-ALL cases and cause deregulation of basic helix-loop-helix (bHLH) family members, such as *TAL1,*[8–10] *TAL2,*[11] *LYL1,*[12] *BHLHB1;*[13] LIM-only domain (LMO) factors, such as *LMO1* and *LMO2;*[14–16] *TLX1/HOX11,*[17–19] *TLX3/HOX11L2,*[1,20] *NKX2.5,*[21] and *HOXA* homeobox genes;[22] *MYC;*[23,24] *MYB*[25] and *TAN1*, a truncated and constitutively activated form of the NOTCH1 receptor.[26] An exception is the *TLX3/HOX11L2* locus, which is aberrantly expressed due to translocations that place it under the control of T-cell regulatory sequences in the proximity of the *BCL11B* locus.[20] In addition, some T-ALLs activate these T-cell transcription factor oncogenes as result of alternative genetic rearrangements. For example, small intrachromosomal deletions in chromosome 1p32 result in *TAL1* regulation by the SIL promoter,[27] and cryptic deletions in chromosome 11p13 can lead to aberrant expression of the *LMO2* oncogene.[28]

The complexity of genetic alterations associated with T-cell transformation is completed with a number of rare but recurrent cytogenetic and molecular alterations resulting in (i) expression of fusion transcription factor oncogenes, such as *PICALM/MLLT10/CALM-AF10,*[29] *MLL-MLLT1/MLL-EML,*[30,31] *SET-NUP214,*[32] and *NUP98-RAP1GDS1;*[33] (ii) activation of multiple genetic factors that drive proliferation, including *LCK,*[34] *CCND2,*[35] *JAK1,*[36] *ETV6-JAK2,*[37] *ETV6-ABL1,*[38] *ETV6-ARNT,*[39] *NUP214-ABL1,*[40] *EML1-ABL1,*[41] *FLT3,*[42,43] and *NRAS;*[44] and (iii) loss or inactivation of tumor suppressor genes, such as *PTPN2,*[45] *NF1,*[46] *PTEN,*[47] *WT1,*[48] *LEF1,*[49] *BCL11B,*[50] and *PHF6*.[51]

Constitutive activation of NOTCH1 signaling in T-ALL

NOTCH signaling plays a critical role in establishing cell lineage commitment decisions in multipotent precursor cells.[52] In the hematopoietic system, NOTCH1 signaling drives the initial commitment of progenitor lymphocytes to the T-cell lineage[53] and subsequent intrathymic T-cell maturation.[54] The NOTCH1 receptor, a class I transmembrane protein, functions as a ligand-activated

transcription factor that directly controls gene expression in response to extracellular signals.[55] Activation of NOTCH1 is triggered by binding to the Jagged and Delta-like family of ligands expressed on the surface of a nearby cell. This ligand-receptor interaction induces a double proteolytic cleavage of NOTCH1, first by an ADAM metalloprotease, which cleaves NOTCH in the extracellular heterodimerization domain, and then by the γ-secretase complex, which cleaves NOTCH1 in the transmembrane region. This latter proteolytic processing results in the release of the intracellular domain of the NOTCH1 receptor (ICN1) from the cell membrane into the cytosol.[56] Following cleavage, ICN1 rapidly translocates into the nucleus, where it associates with RBPJ/CSL DNA-binding protein and recruits the MAML family of coactivators to activate the expression target genes, such as *HES1, HEY1, MYC, PTCRA, DTX1,* and *CCR7*.[57–59] Finally, termination of NOTCH1 signaling occurs following phosphorylation of the C-terminal PEST domain of the receptor, which leads to the recruitment of the FBXW7/SCF ubiquitin ligase to the transcriptional complex and triggers the polyubiquitination and proteasomal degradation of ICN1.[55]

Aberrant NOTCH1 signaling was first implicated in the pathogenesis of T-ALL upon characterization of the t(7;9)(q34;q34.3) chromosomal translocation, present in 1% of T-ALL cases.[60] This translocation leads to the expression of TAN1, a truncated and constitutively active form of NOTCH1.[26] However, the role of NOTCH1 in T-ALL was not fully appraised until activating mutations in the *NOTCH1* gene were found in more than 50% of T-ALL cases.[6] *NOTCH1* mutations typically involve specific domains responsible for controlling the initiation and termination of NOTCH signaling. *NOTCH1* mutations located in the heterodimerization domain (HD alleles) and the juxtamembrane extracellular region (JME alleles) of the receptor induce ligand independent activation of NOTCH1 signaling.[6,61] In contrast, truncating mutations in the C-terminal region of the protein, which delete the PEST domain, extend NOTCH1 signaling by impairing ICN1 degradation in the nucleus.[6] Additionally, mutations in *FBXW7* are present in 8–30% of T-ALL cases, and similar to NOTCH1 PEST domain mutations, impair the proteasomal degradation of ICN1.[62,63] Furthermore, because FBXW7 also mediates the degradation of MYC, JUN, mTOR, and cyclin E, increased levels of these proto-oncogenes may also participate in the transformation of T-ALL harboring *FBXW7* mutations.[64] Moreover, aberrant overexpression of ICN1 target genes contributes to the transformation and progression of T-ALL. For example, increasing expression of Ccr7 by aberrant NOTCH1 regulation leads to T-ALL infiltration of the CNS.[59]

The high frequency of *NOTCH1* mutations in T-ALL has sparked an interest in the development of anti-NOTCH1 targeted therapies for the treatment of T-ALL. Thus, γ-secretase inhibitors (GSI), which block the proteolytic cleavage of the NOTCH receptors and preclude the release of ICN1 from the membrane, have been proposed as potential targeted therapy in T-ALL. Early studies revealed that inhibition of NOTCH by GSI treatment results in G1 cell cycle arrest and decreased cell size in T-ALL cell lines harboring activating mutations in NOTCH1.[6,60,65] However, systemic inhibition of Notch signaling with GSIs blocks the function of all four NOTCH family members (NOTCH1–4) and results in gastrointestinal toxicity resulting from accumulation of secretory goblet cells in the intestine.[66,67] Notably, glucocorticoids may abrogate the gastrointestinal toxicity induced by GSIs, while suppression of NOTCH signaling enhances the antileukemic effects of glucocorticoids,[68] suggesting that the combination of GSIs and glucocorticoids may have increased efficacy and decreased toxicity in the treatment of T-ALL. In addition, synthetic peptides targeting the NOTCH transcriptional complex, and inhibitory antibodies specific for NOTCH1 have been proposed as alternative approaches to inhibit aberrant NOTCH1 signaling in T-ALL.[69,70]

Alterations in cell cycle regulators

The *CDKN2A* locus on chromosome band 9p21 contains two tumor suppressor genes: *p16INK4A*, and *p14ARF*. P16INK4A is a direct inhibitor of cyclin D-CDK4/6 complexes, whereas p14ARF antagonizes MDM2, a negative regulator of the TP53 oncoprotein.[71,72] CDKN2A deletions are the most frequent abnormality in T-ALL, present in over 70% of patients.[7] In addition, some T-ALL cases harboring the t(12;14)(p13;q11) and t(7;12)(q34;p13) translocations show aberrantly high levels of expression of the CCND2 cell cycle regulator, which drives cell cycle progression,[35] and chromosomal deletions involving additional negative regulators of cell cycle progression, such as RB1 and CDKN1B, have been reported in the context of copy number alteration studies in T-ALL cases.[73,74]

Activation of transcription factor oncogenes

Overexpression and aberrant activation of transcription factor oncogenes play an important role in T-ALL. These transcription factors typically play key roles in embryonic development and are aberrantly expressed in malignant T-cell lymphoblasts due to chromosomal translocations, which juxtapose them to the promoters of the T-cell receptor genes.

Class II basic helix-loop-helix transcription factors

Alterations in various basic helix-loop-helix (bHLH) transcription factors have been identified in T-ALL. This family of proteins is characterized by a basic domain that mediates DNA binding and two α-helices connected by a loop that are involved in the formation of homodimeric and heterodimeric complexes. In T-ALL, the class II bHLH transcription factor oncogenes *TAL1, TAL2, LYL1*, and *BHLHB1* form heterodimeric DNA binding complexes with E proteins (class I bHLH factors that bind E-box sequences) in order to modulate gene expression.[75] The *TAL1* gene in chromosome band 1p32 is a key regulator of hematopoietic stem cell development expressed in hematopoietic progenitors, mast-cell

lineage cells, and in differentiating erythroid and megakaryocytic cell lineages.[5)] Abnormal expression of *TAL1*, detected in approximately 60% of T-ALL cases, is a result of various rearrangements and deletions.[1,76] For example, in 3% of childhood T-ALL, the translocation t(1;14)(p32;q11) places *TAL1* gene expression under the control of enhancers that drive the expression of TCRA/D. In addition, in 16–30% of aberrant *TAL1* expression, small intrachromosomal rearrangements cause the deletion of the 5' regulatory sequence, placing TAL1 under the control of the promoter of an upstream gene, STIL, which is highly expressed in T-cells.[27] Lastly, biallelic overexpression of TAL1 suggests that additional mechanisms might cause aberrant TAL1 activation.[77] T-ALL cases with *TAL1* expression are characterized by arrest at the late double positive stage of thymocyte development.[1] In addition, *LYL1, TAL2,* and *BHLHB1*, three genes encoding bHLH factors closely related to *TAL1*, are aberrantly expressed as result of chromosomal translocations t(7;19)(q35;p13), t(7;9)(q34;p13), and t(14;21)(q11;q22), respectively.[11–13] These oncogenic class II bHLH factors mainly exerts its oncogenic function through the formation of heterodimers with class I bHLH factors E2A and HEB.[78] In this context, TAL1 and LYL1 act as transcriptional repressors by blocking the formation of E2A and HEB homodimers, which typically drive high levels of transcriptional activation.[79] However, TAL1 can also activate transcription through alternative transcriptional complexes in association with GATA factors.[80,81]

LIM only domain proteins

LIM domain proteins were first associated with the development of T-ALL in cases harboring the t(11;14)(p15;q11) and t(11;14)(p13;q11) chromosomal translocations involving the *LMO1* and *LMO2* genes, respectively.[14–16] Although these translocations only occur in 9% of pediatric T-ALL, aberrant *LMO1* and *LMO2* expression are found in 45% of T-ALL cases, suggesting additional mechanisms of activation. Unlike bHLH proteins, LMO proteins do not interact directly with DNA; instead, they form transcriptional complexes with TAL1 and LYL1 complexes.[1,82] Consistently, activation of *LMO1* and *LMO2* is most frequently coexistent with deregulated TAL1 and/or LYL1 expression.[1]

Homeobox transcription factor oncogenes: TLX1, TLX3, and HOXA

Homeobox (*HOX*) genes are a family of transcription factors that play an essential role in the process of body patterning and organogenesis during embryonic development. These highly conserved transcription factors are becoming increasingly recognized for their function in regulating hematopoiesis and leukemia development.

The orphan homeobox gene *TLX1* was initially identified in T-ALL through the characterization of the t(10;14)(q24;q11) translocation,[17–19,83] which results in aberrant expression of *TLX1* in 5-10% of pediatric and 30% of adult T-ALL cases.[1,84] *TLX3*, a second TLX family member, is overexpressed in 20-25% of pediatric and 5% of adult T-ALL as a result of the t(5;14)(q35;q32) translocation.[20] This chromosomal rearrangement places *TLX3* under the control of strong T-cell regulatory elements in the vicinity of the BCL11B locus.[20] TLX1 and TLX3 leukemias are hypothesized to share a common pathogenic pathway in T-ALL because of their convergent gene expression signatures and their association with cooperating mutations in specific oncogenes and tumor suppressor genes rarely present in non-TLX induced T-ALLs, including the *NUP214-ABL1* fusion oncogene and mutations in the *PTPN2, WT1*, and *PHF6* tumor suppressor genes.[45,48,51,85] *TLX1* expression is associated with a favorable prognosis with low risk of relapse in both children and adults. However, aberrant *TLX3* expression is associated with a less favorable prognosis and a higher incidence of relapse.[1,84,86]

Finally, 3% of T-ALL patients harbor translocations in the *HOXA* cluster of homeobox genes in 7p15 into the *TCRB* and *TCRG* loci, which induce aberrant expression of the *HOXA10* and *HOXA9* genes.[22] In addition, ectopic expression of *HOXA* cluster genes is also noted in leukemias containing *MLL-MLLT1, SET-NUP214*, and *PICALM-MLLT10* rearrangements, suggesting a more general pathogenic role of HOXA miss-expression in leukemia development.[1,22,32,87]

The MYC proto-oncogene

The *MYC* oncogene is activated in about 1% of T-ALLs due to the t(8;14)(q24;q11) chromosomal translocation.[23,24] However, MYC is broadly expressed in T-ALL and functions as a critical NOTCH1 direct target gene, playing a broad and prominent role in promoting cell growth and proliferation in T-ALL.[58,88]

The MYB proto-oncogene

c-MYB, a leucine zipper transcription factor oncogene expressed primarily in immature hematopoietic cells, is translocated to the *TCRB* locus and consequently overexpressed in T-ALL cases with the t(6;7)(q23;q32). This translocation is characteristically found in childhood T-ALLs diagnosed under 2 years of age and is associated with increased expression of genes involved in proliferation and mitosis.[25] In addition, somatically acquired focal duplications of the *MYB* locus are found in about 10% of T-ALLs.[89,90]

Transcription factor fusion oncogenes: MLL-MMLT1, PICALM-MLLT10, SET-NUP214, and NUP98-RAP1GDS1

Although chromosomal rearrangements resulting in high levels of expression of otherwise structurally normal transcription factor oncogenes is a key feature of T-ALL, some leukemias harbor chromosomal translocations that generate fusion transcripts encoding chimeric transcription factor oncogenes. Thus, about 5% of T-ALLs show *MLL* rearrangements as result of the t(4;11)(q21;q23) and t(11;19)(q23;p13.3) translocations,

which produce *MLL-AFF1* (*MLL-AF4*) and *MLL-MLLT1* (*MLL-ENL*) fusion genes, respectively.[91] Notably, even though MLL fusion oncogenes in B-precursor ALL are associated with very poor prognosis, the *MLL-MLLT1* fusion rearrangement seems to confer a favorable prognosis in T-ALL.[1,92] In addition, the t(10;11)(p13;q14) translocation encoding the *PICALM-MLLT10* fusion oncogene is present in 5–10% of T-ALLs.[22,29] Furthermore, a rare cryptic del(9)(q34.11q34.13) deletion generating the *SET-NUP214* fusion gene in T-ALL has been described.[32] A common feature associated with *MLL* rearrangements, and the expression of the *PICALM-MLLT10* and the *SET-NUP214* oncogenes is the aberrant expression of *HOX* genes, suggesting a common mechanism of transcriptional dysregulation in the pathogenesis of these tumors.[1,22,29,32,93] Finally, a number of different rearrangements involving the *NUP98* nuclear pore complex protein gene in 11p15.4 have been associated with the pathogenesis of T-ALL. These rearrangements result in the expression of chimeric oncogenes joining *NUP98* to homeobox and non-homeobox genes.[94–97] Among these, the *NUP98-RAP1GDS1* rearrangement, found in 5% of T-ALL cases, is the most common.[33]

Loss of transcription factor tumor suppressor genes

The Wilms tumor 1 gene

Deletions and mutations in the Wilms tumor suppressor gene 1 (*WT1*) are present in about 10% of T-ALL cases.[48,85] WT1 mutations found in T-ALL are predominantly heterozygous frameshift mutations resulting in truncation of the C-terminal zinc finger domains of this transcription factor. Notably, *WT1* mutations in T-ALL are associated with oncogenic expression of the *TLX1, TLX3*, or *HOXA* oncogenes, suggesting a strong genetic interaction between WT1 loss and aberrant expression of homeobox transcription factor oncogenes in the pathogenesis of T-ALL.[48,85]

The lymphoid enhancer factor 1 LEF1 gene

LEF1, a member of the LEF/TCF family of DNA binding transcription factors, mediates aspects of various signaling pathways, including the WNT, TGF beta, and NOTCH1 signaling. Monoallelic or biallelic microdeletions involving the *LEF1* locus and mutations in the *LEF1* gene are present in about 15% of T-ALLs.[49] Leukemias with *LEF1* mutations and deletions show a characteristic differentiation arrest at the early cortical thymocyte stage of differentiation that resembles that of TLX1 positive tumors.[49]

The plant homedomain finger 6 PHF6 gene

PHF6, a gene encoding a putative epigenetic regulator of gene expression is mutated and deleted about 16% of pediatric and 38% of adult T-ALL cases.[51] Notably, *PHF6* is located in the long arm of chromosome X (Xq26), and PHF6 mutations are almost exclusively found in male patients with T-ALL, which may contribute in part, to the higher frequency of T-ALL in males compared with females.[51]

Genetic alterations in signal transduction pathways

Mutational loss of PTEN

The *PTEN* tumor suppressor gene is a negative regulator of the PI3K-AKT signaling pathway. PTEN specifically terminates signaling through dephosphorylation and inactivation of PIP3, a lipid second messenger generated by the PI3K complex at the membrane and critically required for recruitment and activation of the AKT1 kinase.[98] Chromosomal deletions and/or truncating mutations in *PTEN* occur in 5–10% of T-ALL cases and overall, 17% of T-ALLs lack PTEN protein expression.[47]

ABL1 fusion oncogenes: NUP214-ABL1, EML1-ABL1, and ETV6-ABL1

Although the t(9;22)(q34;q11) translocation and expression of the BCR-ABL1 fusion tyrosine kinase characteristic of chronic myeloid leukemias and adult B-precursor ALLs is rarely found in T-ALL, ABL1 rearrangements have been reported in about 8% of T-ALLs.[99] Thus, about 6% of T-ALL cases show ABL1 amplifications associated with a complex rearrangement generating the *NUP214-ABL1* fusion oncogene[40] from an extrachromosomal element (episome) sometimes reintegrated elsewhere in the genome.[40,100] Interestingly, the *NUP214-ABL1* oncogene is almost exclusively found in T-ALLs expressing the *TLX1* and *TLX3* transcription factor oncogenes.[40] Additional *ABL1* rearrangements present in T-ALL include the *EML1-ABL1* and *ETV6-ABL1* fusion oncogenes.[38,41] Remarkably, small molecule tyrosine kinase inhibitors developed for the treatment of *BCR-ABL1* positive leukemias have proven effective in inhibiting both *NUP214-ABL1* and *EML1-ABL1* fusion oncoproteins, providing a potential targeted therapy for these patients.[101,102]

RAS mutations and loss of NF1

The RAS family of proteins (HRas, KRas, and NRas) is comprised of membrane anchored proteins that propagate extracellular signals that are transmitted through tyrosine kinases, non-tyrosine kinases, and G coupled protein receptors.[103] NRAS mutations, described in 5–10% of T-ALL, result in the accumulation of Ras in its active, GTP-bound confirmation in the absence of stimulation.[44,103] In addition, cryptic deletions and/or mutations in the neurofibromatosis type 1 (*NF1*) gene, encoding a negative regulator of the Ras pathway, occur in 3% of T-ALL.[46]

Activating mutations in JAK1

The Janus kinase-signal transducer or JAK family of protein kinases function as signal transducers downstream of cytokine receptors and play an important role in promoting cell proliferation, differentiation, and survival in hematopoietic precursors.[36] The first evidence linking aberrant JAK signaling with T-ALL came from the characterization of the t(9;12)(p24;p13) translocation, a rare rearrangement encoding the constitutively active *ETV6-JAK2* kinase fusion oncoprotein.[37] In addition, activating mutations in JAK1 have been reported primarily in some adult T-ALLs.[36,104]

FLT3 mutations

The FMS-like tyrosine kinase 3 (*FLT3*) gene plays an important role in stem cell differentiation and proliferation and is frequently the target of activating mutations in acute myeloid leukemia.[105,106] Activating mutations in *FLT3* result from internal tandem duplications in the juxtamembrane domain and point mutations in the activation loop of the receptor.[107] Rare cases of T-ALL harboring *FLT3* mutations typically show an immature immunophenotype characterized by CD117/KIT expression.[42,43]

LCK translocation and overexpression

Lymphocyte specific tyrosine kinase (LCK), a member of the Src family kinases, plays a critical role in proximal T-cell receptor (TCR) signal transduction.[108] Aberrantly high levels of LCK are involved in the pathogenesis of rare cases of T-ALL harboring the t(1;7)(p34;q34) translocation, which juxtaposes the *LCK* locus to the *TCRB* gene.[34]

PTPN2 deletions

PTPN2 (protein tyrosine phosphatase non-receptor type 2) is a cytosolic tyrosine phosphatase that functions as a negative regulator of kinase signaling pathways. Acquired homozygous deletions and mutations in the *PTPN2* locus are present in rare cases of T-ALL (45). *PTPN2* loss is specifically found in TLX1 positive T-ALLs. Mechanistically, loss of PTPN2 increases cytokine sensitivity and promotes cell proliferation in T-ALL cells. Moreover, loss of PTPN2 in NUP214-ABL1 positive leukemias enhances the activity of the *NUP214-ABL1* kinase oncoprotein.[45]

IRS4 translocation and overexpression

The *IRS4* gene in chromosome Xq22 encodes the insulin receptor substrate 4, a cytoplasmic protein that functions as an interface between growth factor receptors with tyrosine kinase activity, such as the insulin receptors, IGF1R and FGFR1, and intracellular signaling molecules containing SH2 domains. A case of a T-ALL patient with a t(X;7)(q22;q34) resulting in the translocation of *IRS4* to the vicinity of the *TCRB* locus with consequent 1000-fold overexpression of the *IRS4* gene has been reported.[109] Notably, forced expression of IRS family members can induce increased cell proliferation, suggesting a pathogenic role for *IRS4* overexpression in T-ALL.

Concluding remarks

In conclusion, T-ALL is an aggressive hematologic cancer for which limited therapeutic options are available for patients with primary resistant or relapsed disease underscoring the need for better treatment stratification protocols and for identifying more effective antileukemic drugs.[2] This imperative is further supported by studies of the long-term effects of intensified chemotherapy in T-ALL survivors, which show that gains in leukemia-free survival have been achieved at the cost of significant increases in rates of acute and chronic life-threatening and debilitating toxicities.[110]

The study of the molecular mechanisms involved in initiation and progression of T-ALL has uncovered a plethora of genetic alterations and aberrant signaling pathways and lead to better insights in the mechanisms that contribute to the malignant transformation of T-cell precursors. In addition, new data continues to arise implicating novel pathways in this disease. For example, microRNAs have emerged as molecules involved in the promotion of malignant T-cell transformation. Specifically, miR-19 was discovered to be involved in the pathogenesis of *NOTCH1*-induced T-ALL, and a newly identified translocation targeting the miR-17-92 cluster coincides with a rearrangement that activates *NOTCH1*.[111] In addition, a recent study showed *Notch1*-dependent T-ALL development in mice lacking the RNA-binding proteins ZFP36L1 and ZFP36L2, suggesting a critical role for these proteins in the prevention of leukemogenesis.[112]

From a therapeutical point of view, the identification of highly prevalent activating *NOTCH1* mutations in T-ALL created enormous interest in developing molecularly tailored therapies and prompted the initiation of clinical trials to test the effectiveness of blocking NOTCH1 signaling with GSIs. Early attempts to inhibit NOTCH signaling with GSIs faced the problem of associated gastrointestinal toxicity; however, the combination of GSIs and glucocorticoids may have increased efficacy and decreased toxicity in the treatment of T-ALL.[68] In addition, the presence of activated kinase oncoproteins in a subset of T-ALLs may offer an additional opportunity for molecularly tailored therapies. Given the efficacy of ABL1 kinase inhibitors for treatment of BCR-ABL1 positive leukemias and the sensitivity of NUP214-ABL1 to these inhibitors,[40,101,113] NUP214-ABL1-positive T-ALL patients may benefit from inclusion of ABL1 inhibitors in their treatment schemes. Similarly, patients with activating JAK1 mutations might benefit from the JAK1 inhibitors currently under development for the treatment of myeloproliferative disorders.[36]

Finally, understanding the pathogenesis of T-ALL is critical for the development of prognostic markers that may identify patients at increased risk of relapse. In light of this, patients with early T-cell precursor (ETP) features[114] or absence of biallelic TCRγ deletion[115] are associated with early treatment failure and poor prognosis.

Overall, the identification and molecular characterization of new oncogenes and tumor suppressors has uncovered much of the mechanisms involved in the pathogenesis of T-ALL. Importantly, this information has started to be translated to the clinic with the development of molecularly tailored therapies for the treatment of this disease.

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Management of acute lymphoblastic leukemia in adults

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The treatment of adults with acute lymphoblastic leukemia (ALL) remains challenging. While there has been a gradual improvement in the overall results over the past 40 years, the long term survival from diagnosis for adults less than 60 years is still no more than 35–40%. The therapeutic options have increased over the past decade to include a better understanding of the indications for transplantation and a broadening of the number of patients who are eligible for such a procedure. New drugs, as well as potential targeted monoclonal antibodies, have opened up novel areas for study in the management of ALL. Sensitive techniques for the detection of minimal residual disease have also opened new strategies for post remission management. This paper will focus on the management of young adults, up to age 60 years, and will include both patients who are Philadelphia chromosome negative ALL, as well as those who are positive. Most of the discussion will focus on newly diagnosed patients.

Prognostic factors

At presentation

It has long been realized that the management of patients with ALL is dependent on the prognostic factors at diagnosis. Patients with known very high risk features are often assigned different post remission strategies, even if the induction therapy is not always altered. Decisions regarding the appropriateness of bone marrow transplantation are also dependent on the prognostic factors at diagnosis. Over the past four decades, there has been a shift in the understanding of the prognostic factors such that morphology[1] and cytochemistry,[2] so dominant in the 1970s, have no prognostic significance nowadays. Even immunophenotyping, which was the dominant determining factor for several decades since the 1980s, is rapidly becoming less important for prognostication, even if its use remains crucial for the initial diagnosis, for the detection of minimal residual disease, and for the application of specific and targeted therapies. Thus, although B- and T-lineage patients are often treated differently, the level of maturity within B-lineage is no longer important for prognosis,[3,4] although among T-lineage patients, there appears to be a difference depending on the level of maturity.[5] At the same time, certain immunologic markers have been recognized as having significant prognostic impact, the most important of these being CD20, which, in several trials, has been reported among adults with B-lineage ALL to have a low complete remission rate and inferior overall survival.[6,7] While these historic prognostic factors clearly have relevance, they have now been mostly superseded by genetic and/or molecular markers leading to a changing paradigm, incorporating cytogenetics and molecular determinants[8] (Figure 1). The importance of immunophenotyping for assigning specific targeted therapies against antigenic determinants for B or T-cell ALL, such as nelarabine, forodesine, or monoclonal antibodies,[9–11] will be fully discussed in the *Therapy* section.

Age has withstood the test of time and remains the most important prognostic factor in ALL that is unlikely to be superseded by any of the new molecular determinants. Not only are there enormous differences in prognosis between childhood and adult ALL, but age also critically affects the prognosis within adult groups. Although most clinical studies have used an arbitrary cutoff of 35 or 40 years,[12–14] the prognostic significance of age, in fact, is a continuum between ages 20 and 60.[14]

The prognostic classification in ALL, much like AML, is now moving rapidly towards cytogenetics and molecular markers. The Philadelphia chromosome, t(9;22) (q34;q11), remains the most frequent and clinically significant abnormality in adult ALL, with an incidence that increases with age, reaching 50% among older adults with B-lineage.[15] The importance of determining the presence of the Philadelphia chromosome at diagnosis, either by cytogenetics or by molecular detection of BCR-ABL, cannot be overstated, as the entire approach to such a patient is different (see *Philadelphia chromosome-positive ALL* section). Among patients who are Philadelphia chromosome-negative, management is also affected by cytogenetics (Table 1) and this categorization has a major impact on choosing the appropriate therapy for ALL.[16] In the past, due to the relative rarity of ALL in adults, the precise prognostic significance of many of the recurring cytogenetic abnormalities could not be determined due to the relatively small numbers. The analysis of cytogenetics among patients

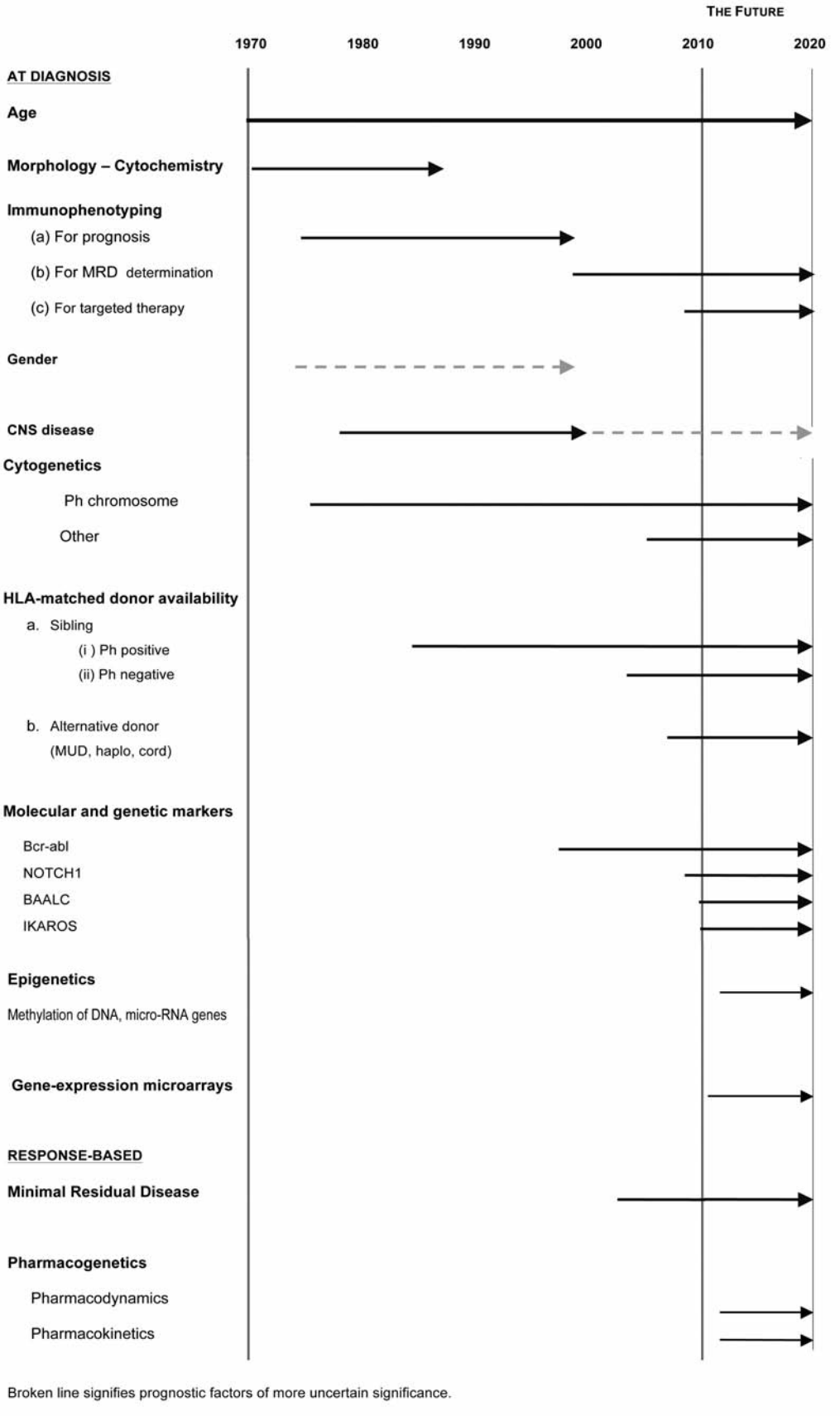
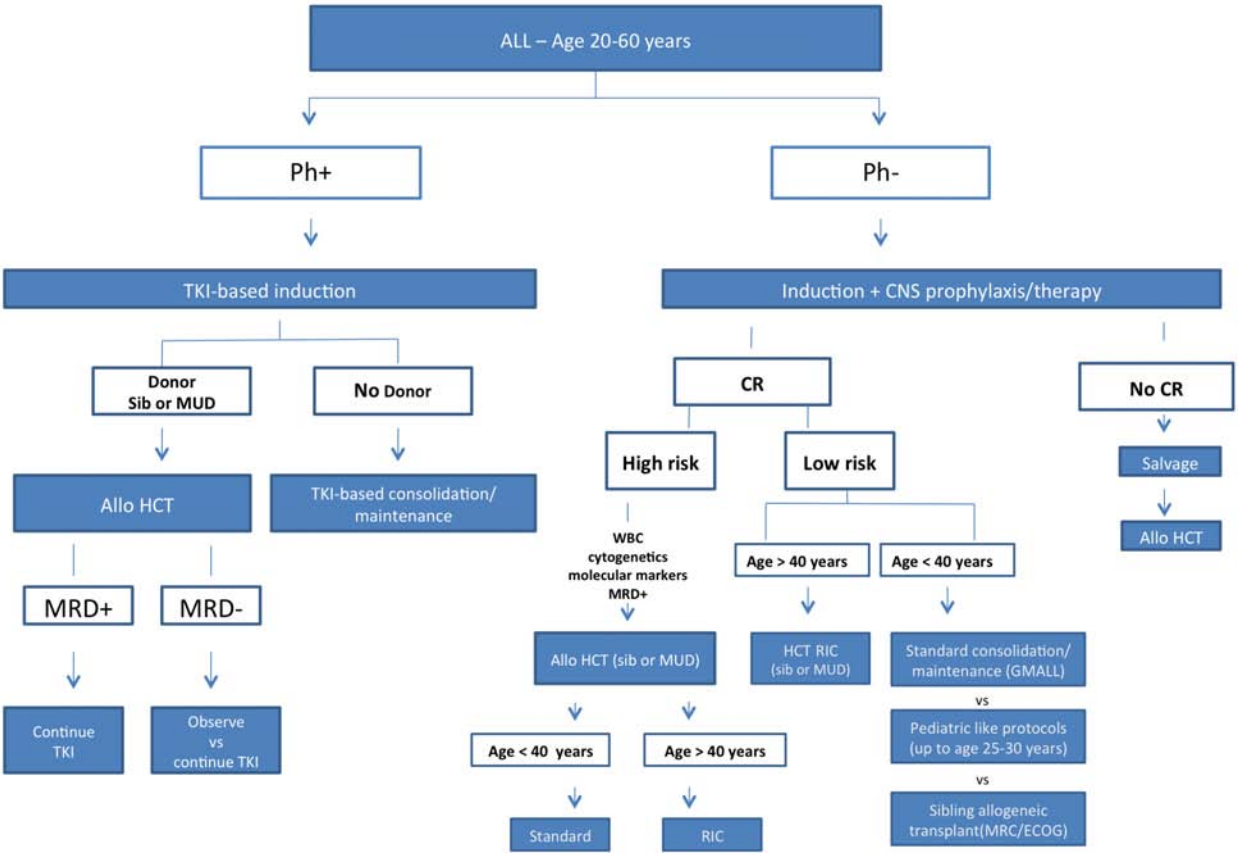


Figure 1. The evolving paradigm of prognostic factors for ALL in adults. Reproduced from Rowe et al, Br J Haematol, 2010, 150, 389-405; with permission.



WBC: White blood cell; MRD: minimal residual disease; RIC: reduced intensity conditioning; HCT: hematopoietic cell transplantation; TKI: tyrosine kinase inhibitors; MUD: matched unrelated donor; SIB: sibling; Standard: standard conditioning.

Figure 2. A suggested algorithm for the management of adults with ALL, incorporating anticipated future direction, not all currently used as standard of care, such as therapeutic stratifications by MRD and the use of RIC transplants for patients over 40 years of age.

Table 1. Cytogenetic, molecular, and immunologic markers of prognostic significance in adult ALL.

Cytogenetics	Molecular Markers	Immunologic Surface Markers	Significance
t(9;22) (q24; q11.2)	BCR-ABL fusion	CD20	Poor prognosis
t(4;11) (q21;q23)	MLL-AF4		
t(8;14) (q24.1; q32)	BAALC gene		
Low hypodiploidy/near triploidy	IKAROS gene		
Complex karyotype			
t(1;19) (q21;p13.3)	NOTCH1		Conflicting data
	FBXW7		
High hyperdiploidy			Better prognosis
Del (9q)			

treated on the MRC UKALL XII/ECOG2993 trial provided for the largest prospective analysis of cytogenetics as a prognostic factor in ALL and put cytogenetics more firmly on the map as having dominant prognostic significance, also for patients who are negative for the Philadelphia chromosome.[16] Molecular markers have recently gained prominence in ALL, although much of the data are emerging. While the presence of the *BCR-ABL* gene has been established as a poor prognostic marker, other markers have been investigated. The *BAALC* gene, a marker of early hematopoietic progenitor cells, has recently been identified as predicting for a poor outcome in both T-ALL, as well as B-ALL[17,18] the *NOTCH1* and *FBXW7* have been fairly extensively studied in T-cell ALL, however, with conflicting results.[19,20] Recent work among B-lineage ALL strongly suggests that the deletions or mutations of the IKAROS family zinc finger 1 gene (*IKZF1*) are associated with a poor prognosis.[21]

Prognostic factors based on response

Time to complete remission has long been recognized as a prognostic factor for adult patients with ALL. Achieving remission within 4 weeks was recognized as a favorable predictor for outcome,[22] although this could not be confirmed in some of the more recent large studies.[14] An assessment of initial response to steroids (within 7–14 days) is becoming a hallmark of pediatric protocols for ALL[23] and has only recently being incorporated by some groups for adult ALL.[24]

The assessment of minimal residual disease (MRD) is a rational test for evaluation of treatment response and provides for a more personalized prognostication, as it

reflects the biology of the disease, as well as the pharmacokinetics and pharmacodynamics in an individual patient. As a relatively new tool, lots of questions come up and the data in the literature are evolving. The main issues are: what is the preferred method? What is the cell number cutoff for positivity? And, most importantly, what is the optimal timing for the evaluation of MRD and the clinical relevance?

There are three methods for MRD testing with advantages and disadvantages for each.[25] The most widely used technique is a polymeranse chain reaction (PCR) analysis of Ig and *TCR* gene rearrangement. Achievement of high sensitivity requires precise identification of junctional region sequences of Ig and TCR genes, which enable the design of clone-specific oligonuleotides. The sensitivity of this method is about 0.01–0.001%, which means detection of 1 leukemic cell in 10,000 to 100,000 normal cells. The second technique is by multiparameter flow cytometry, which relies on the expression of abnormal cell markers on the leukemic cells. Using at least six to eight colors, this approach reaches a sensitivity of 0.01%. In contrast to the PCR method, in this technique, there is a need for a certain amount of cells with similar characteristics. The last technique is a PCR analysis of fusion genes. This method is limited to patients who have a defined genetic abnormality, which means less than one-third of all ALL patients. The most frequent fusion gene is *BCR-ABL* and others include *MLL-AF4, E2A-PBX1,* and *TEL-AML1*. BCR-ABL transcript analysis has a sensitivity of 0.01 to 0.0001%.

Data on MRD assessment began to appear in the pediatric literature[26,27] at the end of the 1990s. A decade later, a major report by the AIEOP-BFM ALL 2000 study in over 3000 patients confirmed that molecular response for treatment, detected by sensitive PCR, in predefined time points is highly predictive for relapse in childhood B-cell ALL.[28] Over the past few years, data regarding MRD in adults are also appearing.

Two publications from the GMALL group assessed the prognostic impact of positive MRD by PCR, at different time points, in standard risk adult patients. They defined three risk groups according to MRD. The lower risk group had rapid MRD decline with negative MRD at both days 11 and 24. The relapse rate at 3 years was 0% in this group. The high risk group had positive MRD until week 16. The 3-year relapse rate in this group was 94%. The remaining patients comprised the intermediate group with a 3-year relapse rate of 47%.[29] Clearly, the greatest uncertainty rests with this intermediate group, which is the largest. It is, therefore, not surprising that attempts at a better predictive value have recently intergraded the use of MRD with pretreatment molecular markers, such as the *IKZF1*.[30] Whereas MRD alone in the intermediate group could identify only 46% of relapses and *IKZF1* predicted for 54%, the integrated use predicted for almost 80% of relapses with 93% specificity.[30] In a subsequent report, the GMALL reported that MRD conversion after 1 year of treatment is a poor prognostic factor, with 61% relapse rate with a median follow up of 16 months. The median time between molecular and hematologic relapse was 9.5 months.[31] The conclusion of this GMALL study was to start reinduction treatment after a relapse detectable only by an occurrence of positive MRD, without waiting for a frank hematologic relapse.

Examination of the B-lineage ALL UK patients of the MRC/ECOG trial revealed that MRD positivity in adults by PCR after phase 2 of induction discriminates best for the outcome. The 5-year relapse-free survival was 15% in the MRD positive patients compared with 71% in the MRD negative ones (P=0.0002). In recipients of allogeneic transplant, the MRD positivity pre-transplant did not adversely affect outcome.[32] In contrast, in the Italian group (NILG), there was a significant prognostic difference between the pre-transplant MRD positive and negative groups. While the cumulative incidence of relapse at 36 months was 0% in patients with negative MRD before transplant, it was 46% in the patients with positive MRD (P=0.027).[33]

Taken together, it is clear that MRD is an important prognostic factor in adult ALL that allows for a personalized prognostication. Both the PCR and the flow cytometric techniques are reasonable options. The most informative time for MRD testing is not clear. Somewhere around the end of induction seems reasonable but the ideal timing still has to be determined and it may well be different for different protocols. The implication of positive MRD after consolidation is uncertain but conversion from negative to positive MRD at any time is a poor prognostic sign.

The importance of MRD cannot be overemphasized, and it is likely that molecular response to the treatment will redefine many pretreatment prognostic factors in ALL, both in childhood and adult ALL.[8]

Therapy

Philadelphia chromosome-negative

Induction

Induction therapy in ALL, as in acute myeloid leukemia (AML), is intended to achieve complete remission (CR), prior to any subsequent post remission therapy. In general, patients who do not achieve remission with initial induction therapy do not do well, even if they subsequently achieve remission through some salvage regimen.[14] There is no single best regimen for induction therapy. Most regimens comprise two phases of induction that include glurocorticoids, vincristine, and an anthracycline. Cyclophosphomide and cytarabine are often also added. With such modern regimens, the CR rate ranges between 75% and 93% for patients less than 60 years, who are Philadelphia chromosome negative (Table 2).

Asparaginase, the mainstay of treatment of childhood ALL for the past three decades (34), is also incorporated in most of the adult induction regimens. The importance of asparagine depletion has been emphasized in a study conducted by the Cancer and Leukemia Group B (CALGB) in the US, with a median survival for patients with asparagine depletion of 31 months compared with 13 months for the non-depleted patients.[35] It is worth noting, however, that the Hyper-CVAD regimen does not include asparaginase as part of the therapeutic regimen, and that the results of induction are similar to other published regimens.[36] Recent data from several prospective studies suggest that the pegylated form of

Table 2. Results of large trials in adult acute lymphoblastic leukemia (ALL)

Study	Year	N	Median Age (Range)	SCT	CR	Early Death	Survival
CALGB 9111, USA (94)	1998	198	35(16–83)	Ph+	85%	8%	40% (3 y)
LALA 87, France (65)	2000	572	33(15–60)	PO	76%	9%	27% (10 y)
NILG 08/96, Italy (95)	2001	121	35(15–74)	PR	84%	8%	48% (5 y)
GMALL 05/93, Germany (96)	2001	1163	35(15–65)	PR	83%	n.r.	35% (5 y)
JALSG-ALL93, Japan (97)	2002	263	31(15–59)	PO	78%	6%	30% (6 y)
Sweden (98)	2002	153	42(16–82)	PR	75%	n.r.	28% (5 y)
GIMEMA 0288, Italy (99)	2002	767	28(12–60)	_	82%	11%	27% (9 y)
MD Anderson, US (36)	2004	288	40(15–92)	Ph+	92%	5%	38% (5 y)
EORTC ALL-3, Europe (70)	2004	340	33(14–79)	PO	74%	n.r.	36%* (6 y)
LALA 94, France (54)	2004	922	33(15–55)	PR	84%	5%	36% (5 y)
GOELAL02, France (3)	2004	198	33(15–59)	HR	86%	2%	41% (6 y)
GIMEMA 0496, Italy (100)	2005	450	16–60	n.r.	80%	n.r.	33% (5 y)
Pethema ALL-93, Spain (71)	2005	222	27(15–50)	HR	82%	6%	34% (5 y)
MRC XII/ECOG E 2993, UK-US (64)	2008	1913	31(15–64)	PO	91%	4.8%	39% (5 y)
PALG 4-2002, Poland (101)	2008	131	26(17–60)	PO	90%	n.r.	43% (3 y)
Total of all studies (using weighted mean)		7,701			84%	7%	35%

Abbreviations: Ph+ SCT in Philadelphia chromosome-positive ALL; PO, prospective SCT in all pts with donor; PR, SCT according to prospective risk model; HR, prospective SCT in a study for HR patients only; n.r. not reported

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asparaginase (PEG), where the *Escherichia coli* is conjugated to polyethyleneglycol, improved efficiency.[37-40] Given the longer half life and the lower risk of antibody formation,[41] it is likely that this form of asparaginase will replace the native source and will be included in future trials of induction therapy in adult ALL.

As most patients get into morphologic CR, any attempts to improve on this would be directed at reducing the level of minimal residual disease (MRD), in the hope that such reduction will ultimately impact on the overall long term survival. Several new agents have been introduced in the past few years for relapsed disease and are currently being evaluated as adjunctive therapy in induction. These include nelarabine for T-cell ALL,[42] clofarabine for all ALL patient,[43] and liposomal vincristine.[44] Inhibitors of purine nucleoside phosphorylase, such as forodesine, are in early stage of development and have the potential to improve the outcome, especially in T-cell ALL.[45]

Monoclonal antibody therapy is an attractive option to consider in induction therapy for ALL. Some encouraging data have been reported with the use of rituximab, a monoclonal antibody to CD20, in the management of B-cell ALL.[46] Epratuzumab, a monoclonal antibody to CD22 is thought to act by an immunomodulatory action and is an important potential agent also for B-cell ALL.[47] Alentuzumab, an antibody directed against CD52, is a monoclonal antibody with broad expression on most T- and B-cell ALL patients. However, to date there are only scant data on this in ALL.[48] Perhaps, the antibody with the greatest potential is blinatumomab, which belongs to a new class of bi-specific T-cell engagers (BiTEs). This compound is a monoclonal antibody combining two binding sites: a CD3 site for T-cells and a CD19 site for the target B-cells. The drug works by linking these two cell types and activating the T-cells to exert cytotoxic activity on the target cell. Although preliminary, some extraordinary results have been reported both in pediatric ALL,[49] as well as in adult ALL.[50] In fact, the Eastern Cooperative Oncology Group (ECOG) will shortly embark on a prospective randomized study evaluating blinatumomab in induction, with the aim of achieving a greater degree of MRD negativity.

Mortality from induction, occurring in 3–8% of patients, is also an important issue as this is due mostly to opportunistic infections, such as Aspergillus.[14] This is, in part, related to the prolonged exposure to glucocorticoids. Substituting dexamethasone for prednisone and reducing the overall period of exposure to glucocorticoids will hopefully reduce the induction mortality further.

CNS prophylaxis

The incidence of CNS involvement at diagnosis is about 4–7%.[51,52] Although such involvement portends for a poor outcome, if treated appropriately at diagnosis, the ultimate prognosis is not different from those patients who presented without CNS involvement.[53,54] CNS involvement at first relapse occurs in about 30% of adults without prophylaxis[55] and in only 5–15% of those who got prophylaxis.[51,52]

As CNS relapse carries a very poor prognosis, prophylaxis is an imperative in the management of ALL. There are three modalities of CNS prophylaxis: intrathecal (IT) therapy, high dose systemic therapy that can cross the blood-brain barrier (BBB), and radiotherapy. The main drugs for the first two modalities are glucocorticoids, methotrexate (MTX), and cytarabine. While the efficacy of prophylaxis was established in the early 1980s,[55] the best modality is still an open issue. In pediatric ALL, dexamethasone was more effective in the prevention of CNS leukemia than prednisome was.[56] Most of the adult protocols currently combine IT MTX with high dose therapy (MTX and/or cytarabine). Radiotherapy is a very effective prophylaxis but is toxic for children and can cause cognitive impairment in adults, especially in patients over 60 years.[57,58] In a Spanish retrospective study, the preventive results of IT plus systemic high dose therapy were as good as other studies, which included radiotherapy in their protocol. Thus, the issue of radiotherapy remains open and most of the current protocols omit radiotherapy as prophylaxis,[59] although some reserve this modality for very high risk patients. The ideal interval between IT treatments still has to be established. A relatively new agent is liposomal cytarbine, which when given IT, maintains elevated levels of AraC in the CSF for at least 14 days,[60] thus requiring less frequent IT administrations.

When a patient has CNS involvement at diagnosis, most protocols attempt to eradicate the blasts from the CNS by more intensive (bi-weekly) IT therapy, usually in combination with radiotherapy, and then continue to follow the regular protocol.

Post-remission therapy

*Consolidation/maintenance. M*ost remission therapy for young adults with ALL remains the most challenging and controversial aspect in the management of ALL. Suffice it to say that there are virtually no long-term survivors if no post-remission therapy is given. An overall suggested schema for the management of ALL is outlined in Figure 2, emphasizing the complexities in post-remission management. Historically, patients were offered treatment based on pediatric regimens of ALL on the assumption that this therapy would lead to results in adults comparable with those in children.[61,62] For those patients in whom an allogeneic transplant is not an appropriate option, standard post remission therapy consists of consolidation therapy followed by maintenance therapy for a total of 2.5 years. Consolidation therapy includes high dose methotrexate intercalating with regimens that include asparaginase and cyclophosphomide.

Maintenance therapy for 2 years after induction and consolidation remains a standard of care for patients not transplanted. Randomized studies, mostly in pediactric ALL, have mitigated against any attempts in adults to omit maintenance therapy, as this invariably leads to an inferior outcome. The backbone of maintenance remains methotraxate and 6-mercatopurine. It has not been demonstrated that including cycles of high dose therapy in the midst of the prolonged maintenance is beneficial.[63]

Allogeneic transplantation. Allogeneic transplantation offers the most potent post remission anti-leukemic therapy. This is achieved through the graft versus leukemia (GvL) effect, which is particularly potent in first remission.[64]

Over the past two decades, several studies of allogeneic transplantation in first remission were conducted and there was general agreement that this was a recommended modality for particularly high risk patients, such as those with the Philadelphia chromosome. Gradually, the concept of high risk was broadened to include some other unfavorable cytogenetics on those who did not respond rapidly in induction. Using such criteria, several studies reported a distinct advantage for high risk patients who had a human leukocyte antigen (HLA)-compatible donor.[3,54,65] A meta-analysis of seven studies of transplants in ALL also reported significant advantages for sibling allogeneic transplantation in high risk ALL patients compared with other therapeutic mordalities.[66] None of these studies found any benefit for allogeneic transplants for patients who were not at high risk.

The International ALL trial, conducted by the MRC in the United Kingdom and ECOG in the US, was the largest prospective study of transplantation in ALL with the aim of defining the role of allogeneic transplant, autologous transplant, and chemotherapy for adult patients in first CR up to age 60 years. The hallmark for this study was that all patients received the identical therapy, irrespective of their risk assignment, a concept that was a deviation from the thrust of most clinical trials of therapy in ALL, which are so called "risk-adapted". The trial recruited of 2000 patients over 13 years and the results are shown in Figure 3. The survival of all patients from diagnosis was 39%, with a significantly improved overall survival for patients who had a sibling donor (53% versus 45% at 5 years p=0.01). Despite the significantly reduced relapse rate among all patients with a donor, the high non-relapse mortality (NRM) for high risk patients abrogated the reduction in relapse, such that a definitive advantage could not be demonstrated among high risk patients (Figure 3D). In contrast, among the 239 patients with standard risk who had a donor, the overall survival was significally improved over the 323 patients without a donor (62% versus 52%, p=0.02) (Figure 3C).

Reduced-intensity Conditioning. Reduced-intensity conditioning (RIC) for ALL is clearly an attractive option for older patients or those with comorbidities. In the international ALL trial, high risk patients had an excessively high NRM of 36% at 2 years, which was almost entirely driven by assigning patients over the age of 35 to the high risk group. For such patients, RIC would be an attractive option. Data have been published indicating that RIC transplants are feasible both in first and second remission ALL, with results that are not significantly worse than using a full intensity transplant. Such data have been reported from the Center for International Blood and Marrow Transplant Research (CIBMTR)[67] and the European Bone Marrow Transplant registry (EBMTR).[68] In the later, the relapse rate was greater for patients undergoing RIC compared with myeloblative transplants but the NRM, as expected, was lower. RIC is currently being prospectively evaluated by the NCRI in Britain but caution needs to be advised in using RIC transplants for fit younger individuals with ALL.[69]

Autologous transplantation. There have been several studies of autologous transplants comparing this with standard therapy. None of the studies showed a significant benefit for autologous transplants over chemotherapy although the study conditions of all these trials are different.[54,65,70,71] The large MRC/ECOG study prospectively randomized all patients who did not have a sibling donor to an autologous transplant versus consolidation maintenance therapy for 2.5 years. Although the mortality from autologous transplant was not greater, the overall survival was significally superior in the chemotherapy arm due to the lower relapse rate among patients who received ongoing consolidation maintenance therapy (Figure 3E). Whether additional post autograft therapy may alter these results is unknown, but it is currently difficult to recommend autologous transplant for any patient with ALL, recognizing that the potential benefit among individual subgroups are not necessarily negated by these findings.

Adolescents and young adults with ALL. Multiple reports have described the results of adolescents and, in some cases, young adults, treated on pediatric regimens and have retrospectively compared this with historical data of patients in similar age groups treated on adult protocols [reviewed in ref. #72)]. As a result of these data, many groups have now incorporated a pediatric regimen, and this is being used even in adults up to age 29

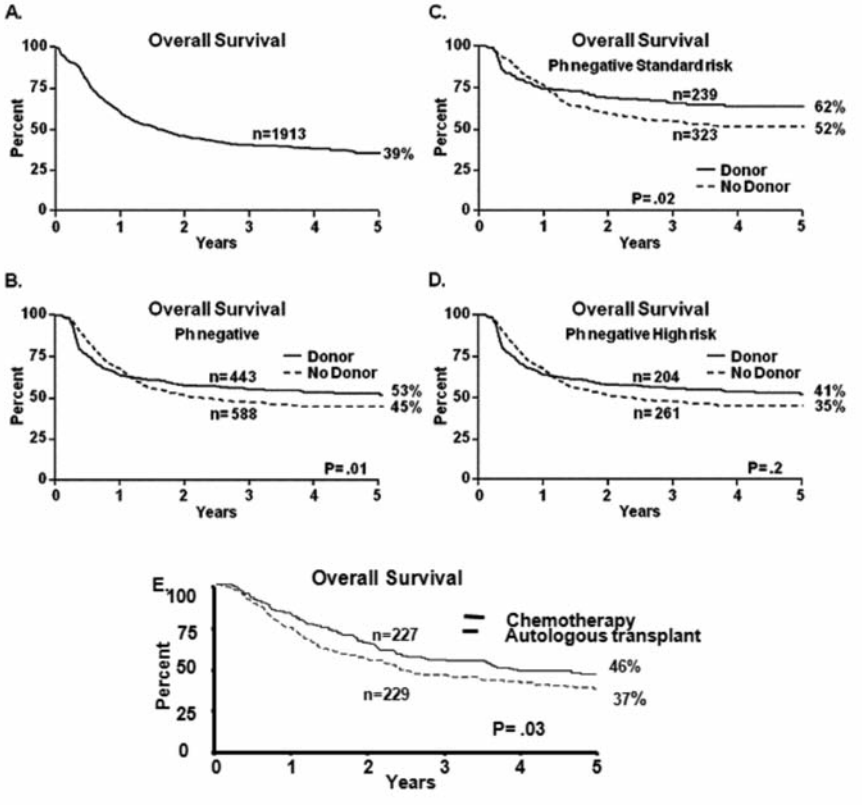

Figure 3. The MRC/ECOG international ALL Trial. Reproduced from Goldstone et al. Blood, 2008; with permission. (A) Overall survival from diagnosis of all patients aged 16–64, including Ph-positive patients. (B) Among Ph-negative patients, there was a significant advantage in the overall survival for patients with a donor compared with those without a donor. (C) Significant survival advantage of Ph-negative patients at standard risk, defined as aged less than 35 years, with a low white cell count at presentation and responding within phase I of induction. (D) Lack of significant benefit for Ph-negative patients at high risk. The reduced benefit for patients with a donor is due almost entirely to excess of transplant-related mortality among patients over age 35 years. (E) Overall survival among patients randomized to receive autologous transplant versus standard consolidation/maintenance chemotherapy.

or higher. It is, however, important to understand that these regimens have not been prospectively studied in young adults; rather they have been adopted. Pediatric regimens are generally more intensive, include far more aspariginase, and reflect a greater protocol discipline, especially about timeliness.[73]

Philadelphia chromosome-positive

Until recently, Philadelphia chromosome-positive ALL (Ph+) was considered the ALL with the poorest prognosis. With chemotherapy alone, most patients did not survive one year.[74–79] Allogeneic transplant improved the survival significantly[78,80–84] and became the gold standard for treatment. The advent of tyrosine kinase inhibitors (TKIs) has completely revolutionized the treatment of Ph+ ALL and has become a part of a new gold standard.

The most important issues regarding the treatment of Ph+ ALL in the TKI are:

Induction
- How to combine TKI with chemotherapy?
- Which TKI to choose?
- TKI and CNS

Postinduction
- In the TKI era, is allotransplant still a must?

Maintenance
- TKI after transplant?

Induction. The combination of conventional chemotherapy and imatinib mesylate has improved the CR rate and the OS compared to historical controls [reviewed in ref. #85)]. The optimal dose of imatinib is not known, as studies have used different doses between 400–800 mg without direct comparisons. The GMALL phase 2 multicentric prospective trial[86] studied the best way to combine chemotherapy and imatinib – concurrent versus alternative administration. The coadministration group resulted in higher rates of PCR negativity (P=0.01) with somewhat higher toxicity but without significant improvement in survival.[87] The MRC/ECOG study emphasized the importance of early administration of imatinib; not later than phase II of induction.

For patients who undergo allogeneic transplantation, it is not clear whether aggressive chemotherapy is needed for induction. The primary data from the GRAAPH-2005 study[88] reported comparable results of two groups, which received either aggressive induction (Hyper-CVAD) or non-aggressive one (vincristine and dexamethasone) combined with imatinib prior to allogeneic transplant.

Most of the studies included imatinib as the first known TKI. Dasatinib, a second generation TKI with a broader spectrum of tyrosine of kinase inhibition, has the potential to be more effective than imatinib but this has only recently been evaluated as first-line therapy. A recent study confirmed the safety and efficacy of dasatinib as first line therapy when given with steroids only.[89] In another European study,[90] dasatinib was given together with low intensity chemotherapy as first line therapy for 71 elderly patients. The CR rate was 90% and the relapse free survival was 22.1 months. Another advantage of dasatinib is its good penetration to the CNS compared with imatinib.[91] Currently, there is no

information about the use of nilotinib as first line therapy for Ph+ ALL.

To summarize, TKI with chemotherapy has become a new standard of care for Ph+ ALL. Imatinib at a dose between 400–800 mg is still the conventional first line treatment but the emerging data may shift this, in the near future, to second generation TKI. Dasatinib is clearly better for CNS disease.

Postinduction. In three different studies[85-87] allotransplant after chemotherapy plus TKI had better results than chemotherapy plus TKI alone. Thus, allogeneic transplantation is still recommended for Ph+ ALL, even in the TKI-era. TKIs improve the CR rate and extend remission duration, thus allowing more patients to undergo allogeneic transplant.[87,92] Still, the follow-up are not long enough and data are lacking, particularly about second generation TKIs and transplant.

Maintenance. Those patients who do not undergo allotransplant need to continue taking TKI therapy indefinitely. A prospective study[93] on 27 Ph+ ALL patients demonstrated that receiving imatinib upon detection of residual bcr-abl transcripts after allotransplant resulted in bcr-abl negativity in 52% of patients. Whether TKIs need to be administered post-allo transplant to bcr-abl negative patients remains to be determined in prospective studies.

Conclusions

While enormous progress has been made in the treatment of childhood ALL, the cure rate in adults remains unsatisfactory. For several decades, beginning in the 1970s, there was a stagnation of new ideas in adults. However, over the past decade, emerging data in transplantation and the development of specific and potential targeted agents have altered the current landscape, as well as the future potential for ALL in adults. Advances in supportive care have also reduced the therapy-related morbidity and mortality. The emphasis on progress can only be made through carefully conducted prospective randomized studies, and because of the relative rarity of ALL in adults, this requires collaborative efforts both nationally and internationally. Much further progress is necessary to improve therapy of adults, alleviate the toxicity, and improve the overall efficacy.

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Acute lymphoblastic leukemia in older patients

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A B S T R A C T

Treatment of older patients with acute lymphoblastic leukemia (ALL) is an unmet medical need. Little improvement has been achieved in the past decades. Older patients with ALL have a significantly lower complete remission (CR) rate, higher early mortality, higher relapse rate, and lower overall survival compared with younger patients. This is explained by a higher incidence of poor prognostic factors on the one hand, and a higher mortality and less intensive chemotherapy due to poor tolerability on the other. Outcome is influenced by conventional prognostic factors but also by age-specific factors, such as general condition and comorbidities. Best results are achieved with protocols specifically designed for older patients with CR rates of 71% (43–90%) and survival rates of 33% (16–71%). For the future optimized supportive care, targeted therapies, intensified consolidation, and reduced intensity stem cell transplantation are promising approaches. One of the most important issues is to differentiate between fit and unfit older patients to offer both groups optimal treatment regarding toxicity and mortality risks, quality of life, and long-term outcome. Therefore, prospective trials specifically designed for older patients are urgently needed.

Introduction

Acute lymphoblastic leukemia (ALL) is often perceived as a mainly pediatric malignancy, which is due to the peak incidence of ALL at the age of one to four years.[1] However, above 60 years, the incidence of ALL increases up to 1.7/100.000 in patients older than 85 years. In the United States (US) the proportion of ALL cases diagnosed in patients older than 55 years (17%) nearly equals the proportion of patients diagnosed at the age of 21 to 54 years (22%).[1] In Western countries, the population is aging and therefore, an increasing number of older individuals with ALL has to be expected.

In retrospective studies, the proportion of elderly patients (> 60 years) referred to specialized centers varied from 18 to 30%.[2–4] The existence of this large proportion of older ALL patients is not reflected by multicentre trials, where the median age was between 25 and 43 years, and the upper age limit is mostly defined between 50 and 65 years.

In addition, older compared with younger patients are far less frequently included in clinical trials. A population based study from the United Kingdom (UK) has demonstrated that the proportion of patients included in clinical trials decreased from 74% in children to less than 10% in patients older than 60 years.[5] There are a number of potential reasons for this. Few studies are offered specifically for the older patient population, and physicians most frequently treat patients 'according' to protocols for younger patients with a number of individual modifications. Older patients themselves may be reluctant to be transferred to specialized centers, and physicians may make less efforts due to their negative expectations regarding the outcome of older leukemia patients.

Clinical features in older acute lymphoblastic leukemia patients

Disease characteristics

B-lineage ALL has a proportion incidence of 75–89% in patients above 60 years compared with 59–66% in younger patients, whereas the incidence of T-ALL is lower in older (8–12%) compared with younger (29%) patients.[4,6] Within T-ALL, the proportion of thymic T-ALL, as a more favorable subgroup, seems to be lower in older compared with younger patients (3% versus 14%).[6] There may also be a higher incidence of mature B-ALL of 10% in older compared with 6% in younger patients.[4,7]

A population based study in Northern England showed that the proportion of patients in whom cytogenetics were attempted was lower in patients older than 60 years (73%) compared with younger patients (85–91%). The proportion of patients with t(9;22), t(8;14), t(14;18) or complex aberrations appeared to increase with age.[7] Within B-precursor ALL, the frequency of Ph/bcr-abl-positive (Ph+) ALL increases from 13% in adolescents to more than 40% in patients older than 40 years.[8] The overall incidence of Ph+ ALL was 15–19% in patients younger than 60 years, compared with 24–36% in older patients.[3,4]

Most studies report a lower frequency of features associated with large tumor mass,

such as high white blood cell count,[4,6] and a lower proportion of males in older (49–54%) compared with younger (58–64%) ALL patients.[3,4]

Comorbidities and geriatric assessment

Often studies describe a higher frequency of poor performance status[3,4] in older patients. In two studies, 30–43% of the patients older than 60 years had a performance status of 2 or more compared with 18–22% in the younger patient group.[3,4] Furthermore, 60–70% of older patients suffer from comorbidities.[9–11] The German Multicentre Study Group for Adult ALL (GMALL) has prospectively evaluated comorbidity according to the Charlson Score in a trial specifically designed for older ALL patients. Comorbidities were described in 84% of the patients. Diabetes (46%), vascular disease (18%), heart failure (15%), and chronic lung disease (12%) were the most frequent ones.[9] Eight to sixteen percent of the older ALL patients had a history of prior malignant disease.[4,9]

Complete geriatric scoring has not been reported from prospective studies in ALL so far, although it had a significant prognostic impact in patients with aggressive lymphoma older than 65 years. All patients considered fit according to geriatric assessment and treated intensively had an excellent outcome with 78% survival. Patients considered unfit had a poor survival, independent of whether they received intensive (20%) or palliative treatment (26%).[12] A complete geriatric assessment, however, is very time consuming and cannot be realized in daily practice in many hospitals. A reduced number of tools including comorbidity score, activities of daily living, geriatric syndromes, and assessment of depression may be suitable for prospective evaluation in clinical trials.

Management issues in older patients

Treatment of acute leukemia in older patients is often complex, not only due to more frequent comorbidities and resulting use of multiple medications with risk of drug interactions but also to physiologic differences related to age. Hepatic and renal function may be impaired or less flexible. Hematopoietic recovery occurs more slowly due to a lower number of hematopoietic stem cells. This also applies to the regenerative capability of tissues, such as mucosa. Specific drugs in ALL treatment are associated with a higher risk of toxicities in older patients. This includes polyneuropathies and constipation associated with vincristine, diabetes, and hyperglycemia associated with steroid application, cardiac toxicities of anthracyclines, and liver toxicities induced by several drugs, such as asparaginase, methotrexate, or purine analogues, among others.

Induction therapy is the most critical phase for management. Seven to ten percent of the elderly patients even die before initiation of any chemotherapy.[4,11] Cause of death in induction is most frequently infection.[13–15] In the ECOG-MRC studies, patients older than 55 years had a significantly higher rate of infections during induction (67%) compared with younger patients (45%).[16] The application of G-CSF during chemotherapy at least may attenuate neutropenia. A randomized, placebo controlled study of G-CSF during induction chemotherapy demonstrated a significant reduction of induction death from 31% with placebo to 5% with G-CSF support in patients older than 60 years.[17] Often it is not possible to distinguish between hematologic toxicity and insufficient treatment response as causes for infectious complications. A high rate of early mortality and complications is also reported for palliative therapy approaches in older patients[2] (Table 1). Both hematological and non-hematological toxicity often results in incomplete drug administration and extended intervals between treatment cycles; this may contribute to inferior long-term results in older ALL patients.

Prognostic factors in elderly ALL patients

Age, beside response to treatment, is one of the most relevant prognostic factors for outcome of ALL. In the age group younger than 60 years, the survival rates decrease significantly with increasing age[18] and this effect continues in the older population.[3,11]

It can be assumed that prognostic factors described for younger ALL patients[19] are also valid in the older ones. There are, however, additional factors with specific relevance in older patients. Bleeding or infection at diagnosis were associated with poorer overall survival.[4,11] In a multivariate analysis, the most significant prognostic factor for achievement of CR and survival was performance status with induction mortality of 35% for patients with WHO performance status above or equal to 2, compared with 4% in those with better performance status.[3]

In the GMALL study for older patients, body mass index, Charlson comorbidity score, age, and ECOG status before onset of leukemia were significantly associated with early death rate. Age and ECOG status also significantly influenced overall survival. Patients older than

Table 1. Outcome with different treatment approaches in older patients with ALL.

Approach	Age (Range, years)	Studies (N)	Patients (N)	CR* (%)	Early death* (%)	Survival **
Population based studies[1,7,22]	>65	3	n.r.	n.r.	n.r.	6–30%
Palliative treatment [2,3,23,24]	60–91	4	94	43 (34–53)	24 (18–42)	7 mo (3–10 mo)
Intensive chemotherapy[4,10,11, 14,25-31]	60–92	15	519	56 (40–81)	23 (6–42)	14% (3–29%)
Prospective studies for older ALL patients [9,10,34,32–37]	55–81	9	447	71% (43–90)	15 (0–36)	33% (16–71%)

70 years with ECOG status above 2, compared with those aged 55–70 years with ECOG status of 2 or better, had an early death rate of 30% versus 9% and a survival rate of 11% versus 37% respectively.[9] A scoring system reported for older patients with acute myeloid leukemia (AML) based on clinical and biologic factors was able to predict the risk of early death in a range from 7 to 63%.[20] On the other hand, population-based data in AML demonstrated that although outcome was strongly correlated to age and performance status, early death rate was always lower with intensive therapy compared with palliative therapy.[21]

These data underline that older leukemia patients' general conditions and pre-existing complications, in addition to biologic risk factors, strongly influence the outcome of induction therapy. Although the prospective applicability of scoring systems for decision making is unclear, it may be concluded that in older patients, it is reasonable to stabilize the general condition and control complications, such as infections, before the start of intensive chemotherapy.

Overall the prognosis of older patients is influenced by an increased toxicity of chemotherapy on one hand, and a higher relapse risk on the other. Toxicities lead to higher mortality in induction and in CR and to a higher rate of interruptions and dose reductions of chemotherapy. The relapse risk is associated with higher incidence of poor prognostic features, possible differences in drug pharmacology, and with less effective chemotherapy. This dilemma may be solved by carefully designed age-specific protocols.

Treatment results in older ALL patients

Population-based studies

Population based studies give an impression of the outcome of an unselected older patient population. In the US, a relative survival of 10% in patients older than 65 years and 6% in those older than 75 years was reported.[1] In Northern England, patients older than 60 years had an overall survival of approximately 20% at 2 years and 12% at 5 years.[7] Survival rates, however, were calculated only for those patients considered fit enough for active treatment. The proportion of patients meeting this definition was 94% below the age of 60 years and 31% in those above 60 years.[7] In Sweden, survival rates were approximately 10% after 2 years for patients older than 75 years and approximately 25% for those aged between 65 and 74 years.[22]

Palliative treatment compared with chemotherapy

Four groups have retrospectively compared results from palliative treatment to chemotherapy approaches. Decision criteria or details of therapy have not been defined. Thirty to fifty percent of the older patients were allocated to palliative therapy mainly due to poor performance status.[2,3,23] Most studies showed an advantage of chemotherapy, such as a significantly higher CR rate, remission duration, and median survival.[2,3,23] Also, early death did not occur more frequently with intensive therapy compared with palliative therapy (18% versus 24%) (Table 1).

Treatment according to protocols for younger ALL patients

The majority of published data is based on results from older patients treated within protocols designed for younger patients. It can be assumed that these are selected patients and that a number of treatment modifications became necessary. In 519 patients older than 60 years, the remission rate was 56%. The rate of early death was 23% with a range from 6 to 42%. The median duration of remission was only 9 months and the probability of survival 14% (Table 1).

Prospective studies designed for older ALL patients

Only few prospective trials with specific protocols for older patients were reported during the past two decades (Table 2). One approach is the use of idarubicine in induction therapy based on a supposed lower cardiac and hepatic toxicity compared with other anthracyclines. It was combined with vincristine, prednisone, and asparaginase in induction and followed by a flexible post-remission schedule aiming to treat the patients on outpatient basis. The authors reported a moderate induction mortality of 18% and a CR rate of 59%.[32]

In a small trial, the use of liposomal daunorubicine was associated with an improved remission rate (41 to 76%).[10] When vincristine was replaced by vindesine in another study, no effect on the incidence of neurotoxicity was observed.[33] Specific data on the use of liposomal vincristine in older patients are not available.

A paediatric protocol modified for patients older than 60 years included asparaginase during induction and consolidation. The CR rate was 71% with 29% induction mortality. The authors reported a number of complications during induction, such as infections (71%), cardiotoxicity (18%), and hyperglycemia (24%). Complications during intensifications were also frequent. The survival after 1 year was 71%.[35]

The Spanish PETHEMA group reported the results of an intensive induction regimen based on vincristine, daunorubicine, prednisone, cyclophosphamide, and repeated cycles of asparaginase. The induction mortality was rather high in this study (36%). In most cases, the cause of death was infection. This omission of asparaginase and cyclophosphamide from induction led to a significant reduction of the early death rate from 70 to 22% and also translated into an improved overall survival.[34]

In a prospective GMALL study, dose reduced induction with idarubicine, steroids, vincristine, cyclophosphamide, and cytarabine was followed by alternating consolidation cycles for 1 year and maintenance. Patients with CD20 positive ALL received rituximab in combination with chemotherapy. In 146 patients, the CR rate was 73%, with 18% early deaths mainly due to infections. The overall survival at 4 years was 24%. Since the mortality in CR was still acceptable (7%), the group decided on further intensification predominantly during consolidation therapy.[9]

The French study group reported a trial, which aimed to assess a new induction regimen with continuous infusion doxorubicine and vincristine compared with pegylated doxorubicine and standard vincristine accompanied by dexamethasone and cyclophosphamide. Induction was repeated and followed by consolidation and maintenance. Despite lower rates of hematologic toxicity, infections, and cardiotoxicity with pegylated

Table 2. Outcome from prospective trials designed for older ALL patients.

Author	Year	Age	Ph+	Patients (N)	CR rate	Early death	Failure	CCR*	DFS*	OS**
Bassan et al[32]	1996	60–73 (64)	yes	22	59%	18%	14%	12	9	20% (2 y)
Delannoy et al[33]	1997	55–86 (67)	yes	40	85%	n.r.	n.r.	n.r.	14	16% (2 y)
Delannoy et al[42]	2002	65 (55–81)	yes	58	43%	10%	47%	5	10	n.r.
Offidani et al[10]	2004	69 (61–79)	yes	17	76%	17%	6%	20	21	38% (2 y)
Sancho et al[34]	2007	65 (56–77)	no	33	58%	36%	6%	46% (2 y)	7	39% (1 y)
Gökbuget et al[9]	2008	67 (55–81)	no	146	73%	18%	8%	33% (4 y)	n.r.	24% (4y)
Kao et al[35]	2008	66 (60–78)	yes	17	71%	29%	0%	82% (1 y)	n.r.	71% (1 y)
Gökbuget et al[36]	2008	66 (56–73)	no	54	85%	0%	15%	9	n.r.	61% (1 y)
Hunault-Berger et al[37]	2010- Arm 1	68 (55–77)	no	31	90%	7%	3%	32% (2 y)	n.r.	35% (2 y)
Hunault-Berger et al[37]	2010 - Arm 2	66 (60–80)		29	72%	10%	17%	52% (2 y)		24% (2y)

Abbreviations: Ph+, Ph/bcr-abl positive ALL included yes or no; Arm 1 continuous infusion Doxorubicin; Arm 2 pegylated Doxorubicin; CCR continuous complete remission; DFS disease free survival; OS overall survival; *median months or probability; **probability.

Table 3. Treatment older patients with Ph+ ALL.

Author	**Year**	**Age** **Median (Range)**	**Induction**	**Consolidation**	**IM dose**	**N**	**CR**	**CCR****	**Survival****
Vignetti et al[38]	2007	69 (61–83)	**IM** + PRED	**IM**	800	29	100%	48% (1 y) 8 mo	74% (1 y) 20 mo
Delannoy et al[39]	2006	65 (58–78)	PRED CH	**IM** + PRED CH **IM**	600	29	72%	58% (1 y)	66% (1 y)
Ottmann et al[40]	2007	66 (54–79) 68 (58–78)	**IM** *rand.vs CH	**IM**+CH	600	28 27	96% 50%	70% (1 y) 17 mo 74% (1 y) 20 mo	57% (1 y) 41% (1 y)
Rousselot et al[41]	2010	69 (58–83)	**DASA** + CH	**DASA** + CH (alternating)	140	71	90%	22 mo	27 mo

Abbreviations: IM, imatinib; PRED, prednisolone; DEXA, dexamethasone; CH, chemotherapy; DASA dasatinib; CCR continuous complete remission; mo months. * preceded by prephase with DEXA+CP in both arms. **Probabilities and median duration.

doxorubicine, there was a trend towards a higher CR rate (72% versus 90%) and a lower rate of relapses (32% versus 52%) with conventional doxorubicine.[37]

Based on protocols developed by French and German ALL study groups, a joint European treatment protocol for older patients with ALL was defined by the European Working Group for Adult ALL (EWALL). The 4-week-induction comprises dexamethasone, vincristine, idarubicine in phase I and cyclophosphamide and cytarabine in phase II. Consolidation consists of six alternating cycles with intermediate dose methotrexate and E.coli asparaginase and high-dose cytarabine followed by maintenance with mercaptopurine, methotrexate, and vincristine/dexamethasone pulses. Overall, 85% of the patients achieved a CR, and no death in induction was observed. At 1 year, 61% of the patients were alive and 49% in continuous complete remission.[9] The moderate intensity consolidation treatment was feasible. It was of interest that asparaginase during consolidation was well tolerated whereas other studies had indicated a lower tolerability during induction.[34,35] Further intensification of consolidation therefore appears to be possible. Furthermore the EWALL protocol represents a basis for investigation of new drugs either in a randomized comparison or based on historic controls.

Targeted therapies for older patients with ALL

Tyrosine kinase inhibitors for older patients with Ph+ ALL

The use of tyrosine kinase (TK) inhibitors is a very promising approach for the large proportion of older patients with Ph+ ALL. Historic results with CR rates

below 50% and survival below 5% could be improved considerably by the use of imatinib or dasatinib in induction therapy and/or consolidation therapy (Table 3).

The GIMEMA group used imatinib at a dose level of 800 mg in combination with steroids for a period of 30 days, followed by imatinib as single drug treatment. The remission rate was 100% and the overall survival after 1 year was 74%. However, a number of patients relapsed with a median remission duration of 8 months and after 1 year, 48% of the patients remained free of disease.[38] In a French study, patients received a conventional chemotherapy induction. Afterwards, imatinib was combined with prednisone and given alternating with chemotherapy during consolidation. The remission rate with chemotherapy alone was lower than with imatinib single drug treatment (71%). The overall survival of 66% at 1 year was significantly improved compared with a historic control without imatinib (43%). A similar improvement was achieved for relapse free survival with 58% versus 11% after 1 year in the historic control.[39]

In a prospective randomized trial from the GMALL study group, older patients with Ph+ ALL received a 4-week treatment with imatinib only compared with a 4-week chemotherapy induction without imatinib. The remission rates were 96% and 50%, respectively. After induction patients received consolidation chemotherapy parallel with imatinib for at least 1 year. In both arms, the relapse rate was high. Despite the significantly higher CR rate with imatinib therapy in induction, after 1 year no difference in terms of survival was detected.[40]

The most recent and largest study in older patients with Ph-positive ALL is based on the EWALL chemotherapy backbone. Induction consisted of vincristine, dexamethasone, and dasatinib at a daily dose of 140 mg. Consolidation and maintenance chemotherapy was combined with intermittent dasatinib applications. In 71 patients, the CR rate was 90%. The regimen was feasible, and the median duration of remission (22 months) and overall survival (27 months) was promising. Persistent minimal residual disease (MRD) above 0.1% after induction and consolidation was associated with a poorer remission duration of only 5 months. Overall, dasatinib showed favorable antileukemic activity. However 13 of 16 patients with relapse showed a mutation in the TK domain, which, in most cases, was a T315I mutation.[41]

Overall, single drug treatment with TK inhibitors can be used successfully for induction therapy in older patients with Ph+ ALL. The major advantage is the low early mortality. For maintenance of remission consolidation, chemotherapy is essential. It remains open to question whether continuous or intermittent treatment with TK inhibitors is superior. At relapse, most patients show mutated clones with resistance to TK inhibitors. Alternating treatment with different TK inhibitors or the use of third generation inhibitors may be useful to avoid this process. For future improvement, identification of poor molecular responders and change of TK inhibitor might be an approach. Furthermore, older patients with Ph+ ALL are candidates for dose reduced stem cell transplantation.

Rituximab for CD20 positive B-precursor ALL

Outcome of younger and older patients with mature B-ALL or Burkitt's lymphoma has been significantly improved by the combination of dose dense chemotherapy with Rituximab.[43,44] Also in younger patients with CD20 positive B-precursor ALL, the addition of Rituximab to chemotherapy improved the overall survival considerably.[45,46] No improvement, however, could be demonstrated for patients older than 60 years treated with the Hyper-CVAD regimen and rituximab.[46] The reason was apparently a higher mortality in CR due to infections underlining the need for intensive supportive care for older patients throughout the whole treatment period.

Other antibodies

ALL blasts express a number of intracellular and surface antigens, such as CD33, CD19, and CD52, which could be a target for antibody therapy; however so far, few data on clinical application are available.[47] A new promising approach is the use of a bispecific CD19 antibody, which has the potential to engage cytotoxic T cells in patients for lysis of CD19 positive leukemia cells.[48] In 19 evaluable patients with persistent minimal residual disease, the molecular remission rate was 84%. A number of older patients, who were not able to receive a stem cell transplantation, remained in remission for more than 1 year.[49]

Stem cell transplantation in older patients with ALL

In patients older than 55 years, the indication for stem cell transplantation (SCT) is rarely made due to the expected high transplant related mortality (TRM). It remains to be determined whether SCT with dose reduced conditioning (RIC) could be a promising alternative for older patients with high risk features. For the interpretation of published results, it has to be considered that patients treated with reduced intensity conditioning are negatively selected according to age and comorbidities. Retrospective analyses and registry data show in older patient populations with a median age of 38 to 56 years, overall survival rates after RIC between 18 and 48%, relapse incidence rates of 36% to 50%, and TRM rates between 21 and 41%.[50-53] RIC compared with full intensity conditioning in patients above 45 years showed a higher relapse incidence, a lower mortality, and overall comparable results.[53] Full conditioning was associated with a considerable mortality up to 36% in patients older than 60 years. With RIC transplantation, however, survival rates of 48% in patients aged 50 to 60 years and survival rates of 32% in those older than 60 years were reported with mortality rates around 20%. Therefore, prospective evaluation of RIC in older ALL patients with high risk features is of interest.

Future treatment of older patients with ALL

The term of being old has not been well defined in the context of acute leukaemias. Chronological age alone is not suitable for decision making due to broad variability

of the aging process with respect to organ function, cognitive, and functional status, as well as social resources. Comorbidity and geriatric assessment may help to screen these dimensions systematically and to develop better models for decision making. It also helps to detect unknown health problems and social factors, such as dependency and depression.

For practical reasons, different steps for an age-based stratification can be defined, such as the age of 55–60 years as a cut point for very intensive chemotherapy and SCT with full conditioning. The cut point of 75 years is often used for the definition of old age.

For future treatment of older ALL patients, it will be essential to distinguish between frail or unfit patients in whom an unacceptable high mortality of induction therapy has to be expected and fit patients who can tolerate intensive chemotherapy. A third group are patients with good general condition before onset of leukemia but the presence of leukemia associated complications, who may benefit from an extended pre-phase treatment with intensive supportive measures in order to stabilize their general condition. For decision making, the patient's wish and individual status, disease characteristics, and the expected outcomes regarding early mortality and long-term survival have to be considered and discussed.

The major risk for older ALL patients treated with chemotherapy is death due to infections. It is therefore essential to provide intensive supportive care, including G-CSF, anti-infectious prophylaxis, and environment.

All older patients need a comprehensive diagnostic classification. The identification of the bcr-abl translocation is crucial since even in very old patients, the use of TK inhibitors offers a realistic chance of complete remission with very limited toxicity.

The attempt to achieve a remission should be made whenever possible. Specific risks, such as the prolonged use of steroids or asparaginase in induction, should be avoided. On the other hand, there is still space for intensification of chemotherapy particularly during consolidation for fit patients. This includes the use of asparaginase and RIC transplantation. It will be crucial to identify prognostic factors for older ALL patients to define indications for SCT. In unfit older patients, a minimal induction and consolidation therapy is recommended with the aim to control the disease. In both groups the use of targeted therapies, such as Nelarabine, TK inhibitors, antibody treatment, or new drugs with potentially reduced or alternative toxicity will be essential. Persistence of MRD is one of the most important risk factors in ALL. Therefore, MRD evaluation should also take place in older patients to identify those who could benefit from experimental therapies.

A population based study from the US compared survival data of two periods (1980–1984) and (2000–2004). Most improvement was achieved in younger patients aged 15–29 years, whereas in older patients above 60 years, the survival rates remained nearly unchanged with 8% versus 13% relative survival at 5 years.[54] Whereas the current discussion has a strong focus on treatment optimization in adolescents and young adults, management of older ALL patients remains an unmet medical need. Prospective trials specifically designed for older ALL patients are needed, and patients should, whenever possible, be entered in trials or registries since otherwise, there is no gain of knowledge and no chance of treatment optimization.

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The molecular basis of acute myeloid leukemia

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A B S T R A C T

Acute myeloid leukemia (AML) is a clonal/oligoclonal malignancy distinguished from normal hematopoietic cells by key properties, including differentiation block, enhanced self-renewal, increased proliferation, decreased cell death, dissemination, and genomic instability. Modern research into the pathogenesis of AML involves the elucidation of the role of aberrant chromosomal rearrangements amplifications and deletions, and point mutations and aberrant regulation of gene expression, governed in part by changes in chromatin. New technologies have accelerated the ability to sub-classify AML based upon mutation and gene expression status, and many of the phenotypic properties of AML can be mapped onto underlying genetic lesions. Within several years, a near complete categorization of AML will be achieved, and a variety of new therapeutic targets will be identified. Remaining challenges will be to understand the molecular mechanisms linking genetic and epigenetic changes to leukemia cell growth and the translation of these findings into robust agents designed to target specific mutant or deregulated proteins.

The evolving modes of classification of acute myeloid leukemia

The French-American-British (FAB) system classified acute myeloid leukemia (AML) by morphology and analogy to normal myelopoiesis but it was rather poor information in that only a few subtypes, such as acute promyelocytic leukemia (APL), could be distinguished as having a distinct prognosis. In the past 20 years, conventional cytogenetics, flow cytometry, fluorescence *in situ* hybridization, DNA sequencing, and PCR have more precisely defined prognostically important subsets of AML. This was reflected in the 1999 WHO system that includes cytogenetic and molecular anomalies.[1] The revolution in genomic technology will soon lead to a reassessment of AML and the definition of even smaller prognostic subsets.

Cytogenetics has classically defined three main subsets of AML (Figure 1).[2] The favorable prognosis group includes rearrangement of the retinoic acid receptor t(15;17)(PML-RARα), core binding factor-AML (CBF-AML): t(8;21) (RUNX1-RUNX1T1), and inv(16)/t(16;16)(CBFB-MYH11). These anomalies are more frequent in patients less than 60 years, who typically have higher rates of complete remission and a lower risk of relapse. A number of point mutations in signaling pathway genes, such as KRAS, NRAS, CBL, and JAK2, are also found in CBF-AML that may be involved in disease although their prognostic significance is unclear.[3] Mutation of KIT in CBF-AML found in 25-30% of patients negates the good prognosis of the translocation.[4]

A highly heterogeneous intermediate prognosis group encompasses 50–60% of patients with AML and includes patients with specific chromosomal anomalies, such as trisomy 8 or 21, as well as normal karyotype (NK-AML), characterized by a variety of molecular abnormalities. A subset of NK-AML harbor mutations in genes that confer unfavorable prognosis, including FLT3, MLL, WT1, and RUNX1. FLT3 mutations are found in 28–34% of NK-AML cases, and occur as either internal tandem duplications (ITD) within the region encoding the juxtamembrane domain or within the tyrosine kinase domain, resulting in constitutive activation. Patients can be further subdivided on the basis of FLT3-ITD to wild type ratio, length of the duplication, and insertion site. Patients with higher mutant level had a higher risk of relapse and death.[5] Partial tandem duplications (PTDs) of a single MLL allele are observed in 5–11% of patients with NK-AML.[6] While MLL is a H3K4 histone methyltransferase (HMT) associated with transcriptional activation, AML with MLL-PTD is also associated with increased global DNA hypermethylation relative to patients with wild type MLL, implicating multiple defective epigenetic mechanisms in these patients.[7] Mutations in hematopoietic transcription factors WT1[8] and RUNX1[9] are observed in 10–13% and 33% of NK-AML, respectively.

Mutations in NK-AML associated with a favorable prognosis include those affecting the *NPM1*[10] and *CEBPA*[11] genes. Truncating NPM1 mutations leading to abnormal cytoplasmic localization of this phosphoprotein (NPM1c+) are observed in approximately 45–64% of NK-AML and are associated with a favorable prognosis unless combined with FLT3 mutations.[10] CEBPA mutations affect 10–18% of NK-AML patients and lead to

either a truncated dominant negative isoform or protein with decreased DNA binding or dimerization ability. Approximately 85% of CEBPA mutant AMLs are biallelic so that no wild type protein is produced,[12] and these patients have a particularly good prognosis.[13] By contrast, mutation of a single allele of CEBPA does not affect prognosis. NRAS (9–14%) and KRAS (5–17%) mutations are of uncertain prognostic significance. In NK-AML, overexpression of the *BAALC*, *ERG*, *MN1*, and *EVI1* genes is associated with poor prognosis.[14,15]

Poor prognosis AML includes patients with complex karyotype (harboring at least four unrelated cytogenetic abnormalities).[16] These patients tend to be older and may have an antecedent myelodysplastic or myeloproliferative disorder. Mutations involving the TP53 occur frequently in this group (56–78%).[17] Amongst the adverse prognosis group, multiple studies showed that monosomal karyotype (MK), defined as autosomal monosomy in the presence of another autosomal monosomy or other chromosomal abnormalities, is associated with a particularly poor prognosis, with overall survival rates of less than 5%.[16,18,19]

Single nucleotide polymorphism (SNP) arrays, which can identify small genomic amplifications, deletions, and areas of copy number neutral loss of heterozygosity (LOH), also known as uniparental disomy (UPD), showed that approximately 20% of NK-AML exhibited partial UPD.[20] Later studies revealed that AML is characterized by a high number of small, non-recurrent copy number alterations.[21] SNP arrays identified TET2 as a potential tumor suppressor in AML and other myeloid neoplasms, as TET2 mutations are found in 24% of secondary AML, 19% of myelodysplastic syndrome (MDS), 12% of myeloproliferative neoplasms (MPN), and 22% of chronic myelomonocytic leukemia (CMML).[22] Over the past 2 years, whole-genome sequencing discovered recurrent mutations in AML, such as DNMT3A[23] and IDH1.[24] DNMT3A mutations were enriched in patients with intermediate risk cytogenetics (33.7%) and conferred a poor prognosis. IDH1 and IDH2 mutations were found in 14% and 19% of NK-AML, respectively and the impact of these mutations on outcome is uncertain and may be influenced by the presence of coincident mutations (reviewed by Lowenberg B. in this issue).[25,26] Large-scale sequencing also revealed mutations in ASXL1 in 6% and 53% of patients with primary and secondary AML.[27]

AML may be classified by gene expression, DNA methylation, and chromatin modification patterns. Gene expression profiling can predict FAB subtypes[28] and discern novel prognostic subsets of AML.[29–31] The combination of gene expression and promoter methylation signatures, in which the transcriptional capacity of the genome is ascertained, can further identify AML subtypes.[32] Figueroa *et al.* found 16 different patterns of promoter methylation among AML. While some corresponded to known cytogenetically defined subtypes, there were five methylation-defined AML subtypes with no other defined genetic characteristics. Several of these groups were subsequently shown to correspond to patients with TET2 or IDH1 or IDH2 mutations.[33] The methylation status of 15 promoters could be used as a predictor of survival. Analysis of histone methylation patterns in primary AML specimens may also be useful. Profiling of H3K9 promoter methylation revealed distinct differences between AML blasts and normal CD34+ cells and found that the state of H3K9 methylation was a predictor of prognosis.[34] Unlike DNA methylation profiling, however, H3K9 methylation patterns were not associated with known cytogenetic abnormalities and were not correlated with gene expression.

MicroRNAs play an important role in normal hematopoiesis and leukemogenesis.[35] Genome-wide microRNA analysis in AML identified a correlation between microRNA signatures, specific cytogenetic groups, and prognosis.[36,37] In NK-AML, a microRNA signature was identified for high-risk patients harbor-

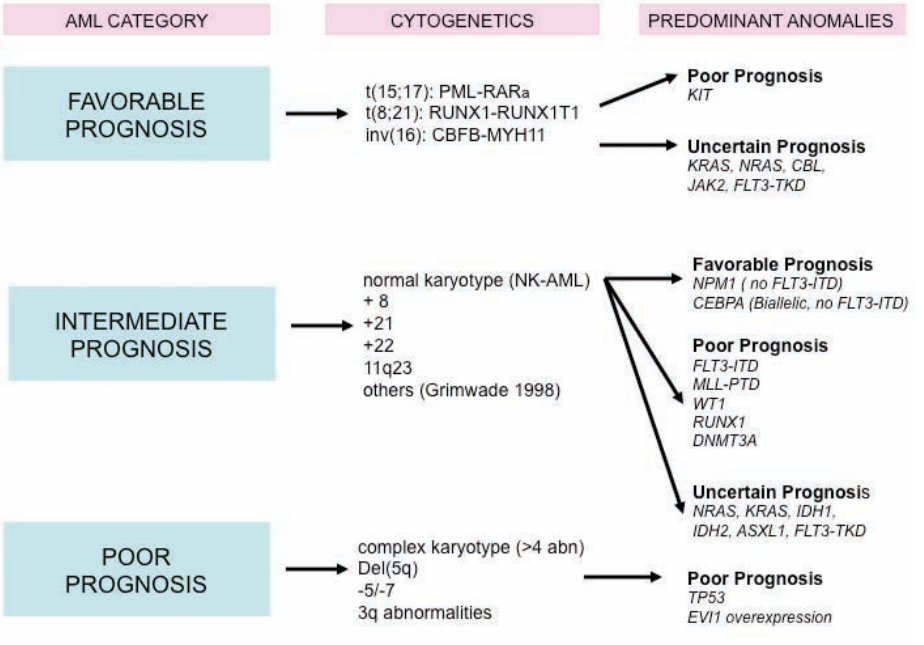

Figure 1. Stratification of Prognostic Groups in AML. AML patients are classified at time of diagnosis into three different prognostic groups based on their cytogenetic profiles. These groups can be further sub-divided on the basis of specific mutations.

ing FLT3-ITD, wild-type NPM1, or both mutations. Expression of microRNAs in these patients was associated with genes involved in innate immunity.[38] Deregulation of specific miRNAs can explain aspects of AML biology, and hence represent new therapeutic targets. For example, miR-126/126*, which is elevated in CBF-AMLs, inhibited apoptosis of AML cell lines and cooperated with RUNX1-RUNX1T1 to enhance self-renewal activity of mouse bone marrow progenitors.[36]

Mutational information in AML is influencing clinical practice. In addition to distinguishing the good prognostic cytogenetic karyotypes from others, clinicians are beginning to stratify AML by virtue of mutations in the genes noted above. Evidence suggests that patients without the FLT3 mutation may benefit from higher doses of anthracycline,[39] and NPM1c+ patients lacking the FLT3 mutation have amongst the most favorable prognoses.[40] Patients with biallelic CEBPA mutation might be spared stem cell transplant consolidation.[41] What is not yet certain is how we can best integrate knowledge of these chromosomal anomalies, point mutations, gene expression, DNA methylation, and miRNA profiles.

Pathogenesis of acute myeloid leukemia

The leukemic stem cell

Over the past decade, work initiated by Dick and colleagues posited the existence of a leukemia stem cell (LSC), perhaps better termed a leukemia initiating cell (LIC). The human LSC was defined as a human leukemia cell with defined cell surface markers capable of causing disease in an immunocompromised mouse. Only a small population of AML cells was found to be capable of initiating and maintaining disease upon xenotransplantion into severe combined immunodeficient (SCID) mice.[42] Similar to HSCs, these cells often express CD34 but not CD38, and possess extensive self-renewal properties. Thus, AML was envisaged as a hierarchy, as in normal hematopoiesis, where a subpopulation of cells can both self-renew and differentiate, while the bulk of the leukemia was more differentiated and lacked this capacity. Indeed, several studies identified a subpopulation of AML cells that display a quiescent phenotype, and these cells were capable of repopulating AML in SCID mice.[43,44]

However, the cancer stem cell hypothesis is controversial and is undergoing re-examination. In melanoma, for example, optimization of xenograft methods showed that a single unsorted melanoma cell can give rise to a tumor in a highly immunocompromised mouse.[45] In the Eμ-Myc mouse leukemia model, a single cell found at a frequency of 1/10 can transmit leukemia in a syngeneic mouse.[46] Since microenvironment factors and the immune system have been shown to affect cancer progression, the xenotransplantation of human AML cells into the mouse microenvironment may not accurately reflect the ability of these cells to cause disease. This may lead to underestimation of the number of leukemia initiating cells, which is likely to affect the design of therapies targeted at this specific population. However, the fact that only a certain fraction of cells can break the xenograft barrier and grow in mice might also reflect inherent heterogeneity and clonal evolution in the leukemia. In the clonal evolution model, mutant clones with a growth advantage become the dominant population, until clones with additional growth and survival advantages emerge. Given that genetic instability is a hallmark of cancer cells, and that LSCs are capable of clonal evolution,[47] it is likely that such clonal diversity plays an important role in AML progression and disease resistance. Recent work from the Greaves laboratory showed that leukemic cells from individual patients with ALL contain multiple genetic abnormalities in addition to the ETV6-RUNX1 founder mutation. These mutations and copy number alterations were not acquired in any specific order, indicating a 'branching' clonal evolution, and leukemia-initiating cells, as assayed by serial transplant into NOD/SCID IL2Rγ^{null} mice, were also genetically heterogeneous.[48] Clearly, targeting cells containing founder cancer initiating mutations in addition to the bulk leukemia, which may be dominated by a subclone with secondary mutations, is required to eradicate the disease.

Despite the controversy surrounding the definition of LSCs and their frequency in different cancers, such debate has fuelled research into pathways that drive aberrant self-renewal in these leukemia-initiating cells. In some cases, induction of these aberrant programs can be directly mapped to specific molecular lesions of AML. For example, expression of the NPM1c+ variant is associated with induction of genes linked to the stem cell phenotype, including members of the HOX gene family, and the Notch1-ligand JAG1 and repression of CDKN2C.[49] The FLT3-ITD mutant confers self-renewal to human CD34$^+$ cells.[50] Wnt signaling has been linked to self-renewal of normal and cancer stem cells.[51] Many of the AML fusion proteins induce the expression of β-catenin and γ-catenin[52] and components of the Jagged/Notch pathway, which are implicated in the regulation of self-renewal.[53]

More recently, efforts have been focused on identifying the gene expression signatures of LSCs to facilitate risk-based stratification of patients and help guide therapy design. A study contrasting expression profiles of paired LSC-enriched (CD34+CD38-) and leukemic progenitor (CD34+CD38+) cells in primary AML patients identified a set of genes, whose high expression was associated with worse overall event-free and relapse free survival.[54] Studies by Wang[55] profiling LSC-enriched populations in MLL-AF9 and HoxA9/Meis1a AML murine models revealed activation of the Wnt/β-catenin pathway. Given that this pathway is not absolutely essential for adult HSC self-renewal, these studies highlight a pathway that may be uniquely targeted in LSCs. These data support the notion that therapies to eradicate disease must be targeted to the heterogenous components of the tumor, including LSC and the cancer cell at large.

Inappropriate proliferation: aberrant signal transduction

Abnormal proliferation is often the result of activating mutations affecting tyrosine kinase signaling pathways. Following the discovery that the tyrosine kinase activity of ABL is essential for BCR-ABL transformation in CML,

and the success in treating these patients with imatinib, mutations in tyrosine kinase signaling molecules have been implicated in the pathogenesis of AML.

FLT3 is a receptor tyrosine kinase (RTK) that plays important roles in hematopoietic stem and progenitor cell survival and proliferation. Mutations of FLT3 occur in approximately 30–40% of all AML and include FLT3-ITD mutations (~20%), inserting into either the juxtamembrane domain or the first kinase domain (TKD1) of the receptor[56,57] and missense point mutations (5–10%) in the activation loop of the second tyrosine kinase domain.[58] These mutations lead to the constitutive activation of the FLT3 signaling pathway and downstream targets, conferring enhanced proliferation and survival.[59] Gain of function mutations of KIT, a member of the type III RTK family, occur in approximately 25–30% of CBF-AML and recent gene expression analyses of KIT mutated CBF-AML revealed deregulation of genes belonging to the NFκB signaling pathway that may affect normal apoptotic mechanisms.[60] The majority of KIT mutations affect the extracellular domain (exon 8), allowing spontaneous receptor dimerization,[61,62] or the activation loop of the tyrosine kinase domain (exon 17), leading to constitutive phosphorylation. The JAK2V617F mutation, characteristic of MPN, is found predominantly in patients with secondary AML following MPN;[63,64] however, it is also present in 6% of t(8;21) AML, suggesting that it may represent a cooperating event.[65] Collectively, these data suggest that activation of RTK pathways may be a universal feature of AML. Subsequent high-throughput re-sequencing of all tyrosine kinase encoding genes has led to the identification of novel mutations in JAK1, Discoidin domain receptor 1 (DDR1), and Neurotrophic tyrosine kinase receptor type 1 (NTRK1) in AML.[66]

RAS oncogenes are a family of guanine nucleotide-binding proteins that are key molecules in signal transduction pathways through RTKs, including KIT and FLT3. Activating mutations of RAS are found in approximately 25% of AML cases and occur most frequently in N-RAS than K-RAS or H-RAS.[67] Several studies found no correlation between RAS mutations and clinical outcome, which at first appears puzzling given the relatively consistent negative prognostic importance of RTK mutations. This might be explained by the fact that RTKs upstream from RAS affect more pathways of cell survival and renewal. However, studies in MDS patients revealed a frequent association between patients with RAS mutations and those who progress to AML.[68]

Inhibition of differentiation: the role of transcription factors

AML cells frequently harbor balanced translocations, leading to the fusion of the DNA binding domain of a transcriptional activator with proteins that function as transcriptional repressors, thus inhibiting the target genes of the original transcription factor, culminating in differentiation block. The CBF complex is a heterodimer composed of RUNX1 and CBFβ and is the target of at least three common translocations in AML: t(8;21)/RUNX1-RUNX1T1, t(3;21)/RUNX1-EVI1, and inv(16) resulting in CBFβ-MYH11. These chimeras act as dominant negative forms of the CBF complex, and can also perturb the function of other important hematopoietic factors. RUNX1-RUNX1T1 silences hematopoietic genes via the aberrant recruitment of histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) to RUNX1 target genes but also directly interferes with RARα function by receptor binding and affects recruitment of cofactors by CEBPε and PU.1.[69] Additionally, point mutations of the DNA-binding domain of RUNX1 can also occur in AML, inducing loss of DNA binding and transactivation activity.[70]

Rearrangements of the RARα gene, encoding a nuclear hormone receptor, are hallmarks of APL, classified as the M3 FAB subtype of AML. In 98% of cases, APL is associated with the t(15;17) translocation generating the PML-RARα fusion protein; however, other rare variants involving the RARα gene and different partners (designated X) also exist.[71] The ability of PML-RARα and other X-RARα fusions to disrupt normal retinoic acid signaling occurs in part, as a result of the aberrant recruitment of chromatin modifying enzymes to endogenous RARα/RXR target genes. The APL fusions appear to repress genes involved in lineage differentiation and DNA repair, whilst activating genes of the Wnt and Notch pathways, such as γ-catenin, leading to differentiation block and increased self-renewal.[53,72,73] Disruption of normal PML function may also contribute to the leukemic progression. The PML protein associates with and stabilizes the DNA damage response protein TopBP1 in response to ionizing radiation. In APL, where the PML nuclear body is disrupted, TopBP1 function is impaired.[74] PML-RARα associated APL has a striking response to all-trans retinoic acid (ATRA). ATRA converts PML-RARα from a repressor to an activator and triggers fusion protein degradation, reversing aberrant gene expression, leading to differentiation of leukemic blasts and transient disease clearance.[75] Currently, the combination of anthracyclines and ATRA leads to complete remission for 90% of patients, however 5–30% of patients relapse.[76] The introduction of arsenic trioxide (ATO) into this treatment strategy led to synergistic elimination of leukemic cells and higher cure rates.[77] Mechanistically, ATO functions by inducing the degradation of the PML-RARα via multimerization and ATO binding, sumoylation, ubiquitylation, and proteosome-mediated degradation.[78] This leads to apoptosis and modest differentiation, effects that are likely secondary to PML-RARα degradation. Given that PML-RARα degradation has been associated with clearance of LICs and eradication of disease in mice, it is likely that oncoprotein degradation, rather than differentiation is the primary mechanism for disease elimination.[79]

Overexpression of HOX genes is strongly associated with AML. In particular, overexpression of HOXA9 is associated with poor outcome in response to chemotherapy.[80] In contrast to other transcription factors, individual HOX genes are only occasionally involved in chromosomal translocations, as in translocations involving the nucleoporin 98 kDa (NUP98) gene, such as the NUP98-HOXA9 fusion in t(7;11)-associated AML.[81,82] More often, HOX expression is increased due to aberrations affecting upstream regulators. One of the most frequent translocations in leukemia involves MLL, and transformation by MLL fusions is associated with the ability to upregulate HOX genes. MLL is a histone methyltransferase (HMT) that specifically methylates histone 3 lysine residue 4 (H3K4), a mark typically asso-

ciated with gene activation, via the SET domain.[83] MLL is fused to more than 50 different partner genes in AML and ALL, the most common being AF4 family members, AF9, ENL, and AF10, which are elongation assisting proteins that recruit H3K79 HMT, DOT1L. These fusion proteins contain the N-terminus of MLL, which can direct the chimeric proteins to bind specific sites on the genome, but lack the C-terminal SET domain and HMT activity. The recruitment of the DOT1L and transcriptional elongation factors by MLL-fusion proteins represents a major a mechanism of upregulation of HOX genes in AML.[84] Indeed DOT1L inhibitors like EPZ01 (Epizyme) specifically inhibited the proliferation of cell lines harboring rearrangement of MLL.[85] Additionally, the caudal-type homeobox transcription factor 2 (CDX2), a regulator of HOX genes during embryogenesis, is overexpressed in 90% of AML patients and closely associated with HOX expression.[86]

Escape from apoptosis, loss of cell cycle control, and genomic instability

High resistance to apoptotic signals is critical to AML and cancer development in general. RTK activation enhances cell survival by activating phosphatidyl-inositol 3-kinase (PI3-kinase) signaling. Downstream targets, such as AKT and mTOR, are often constitutively activated in AML blasts and PI3-kinase inhibitors impair blast survival at least in part through induction of apoptosis.[87] AKT acts as a survival factor that inhibits apoptosis through phosphorylation of the FoxO transcription factors and BAD, resulting in release of the anti-apoptotic regulator BCL-2. High expression of BCL-2, the inhibitor of apoptosis family member survivin, and low levels of the extrinsic death pathway protein FADD, are predictive of poor clinical response in AML.[88] The subversion of apoptosis may also occur due to mutations in the TP53 tumor suppressor gene.[89]

Similarly, disruption of the cell cycle is an important event in malignant transformation. The cyclin-dependent kinase inhibitors (CKIs) INK4 (p15, p16, p18, and p19) and CIP/KIP ($p21^{CIP}$, $p27^{KIP1}$, $p57^{KIP2}$) proteins are key cell cycle regulators that inhibit the activity of the cyclin:CDK complexes, which promote cell cycle progression. Loss of CKI activity is frequently observed in AML, often due to hypermethylation of the promoter region, which occurs at an incidence as high as 40–80% for methylation of p15.[90–93] Loss of TP53 function by mutation, loss of PML nuclear body function as described above, or by disrupting the ability of NPM to sequester MDM2 may also lead to G1 checkpoint abrogation.

Genomic instability is a hallmark of cancer cells and may be partially explained by loss of TP53 functions described above. Indeed, TP53 mutations are highly associated with complex, aberrant karyotype AML, and are a strong prognostic indicator of lack of response to chemotherapy and poor overall survival.[94] However, other oncogenic lesions, such as loss of PML function in APL and dysregulated FLT3 signaling may also contribute to genomic instability. For example, cells overexpressing FLT3-ITD contained elevated levels of reactive oxygen species (ROS), leading to increased double stranded DNA breaks and misrepair.[95] Genome-wide hypomethylation of H3K79 by the CALM-AF10 fusion renders cells more sensitive to γ-irradiation and cells had increased chromosomal instability.[96] Coupled with defects in DNA repair mechanisms, such as non-homologous end-joining, these mechanisms are likely to provide leukemic cells with the genetic variation required for clonal evolution, a key requisite for evading targeted therapies.

Abnormal metabolism

Abnormal metabolism by cancer cells was first described by Warburg, who demonstrated that tumors

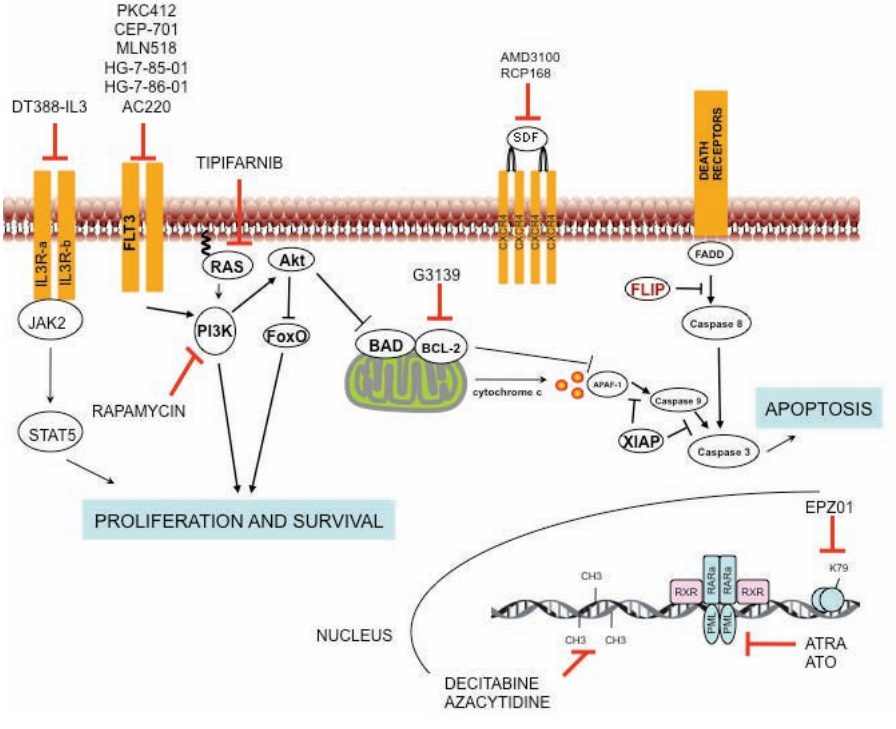

Figure 2. Therapeutic Targets in AML. Therapeutic agents may be directed against proliferative signals generated by signaling receptors (IL3-R, FLT3) or molecules (RAS), signaling pathways (PI3K/AKT), migration (SDF-1/CXCR4), anti-apoptotic molecules (BCL2), or aberrant chromatin modifications (DNA methylation, histone methylation) and oncogenic fusion proteins themselves (PML-RARα).

have a high glycolytic rate and increased uptake of glucose under aerobic conditions.[97] The recent discovery of recurrent mutations in approximately 15–30% of AML affecting two enzymes involved in citrate metabolism, IDH1 and the related mitochondrial homolog IDH2, suggests that dysregulated metabolism may also underlie AML pathogenesis.[24,25,98] Mutation of IDH1 was first identified in glioblastoma.[99] IDH1 mutations are found in 70% of grade II and III gliomas and secondary glioblastomas that developed from these lesions.[100] IDH1/2 are normally responsible for the interconversion of isocitrate and α-ketoglutarate (α-KG); however, heterozygous mutations in IDH1/2 yield a novel enzymatic activity that converts α-KG to 2-hydroxygluturate (2HG).[101] Elevated 2-HG was observed in many patients with NK-AML, and the leukemic cells of these patients displayed lower frequencies of other AML mutations, suggesting that these mutations may be key to AML pathogenesis.[98] The 2HG produced in these patients interferes with enzymes that require α-KG as a substrate. IDH1/2 mutations are mutually exclusive of TET2 mutations in AML due to overlapping function.[102] TET2 is an α-KG dependent enzyme responsible for catalyzing cytosine 5-hydroxymethylation, a possible intermediate on the way to DNA demethylation. Thus, mutations in TET2 presumably lead to DNA hypermethylation, and indeed TET2 mutant AML was associated with a hypermethylation phenotype.[102] Strikingly, IDH1/2 mutant AML was also associated with a hypermethylation signature that overlapped with TET2 and IDH1/2 mutants inhibited TET2 cytosine 5-hydroxymethylation. Another class of α-KG dependent enzymes are the Jumonji-C domain histone demethylases, which like TET2, are α-KG dependent dioxygenases, and 2-HG inhibits the enzymes *in vitro*.[103] Furthermore, enforced expression of glioma-associated IDH1/2 mutations inhibited histone demethylation and led to an increase in HOX gene expression.[103] Given that overexpression of HOX genes is commonly observed in AML, it will be of interest to determine the relationship between IDH1/2 mutations and HOX gene expression and global histone methylation in AML patients.

IDH1 mutants can also induce the HIF-1α pathway.[104] α-KG activates proline hydroxylases that inactivate HIF-1α; therefore a decrease in α-KG in IDH mutant cells may promote accumulation of HIF-1α. Indeed, HIF-1α levels are increased in human gliomas with IDH1 mutations.[104] Hypoxia-inducible factors (HIFs) are responsible for mediating cellular responses to conditions of low oxygen. Recent studies show that adult HSCs have increased levels of HIF-1α, due to stabilization mechanisms induced by the hypoxic bone marrow microenvironment[105] and niche factors, TPO and SCF.[106,107] This leads to increased glycolysis and decreased mitochondrial oxidative phosphorylation maintaining these cells in a quiescent, primitive state. While the exact contribution of increased HIF-1α to AML pathogenesis remains unknown, it is possible that elevated levels of HIF-1α may perturb normal differentiation and HSC cell cycling to promote leukemogenesis. Indeed, HIF-1α regulates genes involved in survival, differentiation, cell cycle control, and those controlling stem cell self-renewal, including Oct-4, Myc, in addition to activating Notch signaling.[108]

Leukemic cell adhesion and migration

The expression of cell surface adhesion proteins is associated with leukemic cell survival and drug resistance. High expression of CD31 relative to CD38 promotes interaction with endothelial cells, and the resultant transendothelial migration may account for the high peripheral white blood cell count in these patients. Conversely, a high CD38 to CD31 ratio leads to retention of leukemic cells in the bone marrow, which may play a role in drug resistance.[109] The SDF-1/CXCR4 interaction also plays a critical role in leukemia cell dissemination. Targeting this interaction with a competitive antagonist of CXCR4 allowed the release of leukemic cells from the protective bone marrow niche and enhanced the efficacy of chemotherapeutic drugs in a murine model of APL.[110]

Therapeutic targets in acute myeloid leukemia

The ultimate goal of understanding and classifying the molecular aberrations underlying individual AML subtypes is to devise targeted, personalized therapy, thereby reducing the risk of relapse and unwanted treatment side effects. Currently, treatment of AML relies on the use of induction chemotherapy using a combination of cytarabine and anthracycline; however, while CR is achieved in 70–80% of patients under 60 years old, risk of relapse is high even after post-remission therapy. Furthermore, the use of intensive post-remission therapy is most appropriate for younger adults who do not comprise the bulk of AML patients. The future of AML therapy may lie in the development and robust testing of compounds directed against specific molecular aberrations or fusion proteins (FLT3, IDH1/2, BCL2, PML-RARα), aberrant epigenetic modifications (DNA methylation, histone deacetylation), and targeting the LIC (surface markers, niche interactions) and factors affecting multi-drug resistance (MDR) (Figure 2). The interplay between various molecular aberrations underlying AML at the level of major signaling pathways suggests that the combined inhibition of several signaling pathways is required to achieve maximum clinical benefit. For example, MEK1/2 kinase inhibition synergizes with the FLT3 inhibitor sunitinib to inhibit proliferation and induce apoptosis in cell lines.[111] As the list of genes mutated and pathways deregulated in AML grows, more targets for therapy will be investigated; whether this can translate into an increase in the still quite guarded prognosis of AML remains to be determined.

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Genetic markers in relation to the therapeutic management of acute myeloid leukemia

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A B S T R A C T

More than any other genetic source of information, cytogenetics has become solidly established in the clinical management of patients with acute myeloid leukemia (AML). Cytogenetic examination furnishes insights into the highly variable clinical biology of AML and allows for diagnostic and prognostic distinctions. The explosion of genomic knowledge in the last decade, accompanied by the introduction of modern technologies that allow for genome-wide analysis (e.g., high-throughput arrays, whole genome sequencing) has generated a wealth of novel markers with diagnostic and prognostic significance. The continuing identification of emerging biomarkers has also created an awareness of the necessity to use these markers in a meaningful way in clinical practice. It remains a respectable challenge to pragmatically integrate new markers arising from ongoing research in decision algorithms. This challenge will not only involve future clinical and biological studies but also bioinformatic and biostatistical developments adapted to high-dimensional data sets. Rather than presenting an exhaustive inventory of numerous markers, this review will highlight selected examples of biomarkers, which may serve as illustrative prototypes of current developments and challenges.

Clinical context

The treatment of acute myeloid leukemia (AML) is among the most dose-intensive approaches in clinical oncology, and treatment outcome varies greatly among patients. Of adults up to the age of 60, on average 40% will have long-term survival prospects; for older patients, this value is only 10–15%. Among these average estimates, there is considerable variation between patients. Clinical factors (e.g., age), hematological factors (e.g., secondary leukemia), and in particular, genetic leukemia-specific changes are the major determinants of outcome. This review highlights some recent developments in the cytogenetic and genomic area that exert clinical relevance.

Cytogenetics: what is new?

It has become standard practice to collect cytogenetic information in patients with AML at initial presentation and derive clinically relevant information.[1–3] Prominent examples of cytogenetically defined subtypes of AML are the core-binding factor AMLs that carry a comparatively favorable prognosis. At the other end of the prognostic spectrum, one can discern the AMLs with monosomal karyotypes indicative of perhaps the worst possible outcome independent of age.[4]

Monosomal karyotype

AML with monosomal karyotypes (defined by at least two monosomies or a single monosomy plus an additional structural cytogenetic abnormality) generally have an estimated long-term survival of 0–10% ('very poor'). The initial assessment of prognosis feeds treatment decisions, for example, as regards the choice to lead a patient with AML to an allogeneic stem cell transplantation and chose the preferred type of allogeneic transplant, or for instance, to discourage intensive chemotherapy in a patient of older age. A major proportion of the complex karyotypes (usually defined as three or more clonal aberrations), for a long time, has been regarded as unfavorable, but this is probably largely due to the fact that they are admixed with monosomal karyotypes. The complex karyotypes appear no more unfavorable than any general cytogenetic abnormality if monosomal karyotypes are taken into account and excluded from the complex karyotypes. The profound negative prognostic effect of monosomal karyotypes in patients with AML is independent of age and holds for adults, young and old alike.[4–6]

Various specific cytogenetic abnormalities

While the area of cytogenetics has been extensively explored during the last 20 years, the impact of specific abnormalities that present at rare frequencies deserves further exploration. In fact, there may be infrequent specific cytogenetic entities representing interesting subtypes, which can only be evaluated if large data sets are available. An impressive effort into this direction has recently been undertaken by the MRC AML Group in almost 6000 patients.[7] The latter study based on robust numbers disclosed or confirmed various important notions. For instance, chromosomal abnormalities additional to the favorable t(8;21) do not modify prognosis. In multivariable analyses, various

abnormalities, some of which are already being well established as unfavorable,[7] predict a significantly poorer outcome, namely abn(3q) (excluding t(3;5) (q25;q34)), add(5q)/del(5q), -5, -7, add(7q)/del(7q), t(6;11)(q27;q23), t(10;11)(p11~13; q23), other t(11q23) (excluding t(9;11)(p21~22;q23) and t(11;19)(q23;p13)), t(9;22)(q34;q11), -17, and abn(17p). The t(9;11) (p21~22;q23), which leads to the MLLT3-

MLL fusion and is now recognized as a distinct disease entity in the WHO classification,[8] was found to have a relatively favorable outcome in accordance with earlier studies.[7] Another disease entity recognized in the updated WHO classification[8] is the t(6;9)/DEK-CAN, which had previously been associated with a very poor prognosis in a large case series and is generally assigned to the adverse cytogenetic-risk group. In the MRC study, there was some evidence of poorer survival in patients (n=42) with the t(6;9)(p23;q34) compared with those with normal karyotype, but the unfavorable effect was not sufficiently strong to emerge in multivariable analysis.

AML with abn3q

These large data sets are also useful for exploring particular known cytogenetic subtypes in greater detail. Another such survey in as many as 6500 patients with AML jointly conducted by the HOVON/SAKK/AMLSG groups has been undertaken with the focus on 3q abnormalities.[9] AML with inv(3)(q21q26) or t(3;3) (q21;q26) is recognized as a WHO entity[8] and is characterized by involvement of the *EVI1* (*ecotropic virus integration-1*) gene and an adverse prognosis (regarding *EVI1* see also below). However, the impact of various other 3q abnormalities remains to be elucidated. In the aforementioned MRC study, patients with inv(3) (q21q26) and t(3;3)(q21;q26) had a particularly bad outcome independent of the presence or absence of monosomy 7. The HOVON/SAKK/AMLSG study has now demonstrated in this unprecedented large series of patients in multivariable analysis, that only inv(3)/t(3;3) but not other balanced t(3q26) or t(3q21), predict for highly inferior relapse-free survival or overall survival. The other balanced t(3q26) or t(3q21) include aberrations, such as t(2;3)(p15~23;q26.2), t(3;12)(q26.2;p13), and t(3;21)(q26.2;q22.1), rearrangements of 3q26 with chromosome 3 bands other than 3q21 (n=10), or balanced 3q21 rearrangements, such as t(1;3)(p36.3;q21.1) and t(3;5)(q21;q31).

Thus, AML patients with inv(3)/t(3;3) are clinically, cytogenetically, and molecularly distinctive, which is in full agreement with their incorporation into the latest WHO classification[8] as a new entity. The inv(3)/t(3;3) population was associated with younger age and higher platelet and white blood cell counts, and they presented with notably high frequencies of monosomy 7 (66%) and N-RAS mutations (28%). Patients with inv(3)/t(3;3) had highly unfavorable 5-year survival rates (OS: 5.7% ±3%; P= .001; EFS:0%; P=.001). The adverse prognostic impact of inv(3)/t(3;3) was enhanced by additional monosomy 7. EVI1 expression levels were significantly higher in patients with additional monosomy 7. There was no difference in survival between patients with classical inv(3)(q21q26.2) and t(3;3)(q21;q26.2) variants.

The latter study also confirmed an earlier study[10] that had suggested that AML with cryptic 3q rearrangements, identified by disproportionate EVI1 and MDS1/EVI1 expression ratios should be included in the WHO entity inv(3)/t(3;3).

The ongoing search for somatic gene mutations

A progressive number of gene mutations have been discovered in AML, some of which occur at relatively common (e.g., >5%) frequencies. This discovery process is expected to continue to yield novel markers and to complement our knowledge about the genomic basis of AML. Whole genome deep sequencing[11] has drawn attention to mutations in the gene *cytosolic isocitrate dehydrogenase 1* (*IDH1*). Subsequently, gene mutations have also been discovered in its mitochondrial homolog *IDH2*. *IDH1/IDH2* mutations are seen, on average, in about 15% of cases of AML (see Table 1). These mutations are more frequent in patients of higher age with AML and are more common among normal karyotype AML and AML with another gene mutation *nucleophosmin-1* (*NPM1*). IDH mutations have been suggested to code for proteins that express neoenzymatic activity leading to gain of function. Already, within a year after the published discovery of this particular gene mutation, a wealth of studies on their biological role,[12–14] demographic relationships, and prognostic significance have appeared.[15–24] However, with additional gene mutations coming up, it becomes clear that the analysis of the prognostic value against the background of numerous other associated gene mutations will be quite complex. Almost all studies deny a prognostic significance of *IDH1* and *IDH2* mutations in unselected adults with AML. Their prognostic impact may depend on the coexistence of other gene mutations (see Table 1) and thus only become apparent in specific genotypic subsets. Current available experimental data remain controversial and show discrepancies between studies that need to be resolved. The reported differences between *IDH1* and *IDH2* and differences of prognostic impact among *FLT3-ITD/NPM1* composite genotypes for the time being cannot be explained by a unifying understanding. Leukemic *IDH1* and *IDH2* mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation.[25] IDH and TET2 have been postulated to act in related promoter methylation pathways. Mutations in *Ten-Eleven-Translocation-2* (*TET2*) itself are common in myeloproliferative disorders but infrequent in AML but *TET2* and *IDH* gene mutations in AML seem mutually exclusive.

Another recently identified gene mutation involves *DNMT3A (DNA methyl transferase 3A*) that appears in about 20% of patients with AML, in particular in the intermediate-risk cytogenetic category (about 30% frequency). *DNMT3A* mutations have been suggested to predict for bad outcome.[26] The latter gene is of special interest because it encodes an enzyme involved in methylation. How *DNMT3A* mutations relate to methylation abnormalities, methylation patterns, and to other gene abnormalities (e.g., mutations), as well as respond to therapies remains to be established.

Prevalence

	IDH1 mutant #	*IDH1* mutant %	*IDH2* mutant #	*IDH2* mutant %	*IDH1/IDH2* mutants %
Marcucci JCO 2010 (19–83 yrs) (ref 15)					
All karyotypes	--	--	--	--	--
CN-AML	49/358	14	69/358	19	33
NPM1 mut+CN	34/234	14.5	31/234	13.2	28
Paschka JCO 2010 – AML, 16–60 yrs (ref 17)					
All karyotypes	61/805	7.7	70/805	8.7	16
CN-AML	41/338	12	35/338	10.4	23
*NPM1*mut	30/203	14.8	20/203	9.8	25
Boissel JCO 2010 - AML, 17–70 yrs (ref 18)					
All karyotypes	50/520	9.6	15/520	3	12.6
CN-AML	34/213	16	12/205	6	~22
*NPM1*mut +CN	34/86	39.5	12/82	14.6	~54
Abbas Blood 2010- AML, 15–77 yrs (ref 16)					
All karyotypes	55/893	6	97/893	11	17
CN-AML	39/380	10%	58/380	15	25
*NPM1*mut +CN	35/266	13	40/266	15	28
Thol Blood 2010 (ref 20)					
CN-AML			32/272	12	
Chou Leukemia 2010- AML, 18–90 yrs (ref 21)					
All karyotypes	27/446	6	54/446	12	18
CN-AML	20/223	9	34/223	15	24
Green Blood 2010- AML, 15–68 yrs (ref 19)					
All karyotypes	107/1333	8			
CN-AML	60/528	11			
*NPM1*mut +/*FLT3*-wt	49/298	16			

Prognostic impact

Marcucci JCO 2010 (19–83 yrs)	
reduced RFS - in CN-AML with *NPM1*mut/*FLT3*-ITDneg	p=0.046
Paschka JCO 2010 – AML, 16–60 yrs	
reduced RFS - in CN with *NPM1*mut/*FLT3*-ITDneg	p=0.02
reduced OS - in CN with *NPM1*mut/*FLT3*-ITDneg	p=0.03
Boissel JCO 2010 - AML, 17–70 yrs	
Reduced RFS - in CN-AML	p=0.01
Abbas Blood 2010- AML, 15–77 yrs	
reduced EFS - in *NPM*wt	p=0.02
reduced EFS - in *NPM*wt/*FLT3*-ITDneg	p=0.005
reduced OS - in *NPM*wt/*FLT3*-ITDneg	p=0.03
Thol Blood 2010- AML,	
No prognostic effect of *IDH2* in CN	NS
Chou Leukemia 2010- AML, 18–90 yrs	
Better OS - in *FLT3*-ITDneg (concerns *IDH2* mut only)	p=0.003
Green Blood 2010- AML, 15–68 yrs	
reduced RFS - in *FTL3*-ITDneg	p=0.008
improved RFS - in *FLT3*-ITDmut	p=0.02

CN – normal cytogenetics; *NPM1*mut: *nucleophosmin* gene mutant
FLT3-ITDneg: no internal tandem duplication of the *FLT3* gene
OS – overall survival; RFS – relapse free survival

Table 1. Prevalence and prognostic impact of IDH1 and IDH2 mutations in selected recent studies in AML.

Gene mutations in *fms-like tyrosine kinsase* (e.g., *FLT3*-internal-tandem duplications, *FLT3*-ITD; 25% of cases), *CCAAT enhancer binding protein alpha* gene (*CEBPA*) mutations (~10% of cases), and mutations in *nucleophosmin 1* (*NPM1*; 35% of cases) were discovered several years ago (for recent review see ref. #3). *FLT3* gene mutations alter the signaling function of the hematopoietic kinase receptor. Various recently developed FLT3 inhibitors are currently in clinical trial with the objective to therapeutically target the mutant receptor on the leukemia blasts. Mutations of the *NPM1* result in delocalization of the protein from the nucleolus towards the cytoplasm. The common incidences of gene mutations in *FLT3*, *NPM1*, *CEBPA*, *IDH1/IDH2*, and *DNMT3A* already indicate that some of these will appear in combination. Multiple studies have revealed the favorable outcome of AML with the composite genotype of *NPM1* gene mutation in the absence of *FLT3*-ITD (*NPM1*^{mut}/*FLT3*-ITD^{neg}). Also, AMLs with mutant *CEBPA* carry a relatively favorable prognosis. Recent evidence has modified the latter view. The favorable effect of *CEBPA* gene mutations was recently shown[27] and subsequently widely confirmed[28–31] to be selectively restricted to the biallelic *CEBPA* mutant subset of AML, prevalent in about 8% of AML.[31] The latter mutations that affect the *CEBPA* alleles on both chromosomes define a highly distinctive form of AML with infrequent additional gene mutations[30,31] and a unique gene expression pro-

file.[27,31] Thus, the double gene-mutant *CEBPA* subtype of AML shares a similarly favorable prognosis as the core-binding factor AMLs. Patients whose blasts exhibit *NPM1*^{mut}/*FLT3*-ITD^{neg}[32] and also those with biallelic *CEBPA* mutations generally are not considered for allo-SCT in first CR.[32]

Several other gene mutations have been implicated in AML, for example, *NRAS, KRAS, CBL, TP53*, and *JAK2* mutations.[33,34] Mutations in *ASXL1* (*additional sex comb-like 1*)[33,34] are apparent in about 10% of cases and seem to confer negative prognostic impact. A recent study that suggested high (~30%) *RUNX1* gene mutations in normal karyotype AML exerting unfavorable prognostic significance awaits confirmation.[35] *TET2* mutations are very common in myeloproliferative disorders but not uncommon in AML and are seen in about 10% of cases, however as yet with neutral prognostic significance.[33,34] Finally several studies have demonstrated that mutations in the *Wilm's tumor* (WT1) gene predict adverse DFS and OS in normal karyotype AML[36–38] but current data are not entirely unanimous,[39] and these mutations carry only limited impact at older age.[40]

Gene expression

The introduction of reverse-transcriptase-PCR and microarray technologies has permitted the analysis of the complex patterns of transcript expression of numerous genes.[41] Several genes with relatively high expression levels (overexpression) confer negative prognostic value among AML that is otherwise classified as intermediate risk. Examples of genes that have reproducibly been postulated for prognostic distinction are *BAALC* (*Brain and acute leukemia, cytoplasmic*) gene, *ERG, WT1,* and *EVI1*. The prognostic value of high expression of *BAALC* and *ERG* has reproducibly been demonstrated and recently also in patients with AML of older age.[42] The *ecotropic virus integration site-1* ($^{EVI-1}$) is an oncogene that was originally discovered by its ability to cause leukemia in mice following retroviral insertion mutagenesis. The *EVI1* oncogene is involved in human AML with the unfavorable cytogenetic abnormality 3q26 (see above in section about cytogenetics). In AML developing in patients with inherited disease after gene therapy the activation of *EVI1* has been implicated.[43] Thus, *EVI1* appears as a true oncogene for AML in mice, as well as in humans. Of special note, in about 5% of AMLs, EVI1 is overexpressed in the absence of the structural 3q abnormality, but nevertheless it predicts for a similarly high relapse rate and adverse survival.[10,44–46] It is currently unknown why EVI1 is highly expressed in these leukemias. Recently, it has become clear that EVI-high AML correlates with a highly specific methylation profile (see below). Altogether, AML with high EVI1 represents an adverse subtype. Clearly, gene expression markers furnish informative additional insights for distinction of AML diversity.

When overexpressed, various other markers have been shown to predict for unfavorable outcome of normal karyotype or intermediate cytogenetic-risk AML. Expression levels of the tumor suppressor WT1 (Wilm's tumor-1) have been correlated with outcome but results are not unanimous (see ref. #3). CD34 and MDR1 are examples of markers for which careful studies have been published. High VEGF-C expression was demonstrated to be involved in chemoresistance and it appears a negative prognosticator in multivariable analysis both in adult and pediatric AML.[47]

Epigenetic abnormalities

Hypermethylation has been recognized as a hallmark of cancer. Deregulated acetylation of genes has similarly been recognized as changes that are typically seen in AML, for example, related to specific cytogenetic abnormalities. This has prompted epigenetic drug development, and various agents with histone deacetylase inhibiting and demethylation abilities have been developed, with several currently being in early clinical trial.

Studies during the last year have revealed the notion of hypermethylation being a highly variable process, which may be characteristic for individual patients or subtypes of AML. Some of these specific methylation patterns correlate with particular known cytogenetic abnormalities or gene mutations and thus disclose that these mutations have a profound impact on the methylation status of specific sets of genes.[48] Examples of gene mutations that correlate with methylation aberrations and have been suggested to impact upon methylation status are *NPM1* and *IDH*. In addition, AMLs with *IDH1, IDH2*, and *TET2* gene mutations share overlapping methylation patterns.[25] In AML with IDH1/IDH2 mutations, a remarkable inverse correlation between the expression and methylation of genes was noted. Also EVI+ AML exhibits a highly specific methylation pattern. The hypermethylated genes in EVI1+ AML are probably *bona fide* target genes as EVI1 is capable of binding to these promoters. EVI1 also forms complexes with DNMT3A and DNMT3B.[46] Other distinctive methylation signatures, conferring prognostic impact, cannot (yet?) be linked to known structural gene abnormalities and the causative mechanism of these methylation patterns remains to be disclosed.[48] Further insights into the mechanisms of epigenetic dysregulation may be of direct importance for guiding and developing epigenetic therapies. For the time being, these characteristic methylated subtypes of AML underscore their pathobiological and clinical relevance.

MicroRNAs represent a class of non-coding RNAs that target mRNAs and influence the translation to proteins. Their relevance in relation to AML has recently been reviewed.[49] Their expression may be diverse and also reveal different patterns. In AML, typical microRNA signatures correlating with genetic subtypes (e.g., NPM1 mutations, core binding factor abnormalities)[50] or predicting prognosis[51] have been discerned. Also, particular individual microRNAs (e.g., miR-181a) have been postulated to predict the outcome of the disease in AML.[52]

Integration into clinical practice

AML is a disease with a multi-genetic basis that emerges following the acquisition of multiple and diverse genetic changes. It is a far from a trivial chal-

lenge to interrelate the prognostic impact of the great diversity of genetic alterations, prioritize them according their clinical value, and place them in pragmatic clinical context. The overwhelming and increasing number of genomic abnormalities in association defines a colorful kaleidoscope of combinations of markers and thus defines a remarkable scale of AML subtypes. We are only beginning to understand the relative qualitative and "quantitative" importance of each of these markers, comprehend their pathobiologic role, and assess the significance of their complex genetic mutated background. Available algorithms will continuously be challenged and enriched when new scientific information becomes available. These markers are also of utmost interest for developing therapies that target specific pathways related to particular abnormalities. These algorithms will be used for individualized treatment decisions. For instance, should a patient be exposed to the risk and advantage of allogeneic stem cell transplantation (AlloSCT), and if so what would be the preferred transplantation modality in that particular individual as regards pretransplant conditioning, source of stem cells, and type of donor. Currently, transplantation strategies are already directed by disease risk in combination with transplantation risk algorithms. Ideally, at some point, these algorithms will be instrumental in assessing the outcome of different treatment procedures and offer guidance as regards the personalized therapeutic agent(s) of choice.

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Transplantation for acute myeloid leukemia

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A B S T R A C T

Acute myeloid leukemia (AML) is an extraordinarily heterogeneous disease, and subsets with widely different relapse risk have been characterized. Important factors for determining relapse risk are baseline cytogenetics and the number of cycles of induction chemotherapy required for attaining complete remission (CR). However, increasingly novel molecular genetic markers, such as gene mutations and deregulated gene expression, can be identified in many patients and can help further to stratify patients into prognostic groups. After achieving first CR, three different prognostic groups can be recognized: those with favorable, intermediate, and poor-risk AML have probabilities of relapse of approximately 30%, 50%, and 80%, respectively. While allogeneic haemopoietic stem cell transplantation (HSCT) from both related and unrelated donors reduces the relapse rate for all groups transplanted in first remission, non-relapse mortality may counterbalance that beneficial effect. Thus, allogeneic HSCT is currently only recommended in those patients whose risk of relapse outweighs the risk associated with the transplant procedure itself, and the decision to undertake a transplant should be made only following a complete assessment of cytogenetic and molecular genetic risk. For older patients, reduced intensity conditioning is less toxic, and there is some evidence that this approach may improve survival at least in some sub-groups. For many patients with refractory or relapsed AML, transplantation is the only curative option.

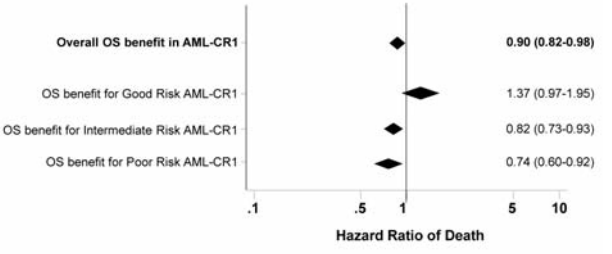
Allogeneic HSCT in first complete remission using sibling donors

Background

Whilst more than 80% of younger patients (<60 years) with acute myeloid leukemia (AML) will achieve a complete remission (CR) after initial induction therapy, a substantial number will relapse. Allogeneic transplantation with myeloablative conditioning is a curative option for younger patients. The indications for allogeneic transplantation have been studied most extensively for patients with matched related donors (MRD). A number of prospective trials have been conducted attempting to define appropriate therapy for patients with AML in first complete remission (CR1).[1-3] In most of these studies, patients were entered at diagnosis, and those who achieved CR and had an HLA -matched family member donor were allocated to allogeneic haemopoietic stem cell transplantation (HSCT), while the others were treated with consolidation chemotherapy. These studies have confirmed that allogeneic HSCT with myeloablative conditioning has a powerful anti-leukemic effect but that the survival benefit is variably offset by the increased risk of death in remission. Single prospective trials have neither shown a definitive advantage nor disadvantage in OS of allogeneic HSCT for patients with AML in CR1. For example, in the MRC AML 10 trial, allogeneic HSCT given after intensive chemotherapy was able to reduce relapse in all risk and age groups.[1] However, due to the competing effects of procedural mortality and an inferior response to chemotherapy if relapse does occur after allogeneic HSCT, there was a survival advantage only in patients of intermediate risk.

However, as a result of these trials, a consensus has emerged that patients with good-risk AML, including acute promyelocytic leukemia (APL), do not benefit from allograft in CR1. Our knowledge of the role of allogeneic HSCT has recently been helped by a meta-analysis of results from four large European co-operative groups encompassing more than 4000 patients in a donor/no donor analysis.[4] This study showed that allogeneic HSCT resulted in an improvement in DFS and OS in patients with intermediate risk and adverse risk cytogenetics who were less than 35 years of age and received a myeloablative HSCT. The authors concluded that there was a benefit for allograft when the relapse risk was greater than 35%. In this analysis, there was no benefit for older patients because of increased non-relapse mortality (NRM). A second meta-analysis of 24 trials analyzed 3638 patients with AML in CR1 in studies conducted between 1995 and 2003 essentially confirmed these findings.[5] The survival advantage for allogeneic HSCT was most obvious in patients with unfavorable-risk cytogenetics, was less impressive in those with intermediate-risk cytogenetics, and was not apparent among patients with favorable-risk cytogenetics (Table 1).

Table 1. Meta-analysis of 23 randomized trials of allogeneic HSCT analysed on a donor no donor analysis. Adapted from Koreth *et al.* JAMA 2009, 301, 2349–61; with permission.



One problem in assessing the role of transplantation in AML, particularly when donor/no donor analyses are used, is the increasing use of unrelated donors in the no donor group and the impact of delayed transplant in CR2 for the donor group. An alternative statistical method is to use the Mantel-Byar approach. This allows for time to transplantation by switching patients at time of allograft in CR1 to the transplantation curve. To illustrate the use of this method, in the MRC AML12 trial, when a Mantel-Byar analysis was used to assess the impact of allogeneic HSCT, transplantation was found to improve RFS and OS significantly in poor risk patients, whereas no benefit was seen in this risk group using a donor/no donor analysis.[6] This was due primarily due to non-compliance with allograft in the donor group in CR1 and the impact of unrelated donor HSCT in CR1 in the no donor group.

Favorable and intermediate risk acute myeloid leukemia

Despite the value of these meta-analyses in guiding practice, the role of HSCT needs to be reassessed in relation to the new information that has accrued concerning the genetic basis of AML and the impact that these have on prognosis. For example, with cytogenetically normal AML (CN-AML), prognostic significance has been shown for mutations in the *NPM1, CEBPA,* and *FLT3* genes alone or in combination in younger adult patients.[7-11] CN-AML patients harboring internal tandem duplication (ITD) of the *FLT3* gene have an inferior outcome compared with cases without *FLT3*-ITD, although there is evidence that outcome may be more related to the level of the mutated allele.[7,8,11] In several studies, the presence of *NPM1* mutation in CN-AML has been associated with higher CR rates and better event-free survival (EFS).[9,10] CN-AML with biallelic mutations in *CEBPA* is another subset that has been associated with a favorable prognosis.[11,12] These findings have led the European LeukemiaNet (ELN) to propose a new classification of AML risk groups, which incorporates this new prognostic information[13] (Table 2). Clearly the value of allogeneic HSCT now needs to be reassessed based on this new classification of AML-related genetic changes. In this context, a study by the German-Austrian AML Group (AMLSG) provided evidence that those AML patients whose molecular genetic profile predicts a favorable prognosis, such as CN-AML with mutated *NPM1* without *FLT3*-ITD, may not benefit from allogeneic HSCT.[14] The adoption of the ELN classification in future studies will be important in aiding the interpretation of trial results, as previously this has been frequently confounded by variation in risk group classifications used by different clinical trial groups.

Table 2. Standardized reporting for correlation of cytogenetic and molecular genetic data in AML with clinical data from ELN AML guidelines.[13]

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/ del(5q); -7; abn(17p); complex karyotype

The role of allogeneic HSCT in the treatment of *FLT3*/ITD positive patients is currently an area of some controversy. These patients were originally included in the meta-analyses of donor versus no donor trials[4,5] discussed above, which showed a superior outcome for allogeneic HSCT in intermediate and poor risk AML. Three groups have recently reported on outcome after allogeneic HSCT in *FLT3*/ITD-positive AML[14-16] and while all three studies showed a strong reduction of relapse with hazard ratios of approximately 50%, only the German study by Schlenk *et al.*[14] showed significantly improved survival by donor availability. In summary, although definitive evidence from prospective trials is not available, allogeneic HSCT should be considered in patients who are *FLT3*-ITD positive, particularly for those patients not involved in clinical trials of FLT3 inhibition.

Further refinement of the assessment of prognosis in AML may be possible in the future by minimal residual disease measurement; for example, by flow cytometry at the end of consolidation chemotherapy. In a recent study of patients with good and intermediate risk cytogenetics, who had evidence of residual disease after consolidation, predicted a significantly higher relapse rate (77% vs. 22%) and reduced OS at 4 years compared with patients with no detectable abnormal cells.[17] Furthermore, patients who had evidence of minimal residual disease and went on to receive an HSCT had a reduced risk of relapse and a superior outcome if they received an allogeneic transplant but this benefit was not observed in patients who had an autograft.[17] The use of minimal residual disease detection and its integration into risk scores, therefore, may inform decision-making concerning transplantation in patients with good/intermediate risk disease in the future.

Overall the choice of post-remission therapy and the decision to undertake allogeneic transplantation should currently be made only following a complete assessment of cytogenetic and molecular genetic risk, and this is particularly relevant for good and intermediate risk AML. This is exemplified by the fact that although no survival advantage has been shown for autologous or allogeneic HSCT in the frontline treatment of good risk AML, there are subsets of core binding factor (CBF) AML that do rather poorly, particularly those with KIT mutations or molecular disease persistence.[18,19] Allogeneic HSCT may be considered in these patients, especially for those with a low transplant risk, although there is no direct evidence to support such an approach, and such a strategy should be investigated within a clinical trial if possible.

Adverse-risk acute myeloid leukemia

For most patients with adverse-risk cytogenetics, outcome remains dismal with conventional consolidation therapy. An allogeneic HSCT from a matched-related donor is currently considered the treatment of choice for these patients. This recommendation is based on results from single studies, as well as from meta-analyses. The US Intergroup Study demonstrated an advantage for allogeneic HSCT for patients with unfavorable cytogenetics with a survival of 44% versus 15% for patients receiving HiDAC consolidation chemotherapy or autologous BMT.[20] Data from the EORTC/GIMEMA AML-10 trial[2] and from trials of the HOVON-SAKK group demonstrated a benefit for allogeneic HSCT among younger patients with adverse cytogenetics.[4] In the UK MRC AML12 trial, allogeneic HSCT significantly improved relapse free survival (RFS) and OS in the 12% of patients who were poor risk (RFS, 39% vs. 9%; P = .0007; OS, 41% vs. 10%; P = .001).[6]

However, other factors apart from cytogenetics can be used to define adverse risk AML in first CR. In the UK, the NCRI AML Working Party have undertaken a retrospective analysis on patients in the MRC AML10 and 12 trials using a Cox proportional hazards model to provide a number of weighted prognostic factors that would be available after treatment course 1, which could provide a risk index for survival from CR. Factors included were age, WBC, performance status, sex, de Novo/Secondary AML, cytogenetics, platelets, BM blasts, and response after course 1 (CR/PR/NR), and of these, age, male sex, *de novo*/secondary AML, status post course 1, cytogenetics, and WBC were included in the final model. Application of this risk score to patients in the MRC AML10 and 12 trials divided patients into three groups, with 5-year survivals of 63%, 47%, and 24%.[21] An important effect when compared with the standard cytogenetic risk definition was to move approximately one-sixth of the patients who were previously standard risk into the high-risk category. The net effect was that an increased proportion (27%) of patients in AML10 and 12 were redefined as high risk compared with 17% previously.[21] When the role of transplantation was examined in the new high risk group, Mantel–Byar analysis shows a significant survival advantage (33% vs. 18%, p=0.01) but no benefit for the new standard risk group. Therefore, the application of this risk score can identify a population of patients that benefits from transplantation, and this comprises a larger population than defined as high risk by previous criteria.[21]

Allogeneic HSCT using alternative donors

Overall, only 30% of patients who are candidates for allogeneic HSCT have a MRD. With the growth of unrelated donor (URD) registries worldwide, the probability that a suitable donor can be identified has increased dramatically particularly for Caucasians, and for a significant proportion of patients, it is possible to find a 10/10 allele matched donor. The risks of graft versus host disease (GVHD), graft failure, and mortality progressively increases with the number of HLA disparities but with the use of high-resolution typing, HSCT outcomes using URD matched at HLA-A, -B, -C, and –DRB1 give results very similar to those expected with a MRD.[22] These studies emphasize the importance of high-resolution HLA typing and the selection of donors with, preferably, no more than one mismatched allele.

Historically the use of URD HSCT in AML has been reserved for patients with relapsed disease; however, recent data have suggested that the results of URD transplants in AML have improved significantly. A recent analysis from the Fred Hutchinson Cancer Research Center compared the results of patients with AML in CR1 (n=226) undergoing myeloablative HSCT from either a matched sibling or unrelated donor selected using high-resolution HLA typing between 1996 and 2007. Survival at 5 years was 63% with matched related donors versus 61% for matched unrelated donors. The 5-year estimates of relapse and NRM were 29.7% and 16.0%, respectively.[23] Failure for each of these outcomes was slightly higher for 10/10 URD than MRD HSCT, although these differences were not statistically significance for any end point. This study led the authors to conclude that their data support the rationale of considering matched or nearly matched URD HSCT for similar indications as currently operative for MRD HSCT for AML patients in first CR.

The German-Austrian trial AMLHD98A prospectively evaluated allogeneic HSCT from matched-related and matched-unrelated donors in younger adults with high-risk AML.[24] High risk was defined by the presence of adverse cytogenetics and/or resistant disease after the first cycle of induction or failure to achieve CR or CRi after double induction therapy. All high-risk patients were assigned to allogeneic HSCT from an MRD, URD, or haploidentical donor. Of the 267 high-risk patients, 162 patients (61%) received allogeneic HSCT, with 51% in CR at transplant. Although patients going on to HSCT were younger (48.9 vs. 55.3 years), multivariate analysis, including allogeneic HSCT as a time-dependent covariable, revealed that allogeneic HSCT significantly improved outcome. Furthermore, there was no difference in outcome between allogeneic HSCT from MRD and URD. This study provides evidence that allogeneic HSCT from either a MRD or a URD improves the outcome of patients with adverse risk AML.

A recent study by the Center for International Blood and Marrow Transplant Research (CIBMTR) supports these results. This study focused on the outcome of 358 patients with AML in CR1 who had unfavorable cytogenetics and who underwent an unrelated donor HSCT between 1995 and 2006, compared with patients receiving transplants from a matched-related donor (MRD).[25] In multivariate analysis, NRM was significantly higher in URD HSCT compared with MRD transplantations: relative risk (RR) = 1.55 (95% CI, 1.02–2.37), P = .04. However, they observed similar LFS and OS with a MRD and 10/10 matched URD (45% and 37% at 3 years, respectively). For recipients of a mismatched (9/10) URD, the OS at 3 years was 29% and was significantly inferior.[25]

Currently, a stem cell source can be found for virtually all patients who have an indication to undergo allogeneic HSCT. For those patients without a suitably matched sibling or URD, haploidentical-related donor or cord blood transplant (CBT) have emerged as a reasonable alternative, and the outcome of these types of transplant is expected to be better than chemotherapy alone in high-risk AML in CR1. Two retrospective reports have compared outcomes of unrelated donor and CBT for adults with acute leukemia.[26,27] In the report from the CIBMTR, the rates of treatment-related mortality and overall mortality were lowest with the use of matched URD transplantation, and were similar with mismatched unrelated marrow and CB transplantation.[27] A report from Eurocord found a similar trend toward improved survival with the use of matched unrelated marrow over CBT.[26] Recently, the use of double CBT (dCBT) transplants has been reported to lead to a reduced risk of relapse in patients undergoing myeloablative transplants for acute leukemia in CR1/CR2 compared with single CBT.[28] Furthermore, a second report, which compared the outcome of dCBT with matched and mismatched URD transplants, showed similar survival for all donor types but with the lowest risk of relapse seen with the use of dCBT.[29] The results of all these studies suggest that CB transplants, despite an increased HLA disparity, are a suitable alternative for appropriate adult patients and that the use of double cords may reduce the relapse risk.

Haplo-identical transplantation using intensive myeloablative conditioning followed by transplantation of high doses of CD34+ T-cell–depleted cells harvested from both PB and marrow has been pioneered by the Perugia group.[30] They have achieved a high-rate of engraftment. In 42 patients with AML in remission, event-free survival was 48%. An alternative approach using a reduced-intensity conditioning regimen and transplantation of T-replete bone marrow and high-dose post-transplant cyclophosphamide to deplete alloreactive T-cells has been developed.[31] In the initial studies of this approach, graft failure, usually non-fatal, was seen in 18%. The cumulative incidences of grades II–IV and III–IV acute GVHD were 35% and 10%, respectively.[31]

In the absence of an HLA-matched donor, both CBT and haploidentical-SCT strategies are suitable options to treat these HR patients, and the option of allogeneic HCT for adults with AML is no longer limited to those patients with matched siblings.

Autologous transplantation

The use of autologous HSCT for AML in CR1 is a controversial area. Autologous HSCT using bone marrow-derived grafts has been evaluated in seven prospective trials in adults with AML in CR1. Most of these studies have described a lower relapse rate for patients undergoing an auto HSCT in comparison with chemotherapy. A meta-analysis of seven trials, including 4410 patients, also indicated that auto HSCT was associated with a reduced risk of relapse associated with a modest improvement in DFS.[32,33] In most prospective studies of auto HSCT, overall survival was not significantly improved, mostly because of the higher mortality compared with consolidation chemotherapy, where bone marrow was used. Another factor is that a smaller proportion of relapsing autologous BMT patients can be salvaged. Mobilized peripheral blood stem cell (PBSC) transplantation offers a much faster hematopoietic recovery with the potential for reduced NRM; however, a higher risk of relapse has been reported with the use of PBSC, particularly if the transplant was performed early after achievement of remission.[34] Furthermore the risk of relapse appears greatest in those patients receiving a high CD34 cell dose of more than $7x10^6$/kg.[35] A recent HOVON/SAKK study has compared autologous transplantation using PBSC with ongoing consolidation chemotherapy in AML in CR1.[36] NRM was 4%, lower than reported in previous studies of autologous transplantation using BMT. However, although the autologous PBSCT treatment arm showed a better relapse free survival at 5 years than the chemotherapy arm (39% vs. 29%), overall survival was not improved due to a greater number of patients of the chemotherapy arm able to go on to receive salvage therapy by allogeneic HSCT.[36]

Hematopoietic cell transplantation for older adults with acute myeloid leukemia

AML patients over the age of 55 years are generally more difficult to treat than younger patients due to intrinsic drug resistance and diminished tolerance to treatment. As a consequence, conventional chemotherapy is more toxic and only curative in a minority of patients. A meta-analysis of myeloablative HSCT in CR1 has shown no benefit in older patients because of the impact of excess NRM.[4] Over the last 15 years, several groups of investigators have developed reduced intensity conditioning (RIC) or non-myeloablative conditioning transplant regimens, less toxic than fully myeloablative transplants, in order to extend the treatment to older patients who might also have comorbidities that preclude full-intensity transplants. These regimens, which are designed to take advantage of an immunological graft-versus-leukemia (GvL) effect to eradicate residual disease, vary in their intensity, including those that are truly non-ablative (fludarabine/low dose TBI) to those that incorporate additional chemotherapy drugs, such as fludarabine/busulfan or fludarabine/melphalan, which are given to provide additional disease control and hence prevent relapse prior to the development of a GvL effect.

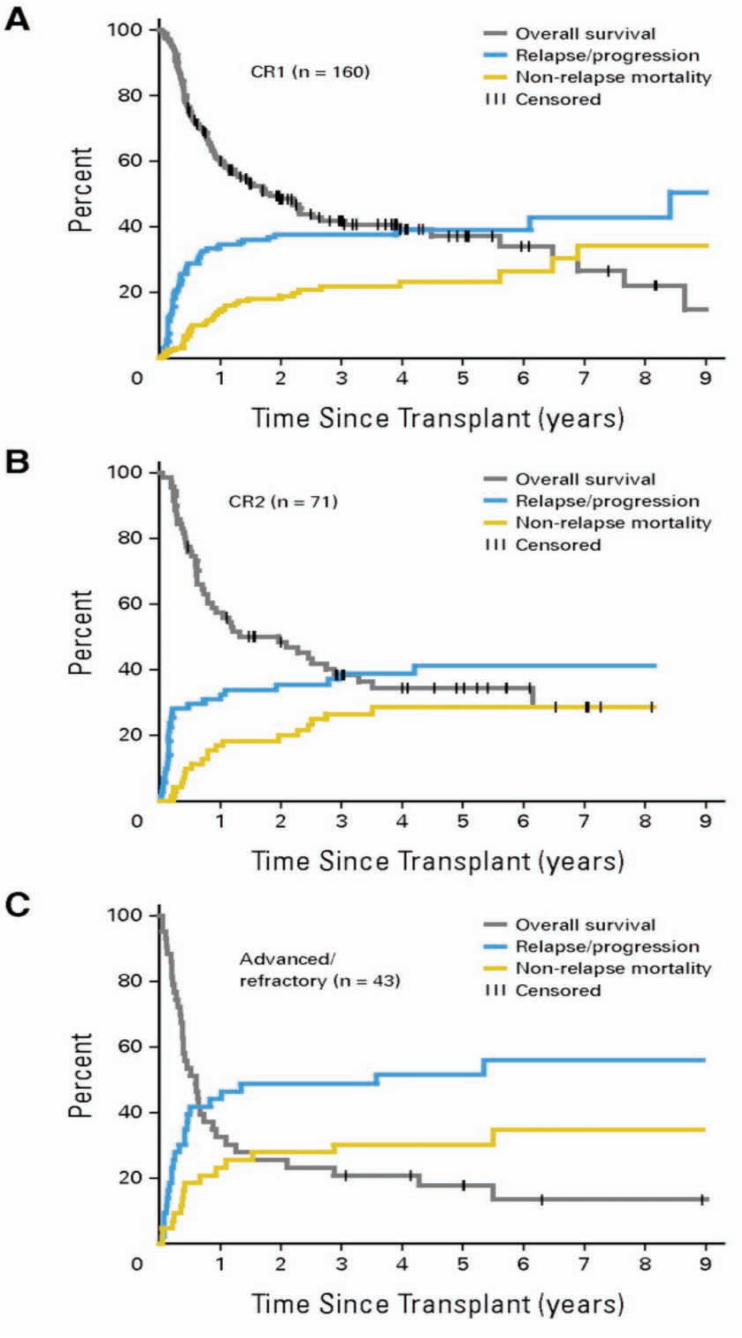


Figure 1. Overall survival, relapse/progression rate, and NRM of patients with AML undergoing RIC transplants in first complete remission (CR1), in second complete remission (CR2), and with more advanced/refractory disease. Reprinted from Gyurkocza *et al*. J Clin Oncol 2010, 28(17), 2859-2867; with permission.

An update from the Seattle consortium[37] of 274 AML patients (median age 60 years) conditioned with the non-myeloablative fludarabine ± low-dose total body irradiation regimen, followed by cyclosporine and MMF had a survival at 5 years of 33%. The estimated 5-year relapse/progression and NRM rates were 42% and 26%, respectively, although the NRM at 100 days was only 4%. The leading cause of NRM was chronic GVHD, and the cumulative probability of extensive chronic GVHD at 5 years was 44%. Unfavorable cytogenetic risk status was associated with increased relapse risk and in this study, chronic GVHD was associated with a significant decrease in relapse/progression. Patients transplanted in CR1 and CR2 had better survival rates than patients with more advanced disease (37% and 34% vs. 18%, respectively) (Figure 1), and best outcomes were seen in those who had favorable/intermediate cytogenetics transplanted in CR.

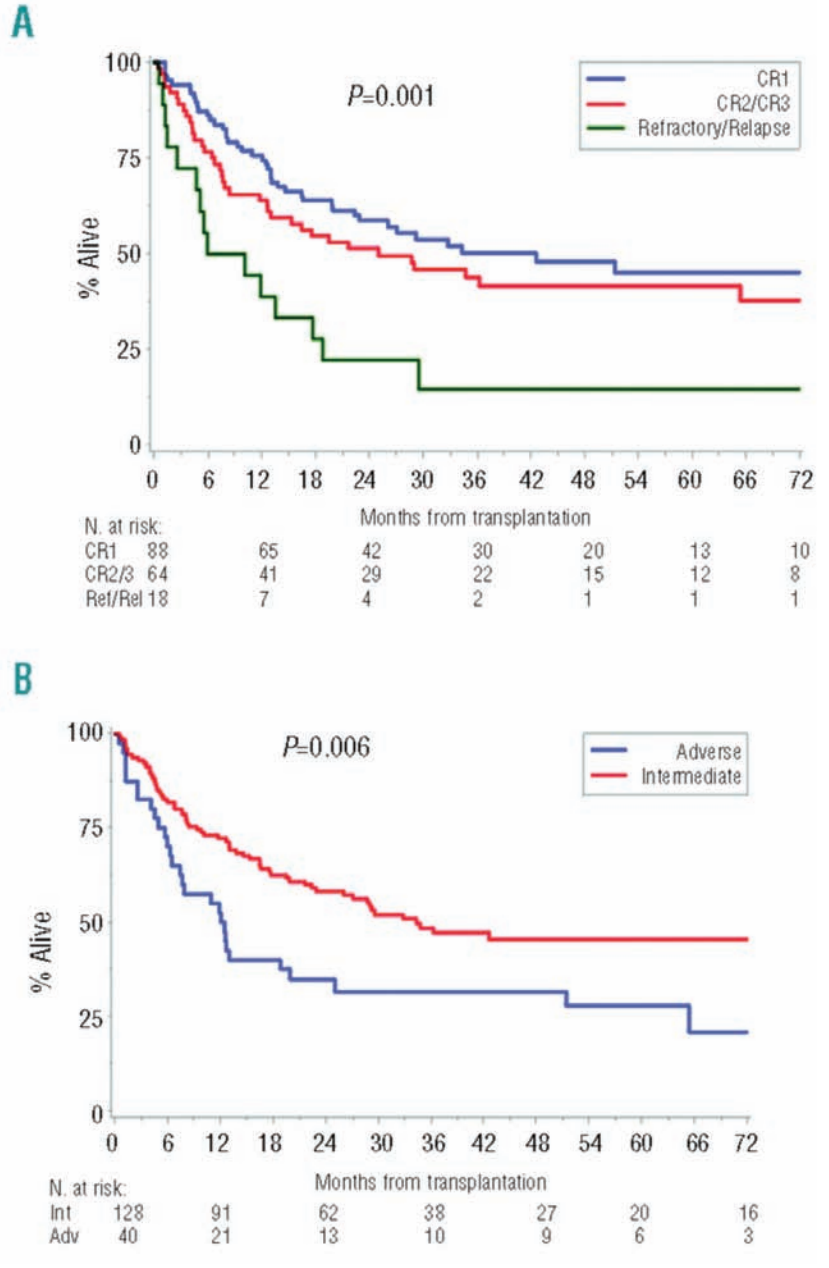


Figure 2. Overall survival after an alemtuzumab based RIC allograft for AML according to disease status at the time of transplant. (A) Overall survival according to cytogenetic risk classification at diagnosis (B). Adapted from, Craddock *et al*. Haematologica 2010, 95, 989-95; with permission.

Patients with HLA-matched related or unrelated donors had similar survivals. These favorable GvL effects, which are counterbalanced by the morbidity and mortality associated from chronic GVHD, has led groups to explore the addition of T-cell depleting antibodies, such as ATG[38] or alemtuzumab (anti-CD52),[39] to the conditioning regimen. These have been shown to reduce the risk of acute and chronic GVHD following sibling and URD RIC HSCT and to be well tolerated in this age group.

Unsurprisingly, pre-transplant factors, such as disease status, cytogenetics, and comorbidity index, affect transplant outcome following RIC transplants in AML. Patients with advanced disease have been reported to have a high risk of relapse compared with those in CR.[37,39] Cytogenetic risk group also affects outcome following RIC HSCT.[37,40] Craddock *et al.*[40] reported that the 3-year DFS for patients with intermediate risk cytogenetics was 47% compared with 32% for patients with adverse risk cytogenetics (Figure 2). Although it has been shown that NRM following RIC was reduced in patients with high comorbidity scores compared with myeloablative HSCT, there is also an adverse effect of comorbidity score on outcome in RIC HSCT. Sorror *et al*. reported that the 1-year NRM was 16% for patients with scores of 0 to 2 and 36% for those with scores of 3 or higher following RIC HSCT (p 0.04).[41] Other pre-transplant factors, such as age, appear less important. A study by the CIBMTR of patients undergoing RIC HSCT for AML found comparable 2-year survival rates and NRM in patients aged 40 to 54, 55 to 59, 60 to 64, and 65 years and older, leading the authors to conclude

that older age alone should not be considered a contraindication to HSCT.[42]

Despite the large number of RIC transplants performed and reported to international registries over the last few years, the true value of this approach in improving survival of older patients with AML remains unproven, and there are few randomized trials or "donor versus no donor" analyses supporting its use. In a retrospective study of a donor versus no donor analysis from a single institution, Mohty *et al.*[43] reported a significant improvement in OS for RIC transplants for AML patients in CR1 using sibling donors. Ninety-five consecutive patients were studied, of whom 35 had a MRD, and 71% of patients with a MRD proceeded to RIC transplant. These patients had a median age of 53 years and were conditioned with a RIC regimen comprising fludarabine, busulfan, and ATG, which has greater dose-intensity than the Seattle low-dose TBI regimen. In an "intention-to-treat" analysis, leukemia-free survival (LFS) was significantly higher in the 'donor' group compared with the 'no donor' group (P=0.003; 60% vs. 23% at 7 years), with a median follow up of 4.8 years.[43]

In the MRC AML15 trial, we attempted to answer these questions in the context of a large multicentre trial evaluating the efficacy of RIC HSCT compared to conventional chemotherapy for patients over 45 years, a group of patients who tolerate a myeloablative SCT less well.[44] In this study, patients older than 45 years with intermediate risk cytogenetics were eligible for RIC allogeneic HSCT in CR1 from a matched-sibling donor. Patients with adverse risk cytogenetics or who had more than 15% blasts in the bone marrow after course 1 were eligible for a RIC HSCT from a MRD or matched URD. Conditioning was primarily with fludarabine, melphalan, and alemtuzumab. Mantel–Byar analysis showed increased deaths in CR for RIC HSCT (HR 6.73; p<0.001) but a lower relapse risk compared to chemotherapy (HR 0.40; p<0.001).There was some evidence of a benefit on OS (HR 0.76; p=0.08). Undertaking an analysis of OS stratified by the presence of a sibling donor and by cytogenetic risk group, a survival benefit of RIC transplant was seen in the subgroup of patients with intermediate risk cytogenetics undergoing a transplant from a matched sibling donor.[44]

Regarding selecting AML patients for myeloablative or RIC conditioning, currently myeloablative HSCT may preferred in patients up to approximately 45–50 years of age with no comorbidities for whom NRM can be estimated as low, reserving RIC for older patients or those with comorbidities. Important unanswered questions remain in relation to RIC HSCT in AML, these include the optimal chemotherapy to be applied prior to transplant, the type of RIC conditioning regimen to be used (reviewed in ref. #45), the benefit in patients with poor-risk cytogenetics, quality of life issues, and the role of URD transplants.

Transplantation for primary refractory acute myeloid leukemia

Unfortunately, even with the best regimens today, approximately 15% of patients have primary induction failure. Primary induction therapy generally comprises two cycles of conventional-dose cytarabine with an anthracycline or one dose of a regimen that includes high-dose cytarabine. For patients who fail to achieve remission with initial induction therapy, allogeneic HSCT is the only form of treatment that offers a chance for cure. Retrospective studies using myeloablative conditioning show outcome is limited by a high relapse rate and a high NRM leading to OS rates of approximately 20% when transplanted from sibling or alternative donors.[46] A recent analysis by the CIBMTR of patients who underwent a first myeloablative allogeneic HSCT in relapse or primary induction failure showed that survival was worse for patients with circulating blasts, those with a mismatched unrelated donor or a related donor other than an HLA-matched sibling, and poor-risk cytogenetics.[47]

RIC transplants have also been explored in this setting; however, an observation common to most studies is that patients with active leukemia at the time of transplant do not benefit due to a high relapse risk. Recently, a strategy has been developed for patients with refractory AML consisting of initial cytoreductive chemotherapy with the FLAMSA regimen, followed by RIC HSCT with prophylactic transfusion of donor lymphocytes.[48] In patients with primary induction failure, results of this approach were impressive, with a low TRM and a 2-year OS and LFS of 62.5% and 62%, respectively. These results demonstrate the importance of initiating a donor search for all transplant eligible AML patients at diagnosis and not just waiting to see their response to first-line therapy. Alternative approaches to refractory AML include the use of novel RIC conditioning regimens. A recent phase 1 trial of high-dose Clofarabine and Busulphan has shown promise in 14 patients transplanted with active chemotherapy-refractory disease. The regimen was well tolerated with durable remissions observed in 9 patients, with 1-year event-free and OS of 53% and 60%.[49]

Transplantation for relapsed acute myeloid leukemia

Treatment of AML in first relapse is associated with relatively low response rates, and if a second CR is attained, the median duration of the second remission is generally considerably shorter than that of the first, and only a minority of patients will survive with chemotherapy alone.[50] Options for post-remission therapy for these patients include allogeneic HSCT using MRD or alternative donors using either myeloablative or reduced intensity conditioning. In an analysis of a cohort of 667 patients with AML in first relapse, Breems *et al.*[51] reported that four factors affected prognosis in CR2: duration of CR1, age at relapse, previous SCT, and cytogenetics at diagnosis. They were able to establish three risk groups with varying prognosis: a favorable prognostic group (OS of 46% at 5 years), an intermediate-risk group B (OS of 18% at 5 years), and a poor-risk group.[51] In all prognostic groups, the best long-term survival was observed in patients treated with allogeneic HSCT. Although selection biases were operating here, these data support the use of allogeneic HSCT in patients in CR2 able to tolerate this treatment.

After allogeneic HSCT, an autologous transplant is possibly the most potent form of post-remission therapy; in this instance, relying solely on the anti-leukemic effect provided by the myeloablative conditioning. Autologous HSCT is therefore an alternative to allogeneic HSCT in CR2, particularly in those patients with a longer first remission. A recent report demonstrated a 5-year OS of 32% in adult patients with relapsed intermediate or good-risk cytogenetics autografted in CR2 and who had a first remission of more than 8 months.[52] The outcome was improved by the use of grafts obtained in CR1 and use of PBSC rather than BM.[52] Excellent results with autologous transplant have also been obtained in APL in CR2 for patients who have achieved a molecular CR following re-induction therapy and who have a graft that is also PCR negative.[53–55] For APL patients not in molecular remission or with a short CR1, an allogeneic HSCT is recommended.[54]

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Unrelated donor transplants

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A B S T R A C T

Unrelated donor stem cell transplantation was introduced into clinical practice in the 1980s and is now more frequently performed worldwide than sibling transplant. The inherent increase in both major and minor histocompatibility increases the complexity of the procedure and in unmanipulated grafts, results in an increase in the risks of graft rejection and graft versus host disease. In contrast, the enhanced alloimmunity leads to a reduction in disease recurrence. Improvements in the methodology of HLA-typing and thus in donor selection, and in patient selection have resulted in steady improvements in outcome since inception. The overall availability of donors and the speed of identifying suitable donors remain a challenge to the success of this technique. On-going controversies include the optimal source of stem cells (blood or bone marrow) and optimal graft versus host disease prophylaxis. More recently, data have become available regarding donor safety, further underlining the need for appropriate patient and donor selection.

Introduction

The issue of matching between donor and recipient has been crucial from the origin of clinical transplant activity, but the concept has evolved over many years, with an increasing level of sophistication (Table 1). The methods of HLA typing have changed from serological identification to molecular techniques. Thus, the definition of a matched donor in the eighties and nineties is different from the definition of a matched donor today. We used to match for the A, B, DR locus at the antigenic, or serologically determined, level, and a 6/6 match would have identified a donor matched with his recipient for six antigens (two on each of the A, B, DR loci). We then started to use molecular methodology for HLA-DR typing, but were still calling this a 6/6 match. A 5/6 match would have been a donor with one of the antigens mismatched.

Currently, we are matching for A, B, C, DRB1 at the allelic level, that is with molecular subtyping of the antigens, looking for 8/8 matched donors (Table 1). Some centers are typing also for DQ looking for a 10/10 match and some others also for DP, looking for a 12/12 matched donor. In an elegant paper, Petersdorf *et al*. have shown that beyond allelic matching, one can also match for haplotypes. A donor and recipient can be matched by phenotype (presence of the same antigens on locus A, B, C, DR) but mismatched by haplotypes. This study showed that matching for haplotypes significantly reduces the risk of GvHD, and this is probably due to the fact that haplotype matching indicates that a greater part of chromosome 6 is matched between donor and recipient. Finally, mismatching can be permissive or non permissive: some studies have shown that mismatching at specific aminoacidic positions may be conductive to more GvHD.[2,3]

Bone Marrow Donors Worldwide (BMDW), based at Leiden University, compiles different national databases (registries) in one single file. The initial resistance to the creation of one single registry rather than individual national registries was won by the scientific stature of the founder, Jan van Rood. Today, BMDW is a potent, fast, and up-to-date tool, comprising over 15,000,000 volunteer donors. It has proved to be clinically useful (a preliminary search is done on line in "real time"), but is also scientifically relevant, since the large number of individuals represented allows for genetic insight in different populations.

Challenges to unrelated transplantation

Finding a donor

The time to identify a donor depends on the HLA of the patient (a common HLA will lead to larger number of potential donors), whether there is a fast lane for a specific disease (acute leukemia) or disease phase, and whether the transplant center is willing to accept a donor who is less than a perfect match. The time to identify a donor is as little as 30 days to as long as many months. An unrelated donor transplant should ideally be considered early in the course of the disease.

Table 1. Matching criteria during different time periods (intervals are artificially set at decades).

	HLA loci	Methodology	Best match
<1990	A,B,DR	Serology	6/6 Ag
1990–1999	A,B,C,DRB1	Serology + molecular	6/6 class I Ag+ DRB1 matched
2000–2006	A,B,C,DRB1, DRB2,B3,DQ	Molecular	8/8 alleles 10/10 alleles
2007	A,B,C,DRB1	DNA strand isolation	Haplotype matched
2010	A,B,C,DRB1+ DP	Functional matching for shared T cell epitopes (TCE) defined by *in vitro* alloreactive cross-reactivity patterns	DP matched or permissive DP-mismatch
2010	A,B,C,DRB1 + KIR haplotypes	Single nucleotide polymorphism	Cen B gene content >2

Donor safety

Although not unique to UD transplants, the issue of safety in HSC donation has gained momentum in the past decade. This is because with the increasing number of donations, probably exceeding 200,000 worldwide, potential risks for the donor have become evident. Because collection can be from the bone marrow (BM) or from the peripheral blood (PB), the procedures and the associated risks vary considerably. BM donation is mainly, but not exclusively associated with the risk of general anesthesia, whereas PB donation carries the risk of vascular complications associated with the leukocytosis induced by growth factor (usually granulocyte colony stimulating factor G-CSF) and stem cell pheresis procedures. The EBMT has recently published a report on 51,024 donations (27,770 marrow and 232,254 peripheral blood) showing approximately 1:100 non severe complications, 1:1.000 severe complications, and 1:10.000 deaths. Deaths were not reduced in PB donations (1.72/10.000) compared to marrow (0.36/10.000). The probability of a second tumor was 0.4/10.000 person years for marrow and 1.2 /10.000 person years for blood.[4] This study contradicts the commonly held notion that PB donation is safer. It should be said that all fatalities occurred in family donors and none in unrelated donors, suggesting that accurate selection is a main factor to prevent severe adverse events. These data support the current efforts of regulatory agencies to trace adverse events, and suggest that great care should be given to the identification of donors suitable for donation.

Graft versus host disease (GvHD)

The conventional prophylaxis for graft versus host disease (GvHD) is cyclosporin (CsA) and methotrexate (MTX) or tacrolimus (FK) and MTX. With a two drug combination, acute GvHD grade III-IV is seen in 10–30% patients. The Boston group has reported an incidence of grade III-IV acute GvHD of less than 5%, using the combination tacrolimus and sirolimus, in a relatively large group of related and unrelated transplants.[5] The addition of a polyclonal antibody, such as anti-thymocyte globulin (ATG), will also significantly reduce acute GvHD and chronic GvHD.[6,7] Both these studies have failed to show a survival advantage for patients receiving ATG compared with controls, although in the long term, chronic GvHD may cause an excess of late mortality[8] and in the short term, quality of life is improved. Alemtuzumab, though not tested in a prospective trial, would seem to reduce the incidence of acute and chronic GvHD.[9] The prophylaxis of GvHD should be designed according to the center's experience and ongoing protocols. It is unlikely that a given regimen will be totally successful, especially because the incidence and severity of acute GvHD depend not only on *in vivo* prophylaxis, but also on factors, such as disease phase, patient age, intensity of the conditioning regimen, stem cell source, and graft composition. Agents or maneuvers that reduce the incidence of GvHD have been shown to increase the risk of relapse, and this must be factored in the choice of a given combination.

Approaches to overcoming these challenges

HLA matching and clinical outcome

HLA typing and donor selection have changed over the past 2 decades (Table 2). In a study by the Seattle group,[10] in chronic myeloid leukemia (CML) in first chronic phase (CP1), patients with a 10/10 allelic HLA match, had a significantly improved survival (70%) compared with patients with a 9/10 match (40%). The effect was not seen in patients with CML in accelerated or blastic phase. A mismatch for one single C allele had the greatest impact on survival (RR 3.18).

The CIBMTR-NMDP paper analyzed 3857 allelic typed transplants, including acute myeloid leukemia (AML) acute lymphoblastic leukemia (ALL), myelodisplastic syndromes (MDS), and chronic myeloid leukemia (CML):[11] again the impact of a single allelic mismatch over a full allelic match was greater in patients with early disease (Table 2). Actuarial 5-year survival in patients with early disease was 50% for 8/8 matched pairs, decreasing to 39% for 7/8 matched pairs and 28% for 6/8 matched pairs. In patients with intermediate disease, these three figures were 32%, 27%, and 15%, respectively, and in patients with advanced disease, 17%, 15%, and 10%, respectively. Again an effect of HLA mismatching was shown, but most prominent in patients with early disease. A study from

Table 2. The effect of HLA mismatch in unrelated donor transplants: representative studies.

Study	Reference	No. of patients	Diagnosis	Groups in which mismatching confers survival disadvantage	Mismatches to be avoided
Seattle	10	1249	Leukemia	Early disease	C locus
NMDP CIBMTR	11	3860	AML ALL MDS CML	Early disease	A or DRB1
JMDP	3	5210	Malignant and non malignant	All patients	C locus Non permissive mismatches positions 9,77,80,99,116,156
Seattle	1	246	Leukemia	All patients	Haplotype Mismatched
GITMO	13	537	Leukemia	All patients	DP non permissive mismatch
CIBMTR	14	1409	Leukemia	AML	Donor Cen B gene content <2

the Japanese Donor Registry (JMDP) on 5210 patients, found six specific amino acidic substitution positions in the HLA class I that were associated with severe acute GvHD: the authors refer to these as "non-permissive mismatches".[3] The positions are 9, 77, 80, 99, 116, and 156 (Table 2). One of these (116) had already been reported as risk factor for GvHD.[2] Patients with a fully matched donor (n=712) have the same risk of GvHD as patients without non-permissive mismatches (n=2670), but significantly less GvHD compared with patients with one non-permissive (n= 602) or two non-permissive (n=66) mismatched donors.

Petersdorf *et al*. showed that matching donor and recipient for haplotypes reduces GvHD and TRM[1] (Table 2). In keeping with this result, survival in excess of 90% has been reported for thalassemia patients, prospectively assigned to receive transplants from UD, matched by ancestral haplotypes: this study was possible because the patients were from the island of Sardinia, known to be homogeneous by HLA typing, and ancestral haplotypes are common in the population.[12] Because of the protective effect of GvHD on leukemia relapse, haplotype matched pairs experienced more leukemia recurrence and survival was similar overall in matched/mismatched pairs.[1] However, in patients with early disease, or in patients with non malignant disorders, one may wish to transplant from haplotype matched donors with little risk of GvHD and TRM. In patients with advanced disease, a partially mismatched donor may also be acceptable, or perhaps preferable.

An innovative approach to hematopoietic stem cell donor-recipient matching consists in taking the conventional allele-matching further to functional matching for shared T cell epitopes (TCE) defined by *in vitro* alloreactive cross-reactivity patterns. In this setting, allelic mismatches not involving TCE disparity (i.e., the mismatched alleles in both patient and donor are positive or negative for the shared TCE) are considered to be permissive, while allelic mismatches involving also TCE disparity (i.e., the mismatched allele in the patient is negative and in the donor is positive for the TCE, or vice versa), are considered to be non-permissive in host versus graft or graft versus host direction, respectively. In a recent study coordinated by IBMDR/GITMO, non-permissive mismatches for HLA-DPB1 defined according to this model were shown to be significantly associated with non-relapse and overall mortality, in unrelated transplantation from 10/10 and 9/10 matched donors.[13] This finding, made in 537 Italian transplants, was recently also confirmed at the international level in over 9000 unrelated transplants (manuscript in preparation).

Finally KIR haplotype typing may permit the selection of donors capable of protecting against relapse.[14] AML patients grafted from Cen-B homozygous unrelated donors have a 16% cumulative incidence of relapse, compared with 37% for patients grafted from Cen- A/A donors ($p<0.001$).

From these studies we can derive the following conclusions on HLA matching and outcome:

1. Better matching leads to better outcome.
2. The effect may be different according to the disease phase (greater effect in early disease).
3. Some specific mismatches can be considered as "non-permissive" as compared with others considered "permissive".
4. Functional matching for shared T cell epitopes may further improve outcome.
5. Particular donor KIR haplotypes may protect recipients against relapse and thus improve survival.

Patient selection and indications

The eligibility for an UD transplant includes both patient related and disease-related variables. The age of patients in pediatric centers ranges from newborn to 18 years, and in a centre transplanting adults it will vary from 18 to 70 years. Patients come to transplant in different clinical conditions, depending on their age, disease phase, number of courses of chemotherapy, and/or radiotherapy and with a variable number of comorbidities, some of them age dependent, such as hypertension, heart failure, chronic lung disorders. A study from the Seattle group[15] has identified a scoring system that quantitates comorbidities: patients with a low score have a significantly lower transplant related mortality (TRM).

Patient eligibility also raises regulatory and insurance issues: the high cost of an UD transplant may not be

covered in certain advanced disease phases because of the very low chance of success. This may also be true for some diseases in which evidence of efficacy of HSCT is lacking (such as solid tumors). The EBMT has attempted to categorize indications for allogeneic sibling and unrelated transplants in different diseases and disease states.[16]

The decision to proceed with an UD HSCT resides ultimately with the patient, his hematologist, and the transplanter. The side effects and toxicities are usually weighed against the risk of the underlying disease. The current distribution of diagnoses in patients transplanted from UD facilitated by the Italian Bone Marrow Donor Program are as follows: AML 27%, ALL 25%, lymphoma 15%, MDS 7%, immune deficiency 9%, Myeloma 8%, CML 4%, aplastic anemia 2%. Therefore, over 50% of all patients undergoing an UD HSCT are being treated for acute leukemia.

Despite the great variability of donor matching criteria, of conditioning regimens, of stem cell sources, and of GvHD prophylaxis regimens, two variables maintain their prognostic relevance: age and phase of the disease. The outcome of 1993 children (under the age of 18) and of 5917 adults (18 years and older) undergoing an UD HSCT in Europe between 1994 and 2007 have been studied within the registry of the acute leukemia working party (V Rocha, personal communication). Leukemia free survival at 5 years for children in CR1, CR2, or later is 55%, 47%, and 27%, respectively. For adults, these figures are 49%, 37%, and 18%, respectively. The effect of age is also evident: children have superior survival in all phases of disease, although the greatest advantage seems to be in CR2; it should also be noted that CR2 is the largest group of patients in the pediatric age, this being the major indication, whereas in adults there are more patients in CR1.

Apart from acute leukemia, the other major indication for unrelated transplants is myelodysplastic syndromes (MDS), a very heterogeneous group of diseases, ranging from chronic anemia to aggressive acute leukemia. When compared with matched sibling donor (MDS) transplants, UD grafts tend to have higher TRM,[17] but lower risk of relapse:[18] this leads to similar final outcomes in MSD and UD transplants. The expected long-term disease free survival would be an overall 30%, with disease phase being the major predictor.

Severe aplastic anemia (SAA) has been a strong indication for allogeneic transplants since the early seventies. The problem for these patients is that they come to transplant with significant problems, such as hemorrhage and infections, often invasive fungal infections (due to prolonged neutropenia) or sepsis. Therefore, conditioning regimens need to deliver strong immunosuppression (to prevent rejection) but at the same time, one must consider the fragile clinical condition of the patients. The advent of reduced intensity regimens and the widespread use of the combination fludarabine and cyclophosphamide, has found a successful application in these patients. The current overall survival for UD transplants in patients with acquired aplastic anemia is greater than 70%, with probabilities over 90% for the most recently grafted patients.[19] Currently, UD transplantation can be suggested up to the age of 65 as a second line treatment for patients with acquired SAA, not responding to a first course of immunosuppressive therapy. Whether it can be offered to young patients as first line therapy remains to be determined.

Stem cell source

There is growing use of peripheral blood (PB) as the source of stem cells for transplantation. In the 5-year period 2000–2004, the ratio of PB to BM in unrelated transplants has gone from 763/1191 (0.6) to 1918/919 (2.0). It is unclear why this has happened: from the donor's perspective, the side effects of PB donation are no less worrying than BM donation. Peripheral blood collections certainly reduce the burden of marrow harvesting in the operating room (at least 3 full hours each), and for a unit performing 100 transplants/year, this could result in saving several hundred person days. Peripheral blood collections increase the work load of the blood bank. For the patient, PB grafts give faster hematologic and immune recovery. In the long term, however, there does not seem to be a significant benefit: the CIBMTR compared 331 PB and 586 BM unrelated transplants performed between 2003 and 2006.[20] Engraftment was faster with PB, but PB recipients had more acute and chronic GvHD. Transplant mortality (TRM), relapse, and survival were identical: late TRM was probably increased in patients with early disease. A retrospective study of EBMT compared 388 ALL patients CR1+CR2, receiving UD BM and 337 receiving UD PB: overall leukemia free survival (LFS) was significantly superior for the BM (45%) versus the PB group (36%); this was due to a lower risk of relapse after BMT (Basara, unpublished data).

A prospective randomized trial is well under way within the NMDP comparing BM versus PB for first UD grafts. Until data from that trial are available, it would seem reasonable to continue using marrow for patients with early disease, and peripheral blood for patients with advanced disease. The choice is not always straightforward, because donors may have their preference: usually the transplant center asks for a given source (BM or PB), declares whether only one source, or both will be accepted as suitable, and then the donor must have the ultimate say.

Conclusions

Unrelated transplants should be considered a therapeutic option in patients with both malignant and non malignant hematologic disorders. The choice of using an UD should be considered early in the course of the disease. This is true especially because search for a suitable donor may require weeks and sometimes months, and the shorter this interval the better the outcome. The success of the transplant will depend on a mixture of different factors: donor matching, donor age, conditioning regimen, phase of the disease, recipient age, prophylaxis of GvHD, careful monitoring for infections, and expertise of the transplant center. The increasing numbers of UD transplants performed suggest continuing success and growing confidence in the scientific community.

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Clinical results of unrelated cord blood transplantation

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A B S T R A C T

The use of umbilical unrelated cord blood (UCB) cells as an alternative source of hematopoietic cell transplantation has been widely used mainly for patients lacking an HLA-matched donor. UCB present many advantages over bone marrow or mobilized peripheral blood from volunteer donors, such as rapid availability, absence of risk for the donor, and decreased incidence of acute graft-versus-host disease. However, a significant clinical problem is delayed engraftment, and this is directly correlated with the number of hematopoietic stem cells in a cord blood unit. Currently many approaches have been investigated with the aim of improving the engraftment rate and decreasing transplantation related mortality, including the identification of prognostic factors associated with engraftment, the use of multiple donors, intrabone injection of UCB, *ex vivo* expansion, and co-transplantation with accessory cells. These approaches may increase the quality and availability of UCB for transplantation.

Introduction

UCBT has extended the availability of allogeneic hematopoietic stem cell transplantation (HSCT) to patients who would otherwise not be eligible for this curative approach. In comparison with other sources of allogeneic HSCT, UCB offers substantial advantages,[1] including: i) significantly faster availability of banked cryopreserved UCB units, with patients receiving UCBT in a median of 25–36 days earlier than those receiving an unrelated bone marrow graft; ii) extension of the donor pool due to tolerance of 1–2 HLA mismatches out of 6; iii) lower incidence and severity of acute graft-versus-host disease (GvHD); iv) lower risk of transmitting infections by latent viruses such as cytomegalovirus (CMV) and Epstein–Barr virus (EBV); v) lack of risk to the donor; vi) higher frequency of rare haplotypes compared with bone marrow registries, since it is easier to target ethnic minorities.

However, the main problem of using UCB for transplantation is the relatively low number of hematopoietic progenitor (HPC) and stem cells (HSC) in UCB compared with bone marrow (BM) or mobilized peripheral blood (PB) grafts, which translates into an increased risk of graft failure, delayed hematopoietic engraftment[2,3,4,5,6]and delayed immune reconstitution.[7,8] The cumulative incidence of non-engraftment after UCBT varies from 10 to 20% and the median time to neutrophil recovery varies from 22 to 27 days. Many approaches have been investigated to enhance collection of HSC and HPC in cord blood units. Examples include injecting cord blood cells directly into the bone marrow;[9] *in vivo* or *ex vivo* amplification of cord blood cells;[10,11] use of double unit UCBT;[12,13] use of reduced intensity conditioning (RIC) regimens;[1314,15] co-infusion with a haploidentical T cell depleted graft,[16,17] or mesenchymal stem cells (MSC).[18]

Many prognostic studies for improving engraftment after UCBT have been performed, analyzing factors related to the patient, disease, donor, and transplantation.[5,19,20,21,22,23,24,25] Modifiable factors have been identified, such as HLA, cell dose, and others related to the graft choice or factors related to the conditioning regimen[26] or GvHD prophylaxis.[27]

Challenges of cord blood transplantation

Based on the initial experience the major challenges in UCBT are delayed engraftment, and graft rejection.

In all clinical studies describing the outcome of UCBT, delayed engraftment and a high risk of graft failure have been described. Almost all series of UCBT in children and adults have demonstrated the profound impact of cell dose (measured as one or more of the numbers of total nucleated cells (TNC) prior to freezing or at infusion, colony-forming cells, and $CD34^+$ cells) on engraftment, transplant-related events, and survival.[28] HLA matching was also recognized as an important factor for engraftment. In 1997, the Eurocord group described for the first time the association of TNC dose and HLA match with neutrophil and platelet recovery and survival, in 143 patients, mostly children, given related or unrelated cord blood transplantation.[19] In fact, the TNC dose infused (higher or lower than the median of $3.7x10^7$/kg) was the best predictive factor for

neutrophil and platelet recovery and improved survival rate. Furthermore, better HLA matching (defined as matched or mismatched based on HLA-A and -B low resolution and HLA-DRB1 high resolution typing) was also associated with better engraftment and survival, but due to the small number of patients, the number of HLA disparities associated with outcomes was not studied. Later on, those results have been confirmed in a series of 562 children and adults who received unrelated cord blood cell grafts:[20] higher cell dose and number of HLA disparities (6/6; 5/6, or 4/6, considering the definition of HLA matching above) were independent factors associated with better engraftment and decreased TRM. According to the aforementioned studies, it was clear that HLA matching and cell dose were crucial factors for improving outcomes after UCBT, and probably the number of TNC collected or infused should not be inferior to 2.5 x10^7/kg or 2.0 x10^7/kg (considering a loss of TNC around 20%). Also the number of HLA disparities should be less than or equal to four out of six (following the above definition).

Factors related to the technique of transplantation, such as the conditioning regimen and the nature of GvHD prophylaxis, may also be associated with more rapid engraftment. In a recent Eurocord study, the use of fludarabine in myeloablative conditioning regimens was associated with improved neutrophil and platelet recovery in adult UCB recipients receiving a lower TNC dose.[26] The use of fludarabine in the preparative regimen has also been associated with improved engraftment independently of cell dose and HLA match in UCBT for patients with Fanconi anemia.[29] Conversely, the use of methotrexate (MTX) containing regimens for GvHD prophylaxis has been associated with delayed engraftment and an increased risk of graft failure in patients with hemoglobinopathies transplanted with HLA identical sibling cord blood units.[27] The impact of its use elsewhere in UCBT has not been evaluated. In Europe and the USA, the most common regimen is calcineurin inhibitor-based GVHD prophylaxis alone or in combination with steroids or mycophenolate mofetil. Nevertheless, the Japanese transplant centers have shown interesting results with calcineurin inhibitors in combination with low dose MTX.[30,34] Prospective studies are needed to establish the role of MTX in GVHD prophylaxis in the setting of UCBT.

Approaches to overcoming these challenges

Selection of cord blood units based on the interactions between cell dose, HLA disparity, and diagnosis

The Eurocord group has analyzed the interaction of cell dose and HLA match in 550 UCB adults and pediatric transplantation recipients with malignant disorders.[23] The 60-day cumulative incidence (CI) of neutrophil engraftment for all patients was 74%, whereas the incidences for those with no HLA disparity (6/6) versus 1 or more out of 6 HLA disparities were 83% and 53%, respectively. The number of HLA disparities was correlated with neutrophil recovery with a log-linear relationship between HLA disparity and risk of graft failure, suggesting inferior engraftment with increased disparity. The cumulative incidences (CI) of neutrophil and platelet recoveries were also associated with number of TNC before freezing, and the use of granulocyte colony-stimulating factor.

Eapen *et al*. analyzed the interaction between cell dose and HLA match, comparing the outcome of 503 UCB transplants and 282 matched unrelated transplants (MUD) in children with acute leukemia.[6] The cut-off for cell dose was defined as 3.0x10^7/kg for survival in children with a 5/6 HLA disparity graft, but the minimum cell doses associated with survival of children given 6/6 or 4/6 grafts were not found. The probability of neutrophil recovery by day 42 and platelet recovery by 6 months was similar after MUD or matched UCBT (6/6). However, higher cell doses (>3.0x10^7/kg) resulted in a higher probability of engraftment in 5/6 UCBT but had no effect in 4/6 UCBT, suggesting that cell dose may not be able to overcome the adverse impact of HLA mismatching in the setting of 4/6 UCBT.

Recently, Barker *et al*.[22] analyzed the combined impact of the TNC dose prior to freezing and HLA match upon the outcome of UCBT in recipients of 0 to 3 HLA-A, -B antigen, and –DRB1 allele-mismatched units. A thousand and sixty-one patients were studied, having received a single-unit CBT for the treatment of acute leukaemialeukemia or myelodysplasia, after myeloablative conditioning. The study demonstrated that the best outcome for neutrophil and platelet engraftment, acute GvHD, TRM, treatment failure, and overall mortality was associated with the transplantation of 6/6 HLA-matched units, regardless of the TNC dose. The next best survival outcomes were observed in recipients of 5/6 HLA match with a TNC dose of 2.5×10^7/kg or greater or 4/6 HLA match units with a TNC dose of 5.0×10^7/kg or greater.[32]

In both previous analyses, the interaction between cell dose and HLA were studied in patients with malignant disorders. However, other factors, such as the diagnosis, have an important role in the rate of engraftment and other outcomes. This is because most patients with hemoglobinopahties have a full marrow and have not received chemotherapy or immunosuppression before conditioning, or, in the case of aplastic anemia, have often received previous multiple transfusions or have a severe infection at the time of transplantation, thus increasing the risk of non-engraftment. Recently, in the light of the observation that the requirements of cell dose and HLA matching may differ in malignant and non-malignant diseases,[1] we attempted to construct an algorithm to guide clinicians in choosing the "best" cord blood unit, taking into account the impact of diagnosis, cell dose, and HLA incompatibilities, in patients receiving a single UCBT. If the cell dose with a single unit is not achieved, a double cord blood transplant should be investigated. With this objective, two different cohorts of patients who had received single UCBT between 1994 and 2005 were analyzed: 925 patients had a malignant and 279 had a non-malignant disease (Eurocord, unpublished data 2010). Donor-recipient histocompatibility was determined by serology or antigen typing (low resolution) for HLA-A and HLA-B and by allele typing for HLA-DRB1. In the malignant disease group, we found that cell dose was the most important factor influencing outcome with targets of a minimum cell dose of 3×10^7 TNC/kg at collection and of 2×10^7 TNC/kg

at infusion. We also showed that the number of HLA mismatches increased the risk of delayed engraftment and led to a higher incidence of transplant-related mortality (TRM) and chronic GvHD; however, it decreased the risk of relapse, resulting overall in a lack of influence of HLA mismatching on overall survival (OS) and disease free survival (DFS). The type of HLA mismatch did not influence outcome, but matching for HLA-DRB1 was associated with a better outcome for patients receiving a graft that had two HLA incompatibilities. As stated earlier, an increasing cell dose abrogated the effect of HLA mismatching, but not for grafts with three or four HLA incompatibilities. Thus, patients with non-malignant diseases should receive a higher cell dose to obtain engraftment than patients with a malignant disease; this should not be below 4.9×10^7 TNC/kg at collection and 3.5×10^7 TNC/kg at infusion. In non-malignant disorders, HLA mismatching played a major role in engraftment, GvHD, TRM, and survival, which was partially abrogated by increasing cell dose. A UCB graft containing two or more HLA disparities with a cell dose inferior to 3.5×10^7 TNC/kg should be avoided.

To show the improvement in UCBT following the general recommendations of more than $2.5x10^7$/kg total nucleated cells at freezing and not more than two out of six HLA disparities (or 4/6 compatibilities) in patients with malignant disorders, we conducted a retrospective study in collaboration with the CIBMTR.[33] In this analysis, we compared the results of unrelated cord blood transplants in adults with acute leukemia given a cord blood graft with more than $2.5x10^7$/kg total nucleated cells at freezing and not more than 2/6 HLA disparities, with bone marrow or peripheral blood grafts. Data were available on 1525 patients aged over 16 years with acute leukemia transplanted between 2002 and 2006 using UCB (n=165), PBPC (n=888) and BM (n=472). UCB units were matched at HLA-A and -B at the antigen level and -DRB1 at the allele level (n=10) or mismatched for one (n=40) or two antigens (n=115). PBPC and BM grafts from unrelated adult donors were matched at the allele-level for HLA-A, -B, -C, DRB1 (n=632; n=332) or mismatched at one locus (n=256; n=140). Leukemia-free survival after UCBT was comparable with that observed after allele-matched (at HLA-A, -B, -C, -DRB1) and mismatched PBPC or BM transplantation. Transplant-related mortality was higher after UCBT than after allele-matched PBPC (HR 1.62, p=0.003) or BM (HR 1.69, p=0.003), since the engraftment rate was decreased in UCB recipients. Grades 2–4 acute and chronic GvHD were lower in UCB recipients compared with allele-matched PBPC (HR 0.57, p<0.001 and HR 0.38, p<0.001, respectively). The incidence of chronic but not acute GvHD was lower after UCB compared with allele-matched BM transplantation (HR 0.63, p=0.011).

In conclusion, in spite the similar final outcomes, the engraftment rate in UCBT recipients with a reasonable UCB cell graft content, is still decreased, probably showing that other mechanisms of UCB engraftment is involved. Therefore, if the engraftment rate after UCBT could be improved, we speculate that the results after UCBT may be better than BM or PB transplantation.

Use of double cord blood units

Because the cell dose is considered to be a critical determinant of outcome in UCBT, the Minneapolis group demonstrated that transplantation of two partially HLA matched cord units may overcome the problem of cell dose and make the transplantation of heavier adult adult patients feasible. This strategy has led to an increased number of adult patients receiving UCBT. Results with double cord blood transplantation support the safety of the procedure.[12,13] Chimerism data from these studies reveal that typically only one of the cord blood units engrafts. In spite of the fact that double cord blood transplant recipients are heavier than patients receiving a single unit, the cumulative incidence of neutrophil recovery does not differ statistically between the two groups. This observation suggests a 'booster' effect from the non-engrafting unit.

To date, 7235 related and unrelated cord blood transplants have been reported to Eurocord from 400 transplant centers in more than 40 countries from 1988 to March 2010 (Figure 1). More than 90% of cord blood transplants used an unrelated donor. Of these, 1152 have been performed using two cord blood units (dUCBT) from 1999 to March 2010. Eighty-four dUCBT have been performed for children (n=45) and adults (n=39) with non-malignant disorders, mainly with bone marrow failure syndromes. There is a list of the numbers of dUCBT in children and adults by diagnosis of malignant disorders (n=1068) (Table 1).

Since 2005, the number of adult patients receiving a dUCBT has surpassed the number of adults transplanted with single units reported to Eurocord. The median age of 954 dUCBT recipients was 44 years (range 18–74 years), median weight was 73kg (range: 40–140 kg), and median nucleated cell dose infused was $3.7x10^7$/kg (range: 1–$10x10^7$/kg); 64% of the transplants were performed using a RIC regimen. Median follow-up was 10 months and estimated 1-year overall survival (OS) was 48% after dUCBT (for 838 patients with available clinical data). The estimated 1-year OS of adults transplanted with a double CB unit for malignant disorders by disease status is shown (Figure 2).

Outcomes after dUCBT compared with single UCBT in adults with acute leukemia

Recent data from the Minnesota group suggest that dUCBT is associated with a higher incidence of acute

Table 1. Number of double cord blood transplants in children and adults by diagnosis.

Diagnosis	Children	Adults
ALL	54	180
AML	35	350
Sec AL	2	5
MDS	10	133
CML	2	50
CLL	-	36
NHL	7	106
Hodgkin disease	4	47
Myeloma	-	47
Total	114	954

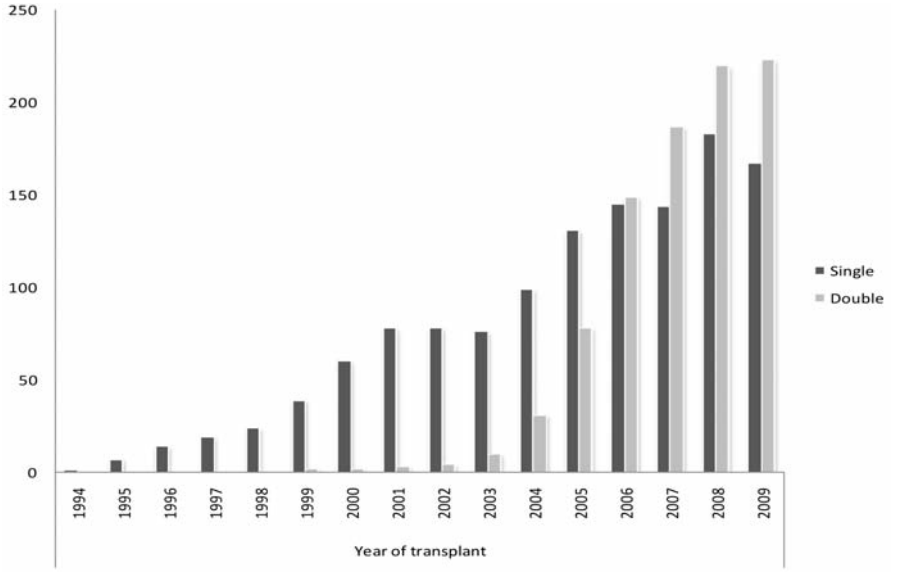

Figure 1. Number of single and double unrelated cord blood transplants in adults with malignant disorders by year, reported to Eurocord Registry.

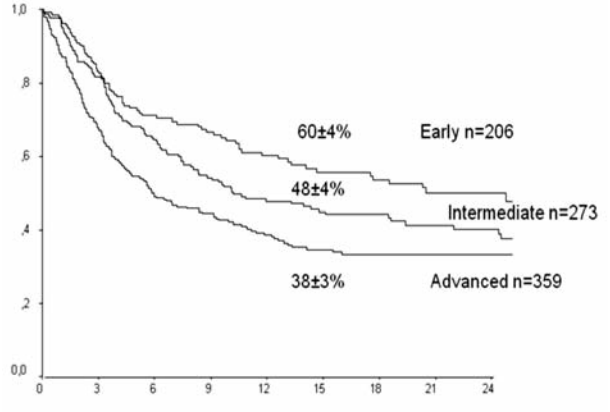

Figure 2. One Year-Overall Survival aftern dUCBT in adults with malignant disorders by status of the disease at transplant.

GVHD compared with that of single UCBT but without an increase in non-relapse mortality. Interestingly, in an analysis of 177 patients with acute leukemia, relapse was significantly lower for early stage (CR1-2) patients who received two UCB units, suggesting a greater GVL effect. DFS was 40% and 51% for single and double unit recipients, respectively (p=0.35).[33]

The Eurocord group has published results of single and dUCBT for patients with lymphoid malignancies.[34] Relapse incidence was reduced for patients transplanted with a dUCBT compared with those patients transplanted with a single unit. Recently, in a preliminary and unpublished analysis, Eurocord in collaboration with the Acute Leukemia Working party of EBMT have compared the outcomes after dUCBT (n=213) with single UCBT (sUCBT=378) in adult patients with acute myeloid or lymphoblastic leukemia in remission. There were some differences between the two groups: dUCBT recipients were heavier (median weight: 69kg vs. 64 kg, p<0.01), tended to be older (median age was 39 years vs. 36 years, p=0.09), transplanted more recently (p<0.01) and more frequently given RIC (55% vs. 32%, p<0.001) and less ATG/ALG (38% vs. 61%, p<0.001) compared with sUCBT recipients, respectively. As expected, dUCBT recipients received a graft containing a higher nucleated cell dose (median of $3.7x10^7$/kg vs. $2.6x10^7$/kg; p<0.0001) and greater number of HLA disparities (4/6:72% vs. 62%, p=0.03) when compared with single UCB grafts. There were no differences in the type of leukemia (AML or ALL) or disease status at transplant (53% were in first CR and 47% in CR2 or more). There were no statistical differences in CI of neutrophil recovery (86± 3%% in dUCBT versus 87±2% in single UCBT, p=0.62); however, CI of acute GVHD (II-IV) was higher after dUCBT (36± 3% vs25± 2%, p=0.004) compared with single UCBT but chronic GvHD was not statistically different between the two groups. Two-year CI of NRM was 37±4% after dUCBT and it was 36±3% after single UCBT (p=0.62), whereas relapse incidence was reduced after dUCBT (18±3% vs. 26±3% after single UCBT) (p=0.05). DFS at 2 years after dUCBT was 45±3% and after single UCBT, it was 38±3% (p=0.05). In a multivariate analysis adjusted for the differences outline above, dUCBT was associated with an increased risk of acute GvHD (II-IV) and decreased incidence of relapse. DFS was not statistically different between dUCBT and single UCBT recipients; however, we observed an improved DFS rate in patients transplanted in first CR with dUCBT. This Eurocord preliminary analysis confirms the previous findings of more GvHD, equivalent NRM, and reduced relapse in adult recipients of dUCBT, mainly for those transplanted in early disease status, showing a higher GVL effect. No impact has yet been observed on DFS, perhaps due to relatively short follow-up at this stage.

Comparison of double unrelated cord blood transplantation with HLA matched sibling and HLA matched and mismatched unrelated hematopoietic stem cell transplantation

Recently, Brunstein, on behalf of the University of Minnesota and the Fred Hutchinson Cancer Research

Center, published an analysis of 536 patients with malignant disease (AML, n=211; ALL, n=236; CML, n=70 and MDS, n=19) transplanted with a HLA 8/8 allele matched related (MRD, n=204) or an unrelated donor (MUD, n=152), 1 allele mismatched unrelated donors (MMUD, n=52), or double UCB (n=128).[35] All patients received myeloablative conditioning with cyclophosphamide and TBI with fludarabine administered prior to dUCBT, and received GvHD immunosuppression with a calcineurin inhibitor and either methotrexate (MRD, MUD, and MMUD) or mycophenolate mofetil (MRD and dUCBT). While patients' weight and sex distribution and proportion with standard risk disease were similar, dUCBT patients were younger (median age 25 years, MRD 40 years, MUD 31 years, MMUD 31 years; p<.01). The proportion of AML and ALL was similar among groups, although more CML patients received a MUD or MMUD. The median follow-up of survivors was 3.1 years (range: 0.3–8.1 years). When transplant outcomes were compared, dUCBT was associated with slower hematopoietic recovery compared with other stem cell sources, with median times to neutrophil and platelet recovery being at least 1 week and 4 weeks longer after dUCBT, respectively. Furthermore, despite greater HLA mismatch, the cumulative incidence of grade II-IV acute GvHD, and chronic GvHD were lowest after dUCBT. Despite a reduced risk of GvHD after dUCBT, the risk of relapse was remarkably low while TRM was elevated, resulting in similar progression free-survival (PFS). In the absence of a MRD donor, using either MUD or double UCB yield encouraging PFS and are promising donor options. These results support the use of HLA 0-2 antigen mismatched dUCBT in patients with hematological malignancies as front line therapy in patients lacking a matched sibling donor.

Reduced intensity conditioning regimens prior to single or dUCBT from unrelated donors in adults

Most studies have tested UCBT in the setting of myeloablative conditioning. RIC before UCBT has been increasingly used in order to decrease toxicity, shorten the duration of aplasia, and extend the availability of cord blood transplantation to the elderly or patients who are not eligible for myeloablative conditioning. The Minnesota group has evaluated the efficacy of UCB in the setting of a non-myeloablative regimen consisting of fludarabine, cyclophosphamide, and a single fraction of total body irradiation (200 cGy) with cyclosporin and mycophenolate mofetil (MMF) for post transplantation immunoprophylaxis. The target cell dose for the UCB graft was $3.0x10^7$ TNC/kg, resulting in the selection of a second partially HLA-matched UCB unit in 85% of patients.[13] One hundred and ten patients with hematologic diseases were enrolled. Neutrophil recovery was achieved in 92% at a median of 12 days. One cord blood unit predominated engraftment and none of the following factors were predictive of which unit eventually dominated: total nucleated, $CD34^+$, and $CD3^+$ cell doses; HLA matching; nucleated cell viability; ABO typing; gender match; or order of unit infusion. The TRM was 26% at 3 years. OS and event-free survival (EFS) at 3 years were 45% and 38%, respectively.

More recently, the Société Française de Greffe de Moelle-Thérapie Cellulaire (SFGM-TC) in collaboration with Eurocord reported results of 155 consecutive UCBT performed using a RIC regimen, with a median follow-up of 18 months (range 2–56).[36] The conditioning regimen was as previously described and GvHD prophylaxis consisted of cyclosporin and MMF. The cumulative incidence of neutrophil engraftment at day +60 was 80±3% with a median time to achieve neutrophils greater than 0.5/L of 20 days; autologous recovery was seen in 14% of the patients. In multivariate analysis, factors independently associated with better neutrophil recovery were the CD34 cell dose (>$1.2x10^5$/kg) (HR 1.51, p=0.04), HLA compatibility (0-1 vs. 2-3) (HR1.5, p=0.05) and a previous autograft (HR1.8, p<0.01). The cumulative incidence of non-relapse mortality (NRM) was 18±3% at 18 months. The estimated probabilities of OS and DFS at 18 months were 62±5% and 51±4%, respectively.

In summary, both studies demonstrated the feasibility of RIC-UCBT and reported encouraging results with this approach. In spite of reducing the duration of aplasia, the cumulative incidence of engraftment remains between 80 and 90%. Once again HLA disparity and cell dose played an important role, and myeloid engraftment was achieved in 94% when patients received well matched (6/6 or 5/6) cord blood unit(s) with a higher CD34 cell dose.[37]

Use of accessory cells to improve engraftment

Co-transplantation of an UCB unit with highly purified $CD34^+$ cells from haploidentical family donors

Phase I-II clinical trials using accessory population(s) to enhance engraftment have shown interesting results. The Spanish group developed a strategy of UCBT with co-infusion of a limited number of highly purified mobilized HSC (MHSC) from a HLA unrestricted third party donor (TPD). Short post-transplant periods of neutropenia were generally observed in adults with haematologicalhematological disorders receiving UCBT with relatively low cell content and 0-3 HLA mismatches after myeloablative conditioning. This shortened neutropenic phase was due to an early and initially predominant engraftment of the TPD-MHSC. After a variable period of double complete TPD + UCB chimerism, final full UCB chimerism was achieved (cumulative incidence >90%) within 100 days. Early recovery of the circulating neutrophils resulting from the 'bridge transplant' of the TPD-MHSC reduced the incidence of serious neutropenia-related infections, also facilitating the use of drugs with myelosuppressive side effects to combat other infections. The observed incidence of GvHD and relapses was low, with overall and disease-free survival curves comparable with those of HLA identical sibling transplants.[17,37,38]

Co-transplantation of an UCB unit with haploidentical parenteral multipotent mesenchymal stromal cells

McMillan *et al.*[18] have reported an attempt to speed hematopoietic recovery in a single-institution phase I-II clinical trial in which *ex vivo* culture-expanded multipotent mesenchymal stromal cells (MSCs) from haploidentical parental donors were infused at the time of UCBT. Fifteen pediatric patients with high-risk acute leukemia were enrolled. Eight patients received MSCs on day 0, with three patients having a second dose

infused on day 21. No serious adverse events were observed with any MSC infusion. All eight patients achieved neutrophil engraftment at a median of 19 days. The probability of platelet engraftment was 75% at a median of 53 days. With a median follow-up of 6.8 years, five patients remain alive and disease free. In another pilot study,[40] *ex vivo*-expanded bone marrow MSC from parental donors were infused at the time of the transplantation or the in case of refractory acute GvHD. Nine patients received MSC immediately after CB transplantation and TPD-highly purified mobilized HSC. Neither immediate adverse effects nor significant differences in CB engraftment or acute GvHD development were observed. Four patients developed grade II acute GvHD, two steroid-refractory. The last two achieved complete remission after therapeutic infusions of MSC.

The results of both pilot studies show that infusion of *ex vivo* culture-expanded haploidentical MSCs into unrelated UCBT recipients can be performed safely. Further studies may investigate the role of co-infusion of MSC in order to improve engraftment after UCBT.

Other experimental approaches

Enhancing collection, homing and expansion of UCB cells

Because of the limiting numbers of HSC and HPC in banked cord blood, the means to: 1) enhance collection of cord blood cells;[39] 2) enhance the homing and engraftment of HSCs /HPCs;[40,41] and/or 3) enhance the *ex vivo* or *in vivo* expansion of these cells could greatly enhance the quality and usefulness of cord blood cells for transplantation.

It is possible to enhance substantially the numbers of HPC collected by perfusing the placental vessels after draining the blood from the cord[41] but the practicality of this method for banking remains to be evaluated. If perfusion of the placenta after collection of blood from the cord is untaken, it would need to be done in selected collection centers with well-trained personnel.

There have been a number of efforts to enhance the homing and engraftment capability of HSCs and HPCs. One proposal is to inhibit the enzymatic activity of CD26/dipeptidylpeptidase IV (DPPIV) with small peptides.[42,43] Other mechanisms are under investigation with the aim of improving *ex vivo* expansion of cord blood cells. Phase I/II clinical trials have started to evaluate the safety and toxicity of infusing Notch-ligand Delta 1 or copper chelator tetraethylenepentamine (TEPA; StemEx) to induce *ex vivo* expansion of cord blood progenitors in patients with hematologic malignancies.[10,11] Interestingly, Notch-ligand Delta 1 has also been shown to have an effect on early T-cell expansion and differentiation.[42] Recently, Delaney *et al*. have described a Notch-mediated *ex vivo* expansion system for human $CD34^+$ cord blood progenitors that results in a marked increase in the absolute number of stem/progenitor cells, including those capable of enhancing repopulation in the marrow NOD-SCID mice.[43] Moreover, a phase I trial is ongoing, involving transplantation of a non-manipulated unit along with cord blood progenitors expanded *ex vivo* in the presence of Notch ligand. Ten patients who underwent dUCBT after myeloablative conditioning for high risk acute leukemia have been enrolled. Significantly, more rapid myeloid engraftment was observed, with the median time to neutrophil recovery (neutrophils 500/µl) being 16 days, faster than would be expected using two non-manipulated units (median time 23 to 26 days or longer in the published literature).[45]

Future efforts to expand HSC/HPC *ex vivo* and *in vivo*, and to enhance the homing and engrafting capabilities of cord blood cells will likely make use of more in depth information on intracellular signaling molecules and their networks involved in HSC and HPC functions, including self-renewal, proliferative, survival, differentiation, and migration.[37] Further information on the bone marrow microenvironment and how HSC/HPC interacts with this microenvironment will permit the development of more effective ways to achieve engraftment.

Enhancing homing capacity with direct intra-bone marrow injection of cord blood cells

The concept of enhancing homing capacity of cord blood cells through their direct injection into the bone marrow environment has led some investigators to apply this approach clinically. In mice, it has been suggested that intra-bone infusion of $CD34^+$ cord blood cells confers an engraftment advantage 15 times greater than after intravenous infusion because cell loss during circulation before homing is circumvented.[44] Recently, a phase I/II study[9] was performed to establish the safety and efficacy of the intra-bone administration of cord blood cells, measured by the donor-derived neutrophil and platelet engraftment. Thirty-two patients presented with leukemia, 14 with advanced disease. HLA-matching was 5/6, 4/6, and 3/6 for 9, 22, and 1 patient, respectively. Most of the patients received a myeloablative conditioning regimen associated with ATG, and only two patients received a RIC-UCBT. Cord-blood cells were concentrated in four 5-mL syringes, and were infused in the superior-posterior iliac crest under rapid general anesthesia. The median transplanted cell dose was $2.6x10^7$/kg. No complications occurred during or after the intra-bone infusion of cells. The median times to recovery of neutrophils and platelets were 23 days (range 14–44) and 36 days (range 16–64), respectively. All patients were fully chimeric from 30 days after transplantation to the last follow-up visit, suggesting early complete donor engraftment. No patient developed grade III-IV acute GvHD. More recently, in a preliminary matched pair analysis comparing patients transplanted with cord blood injected intravenously (IVCB) versus cord blood injected directed into the bone marrow (IBCB) of the iliac crest, IBCB patients (n=50) were matched with 88 IVCB recipients. The cumulative incidence (CI) of neutrophil recovery was 70±5% in IVCB recipients versus 80±6% in the IBCB group (p=0.27). However, patients receiving IBCB had a higher rate of platelet recovery at day 60 (82±5%) compared with the IVCB group (40±5%; p< 0.0001). Strikingly, the incidence of acute GVHD grade II-IV was 12% in the IBCB group compared with 38% in the IVCB group (p=.0001) and the incidence of grade III-IV GVHD was 2% compared with 18%, (p<0.001) respectively. Overall survival at one year was 67±7% compared to 43±5% (p=0.07), respectively. In summary, injection of cord blood cells into the bone marrow appears to reduce significantly the problem of delayed platelet recovery observed after IVCB. The reduced incidence and severi-

ty of acute GvHD observed in IBCB patients is intriguing and promising.[9]

Conclusions

Clinical results after UCBT are improving in the recent years, mainly due to better donor choice (cell dose and HLA matching), improvement in supportive care, greater centre experience. Other approaches that improve engraftment and decrease transplantation related mortality after UCBT are being currently developed with very encouraging results. Those approaches may greatly increase the clinical use of cord blood cells for transplantation.

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Alternative donor stem cell source: haploidentical transplantation

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A B S T R A C T

Initial attempts at haploidentical transplantation with T-cell replete bone marrow were associated with a high transplant-related mortality, mainly caused by severe graft-versus-host disease (GVHD). Previous efforts to prevent GVHD by *ex vivo* T-cell depletion of haploidentical bone marrow were associated with high risk of graft failure and other complications. Improvements in large scale T-cell depletion techniques of haploidentical peripheral blood stem cells helped overcome the HLA barrier by using 'megadose' numbers of stem cells either highly purified by CD34+ positive selection or obtained by negative depletion of T-cells. Furthermore, the use of unmanipulated marrow or blood derived stem cells combined with *in vivo* T-cell depletion, intensive pharmacological prophylaxis of GvHD, or the post-transplant use of high-dose cyclophosphamide have shown promising results. In addition, recent insights into the biology of alloreactive natural killer cells, the permanent availability of the donor post-transplant, and continuous improvements of graft-engineering techniques for the generation of effector cells for post-transplant adoptive transfer facilitate the development of strategies to decrease the regimen-related toxicity through the use of less intensive preparative regimens, to prevent severe infections by rebuilding the immune system, and to decrease the risk of relapse by exploiting the alloreactivity of donor-derived effector cells.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is, for many patients with malignant and for increasing numbers of patients with non-malignant diseases, the only known curative approach. Since only approximately 30% of the patients requiring transplantation have a human leukocyte antigen (HLA)-matched related sibling, matched unrelated donors (MUD), unrelated matched or mismatched cord blood units, or three-loci mismatched haploidentical family donors are used as sources of hematopoietic stem cells. The probability of identifying a matched unrelated donor in the world-wide donor registries is dependent on the diversity of HLA antigens within a population and on the patient's race.[1] In addition, a MUD donor search is time-consuming, and the time from initiation of a donor search to the actual transplant, in most cases, is 4 months or longer.[2] A significant number of patients will progress or even die within the time of donor search, and the potential life-saving allogeneic transplantation will no longer be a therapeutic option.[3] To avoid excessive donor search time and the risk of disease progression, a tool to estimate the probability of identifying a 10/10 HLA allele-matched unrelated donor disease has been described and this will be helpful in guiding therapeutic strategies if the probability is low.[4]

An alternative approach for patients without HLA-matched donors or for patients who are at high risk of disease progression during the donor search is the use of mismatched related family donors. In pediatric patients, these are mainly mismatched parental donors and in adult patients, mainly mismatched siblings. These donors are readily available within few days, are highly motivated to donate large numbers of stem cells, and are also available during the post-transplant course if adoptive immunotherapy is required.

A number of initial attempts using haploidentical non T-cell depleted (T-cell replete) unmanipulated bone marrow (BM) and myeloablative conditioning have been made in the past. Unfortunately, these attempts were associated with early severe and often lethal side effects, including pulmonary edema and multiorgan failure resembling hyperacute graft-versus-host disease (GVHD).[5] In these earlier studies, a high number of engraftment failures were also observed. In a comparison of the outcome of patients after transplantation of bone marrow from HLA-matched and 1-antigen mismatched related donors, the overall survival was similar but the incidence of GVHD was higher in the mismatch group.[6] The small number of patients receiving transplants from a HLA 2- or 3-antigen mismatched related bone marrow donors experienced high and early transplant-related mortality (TRM). Based on these findings, 2- or 3-antigen mismatched transplantation of T-cell replete bone marrow from a family donor using pharmacological GVHD prophylaxis was considered too risky. In a larger series of patients, the risk of treatment failure and

TRM was higher for patients who received a transplant from a 1- or 2-antigen mismatched related donor compared to an HLA-identical sibling.[7]

Since donor-derived T-lymphocytes in the graft are the major cause of GVHD in allogeneic SCT, various attempts were made to deplete T-cells from BM prior to infusion. The first *ex vivo* T-cell depleted bone marrow transplants (BMT) using soybean agglutinin and rosette formation with sheep red blood cells were performed in children with immunodeficiency syndromes.[8] Subsequently, Henslee–Downey and colleagues used intensive total body irradiation (TBI) based myeloablative preparative regimens with partial *in vitro* T-cell depletion of haploidentical BM using anti-CD3 antibodies (T10B9 or OKT-3) with a 1–1.5 log reduction of T-cells and post-transplant pharmacological GVHD prophylaxis.[9] In this large series of 201 patients, 98% engrafted with 5-year cumulative incidences of relapse and TRM of 31% and 51%, respectively. The cumulative incidences of grade II-IV acute GVHD and chronic GVHD were 13% and 15%, respectively. An encouraging survival probability of 20% was seen in patients with advanced hematological malignancies.

Challenges of haploidentical transplantation

Based on the initial experience using unmanipulated or partially T-cell depleted bone marrow and fully ablative conditioning regimens, the major challenges in haploidentical stem cell transplantation turned out to be and still are: (i) the prevention of severe acute and chronic GVHD; (ii) the reduction of the risk of graft rejection; (iii) the acceleration of the delayed immunoreconstitution associated with a high incidence of severe and often lethal infectious complications; and (iv) the implementation of strategies for the selection of the best mismatched donor in terms of natural killer (NK) cell alloreactivity and post-transplant cellular therapeutic strategies to prevent the risk of relapse.

Approaches to overcoming these challenges

Megadose transplantation of positively selected CD34+ stem cells

The 'megadose' concept was first described by Reisner *et al*. in animal models of haploidentical transplantation.[10] It is based on the observation that the transplantation of large numbers of purified hematopoietic stem cells can overcome the HLA barrier of haploidentical transplantation due to the presence of 'veto' cells within the CD34+ selected population.[11,12]

To translate this concept into a clinical setting, Aversa *et al*. devised a strategy for the combination of BM-derived CD34+ stem cells and CD34+ stem cells purified from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells (PBSC) from haploidentical donors using soybean agglutinin and E-rosetting.[13] With this graft-engineering strategy, a 3 log reduction in T-cells was achieved and large numbers of CD34+ stem cells with a low number of graft-contaminating T-cells were transplanted. Primary engraftment was achieved in 16/17 patients with end-stage leukemia. In the absence of any pharmacological GVHD prophylaxis, severe acute GVHD occurred in only one patient and no cases of chronic GVHD were observed. In a subsequent study, the same group then used positively selected CD34+ stem cells from leukapheresis as the sole source of stem cells with a higher degree of T-cell depletion (4 logs) following a preparative regimen composed of TBI, thiotepa, fludarabine, and anti-thymocyte globulin (ATG). The median number of transplanted CD34+ stem cells was $10x10^6$/kg recipient body weight, and the number of co-transplanted CD3+ T-cells was only 2×10^4/kg. The engraftment rate was 95%, and severe GVHD was not seen even in the absence of any pharmacological GVHD prophylaxis.[14] However, due to the delayed immune reconstitution associated with this approach, there was a high rate of infectious complications, and infections were the leading cause of death and the main contributors to a TRM rate of 40%.

The clinical introduction of a semi-automated device for the positive selection of highly purified CD34+ stem cells and the high degree of indirect depletion of T-cells and B-cells to 4.5–5 log and more than 3 log, respectively,[15] led investigators to evaluate haploidentical transplantation using 'megadoses' of highly purified CD34+ stem cells in patients with a variety of diseases. With this extensive T-cell depletion, almost no GVHD was seen even in the absence of any pharmacological GVHD prophylaxis and due to the indirect B-cell depletion associated with CD34+ selection, post-transplant EBV-related lymphoproliferative disorders were also absent in both pediatric[16] and adult patients.[17] Similar results were reported from other investigators using mostly TBI-based myeloablative preparative regimens followed by the transplantation of large numbers of highly purified CD34+ peripheral stem cells without any additional GVHD prophylaxis.[18–22] All these studies in both pediatric and adult patients have in common delayed immune reconstitution, which was associated with a high incidence of severe and lethal infections. In an analysis on behalf of the Acute Leukemia and Pediatric Disease Working Parties of the European Group for Blood and Marrow Transplant (EBMT), of the outcome of 102 children with ALL in remission receiving haploidentical transplants with CD34+ positively selected stem cells, the impacts of the number of transplanted CD34+ stem cells and the transplant center size (in terms of number of haploidentical transplants performed per year) were demonstrated.[23] Patients who received more than 12.4×10^6/kg CD34+ positively selected stem cells had a leukemia-free survival (LFS) of 35±7% compared with 17±6% in patients who received less than 12.4×10^6/kg. Patients who received their transplant at a larger transplant center had a LFS of 39±7% compared with the LFS of 15±6% in patients transplanted in smaller centers.

In a survey of adult patients with high risk acute leukemia in remission at transplantation who received positively selected CD34+ stem cells, the LFS for patients with AML in CR1, more than or equal to CR2 or advanced disease were 48±10 %, 21±%, and 1±1%, respectively. The LFS for patients with ALL in CR1, more than or equal to CR2 and advanced disease were 13±%, 30±8%, and 7±5%, respectively.[24] A reduced

intensity conditioning regimen was employed in 28 adult patients with advanced hematological malignancies who were transplanted with haploidentical CD34+ positively selected stem cells.[25] Engraftment was achieved in 78% of the patients, and the toxicity was mostly infections. The co-transplantation of *ex vivo*-expanded donor-derived mesenchymal stem cells together with CD34+ positively selected stem cells accelerated lymphocyte recovery and reduced the risk of graft failure in pediatric patients.[26]

Transplantation of negatively T-cell depleted stem cells

Depletion of CD3+/CD19+ lymphocytes

While initial attempts to deplete T-cells from BM resulted only in 1–1.5 log reduction of T-cells, semi-automated devices for the concomitant depletion of T- and B-lymphocytes to prevent GVHD and post-transplant EBV-associated lymphoproliferative disease, respectively, allow the effective depletion of CD3+ and CD19+ lymphocytes (CD3/19 depletion) from mobilized peripheral stem cell grafts.[27] The T-cell depletion with this method (3.5–4 log) is less than compared with the CD34+ positive selection method (4.5–5 log). In contrast to the CD34+ positive selection method, large numbers of NK-cells, monocytes, dendritic cells, and other myeloid cells are retained in the graft. In the first clinical study, 20 children with hematological malignancies were transplanted with CD3-depleted PBSC following a TBI-based myeloablative preparative regimen.[28] Of 19 evaluable patients, all engrafted. Six patients (30%) died from TRM and 4 patients (20%) from disease recurrence. Ten patients (50%) are alive and well. A subsequent pediatric study was performed in 25 patients with advanced refractory hematological malignancies (n=9) or patients with relapse after a previous standard myeloablative transplantation (n=16).[29] Since these patients were considered to be at high risk of TRM with a myeloablative preparative regimen, a less intensive non TBI-based conditioning regimen consisting of fludarabine (200 mg/m²), thiotepa (10 mg/kg), melphalan (120 mg/m²), and the anti-CD3 antibody OKT-3 was employed. Two of three patients who did not engraft were rescued by another transplant from their original donor, and one patient had early disease progression without donor engraftment. The cumulative incidences of grade II-IV and grade III-IV GVHD were 44% and 8%, respectively. The cumulative incidence of chronic GVHD was 28%. Thirteen patients died of disease recurrence and four from TRM. No lethal viral infections were seen. Eight patients remain alive with a performance score greater than or equal to 90%.

In a comparative analysis of the immune reconstitution in these two cohorts of patients, a much faster recovery of thymopoiesis, as determined by the rapid increase of T-cell receptor excision circles (TREC), and a rapid return of the T-cell receptor repertoire were seen in the patients who received a non TBI-based less intensive preparative regimen compared with the patients who received myeloablative TBI.[30]

Additional studies were initiated in pediatric and adult patients using the less intensive non TBI-based preparative regimen, consisting of fludarabine (160 mg/m²), thiotepa (10 mg/kg), melphalan (140 mg/m²), and OKT-3.[31] In a study of 38 pediatric patients, primary engraftment was seen in 83%. The major cause of graft failure was rejection. However, after reconditioning and a second transplant with CD3/19 depleted PBSC from the other haploidentical parental donor,[32] engraftment was finally obtained in 98% of the patients. Most importantly, only 1 out of the 38 patients died from TRM. Acute GVHD grade 0-I, II, and III-IV were seen in 73%, 24%, and 3% of the patients respectively. In a recent update, Pfeiffer *et al*. reported an event-free survival of 51% for children with acute leukemia who were in morphological remission at time of transplantation.[33] In a study in adult patients, a similar approach was used with the same preparative regimen.[34] Twenty-nine high risk patients were transplanted and 9/29 patients are alive. The TRM in the first 100 days was 20% and 12 patients relapsed. The immune reconstitution was analyzed in 28 adult patients who received a haploidentical CD3/19-depleted graft. T-cell reconstitution was delayed with a median of 70 CD4+ T-cells/µl on day +100 post-transplant, whereas the NK cell recovery was fast reaching normal values at day +20.[35]

Depletion of TcRαβ+/CD19+ lymphocytes

Compared with CD34+ positive selection, the number of graft-contaminating T-cells is approximately 10-fold higher in CD3/19-depleted grafts, requiring short-term post-transplant immunosuppression with mycophenolate mofetil (MMF).[36] A more effective approach for the negative depletion of T-cells is the recently described negative depletion of T-cell receptor (TcR) αβ+ T-lymphocytes from mobilized peripheral stem cell grafts.[37] With this method, a T-cell reduction of 4.5–5 log can be achieved, which is comparable with CD34+ positive selection. This approach retains NK cells, monocytes, dendritic cells, and γδ+ T-lymphocytes in the graft. γδ+ T-lymphocytes are non-alloreactive lymphocytes with important anti-infectious and anti-tumor properties (for review, see[38]), which might have an impact on the outcome of haploidentical transplantation. In this context, it has been reported that γδ+ T-cells can exert an anti-leukemic effect in partially mismatched HSCT.[39] In this retrospective analysis, patients with a high γδ T-cell count post-transplant had a better 5-year survival than those with normal or low counts (70.8 vs. 19.6%, p=0.0001), while no difference in the incidence of GVHD was observed. The first clinical experience in children undergoing haploidentical transplantation of TcRαβ/CD19-depleted T-cells are promising with a rapid donor engraftment, a rapid expansion of γδ+ T-lymphocytes, and a rapid immune reconstitution with a median time to reach 100 CD3+ T-cells of 34 days (own unpublished observations).

Transplantation of T-cell replete bone marrow and/or peripheral stem cells

The use of T-cell replete BM or PBSCs relies either on the composition of the graft, on intensive pharmacological prophylaxis for GVHD, or on the *in-vivo* T-cell depletion of the donor-derived T-cells after infusion of the graft using polyclonal antithymocyte globulin (ATG) or monoclonal antibodies (Alemtuzumab). Huang *et al*. combined G-CSF-primed BM and unmanipulated PBSC

following a myeloablative preparative regimen in adults.[40] All patients achieved full donor chimerism. Grade II-IV acute GVHD occurred in 78% and chronic GVHD in 73% of the patients. In another large study, HLA-mismatched or haploidentical blood and marrow transplantation achieved comparable outcomes with HLA-identical sibling transplantation.[41] In this setting, the graft composition affected the clinical outcome, and a higher CD4/CD8 ratio in the G-CSF-primed BM was associated with a survival disadvantage and a trend towards relapse, whereas a lower CD4/CD8 ratio in primed BM was associated with a survival benefit.[42] The use of G-CSF mobilized PBSC alone was inferior compared with the use of G-CSF-primed BM and G-CSF mobilized PBSC.[43] G-CSF-mobilized BM alone was used in pediatric patients with hematological malignancies after myeloablative conditioning. For GVHD prophylaxis, ATG, Basiliximab (a chimeric mouse-human monoclonal antibody that recognizes the α chain (CD25) of the IL-2 receptor of T-cells), CsA, and short-course MTX were used.[44] The more than 2-year disease-free survival was 53%. Unmanipulated HLA 2-3 antigen mismatched nonprimed haploidentical BM was transplanted in 30 adult patients with advanced hematological malignancies after myeloablative conditioning and an intensive pharmacological prophylaxis for GVHD.[45] All patients achieved engraftment. Acute GVHD grade II-III occurred in 36.7%, and seven patients died of transplant-related toxicity. The probability of survival at 3 years was 49.9%. Unmanipulated G-CSF-mobilized PBSC were the main stem cell source in a study of 66 adult patients with hematological malignancies after non-myeloablative conditioning regimen.[46] Successful engraftment was seen in 60 patients and only 5 patients developed severe grade III-IV acute GvHD. The overall survival at 6 years was 29.3%. Patients younger than 60 years of age had a better outcome compared with patients older than 60 years of age (43.6% vs. 9.5%, respectively). Patients transplanted from 1, 2, or 3 loci mismatched donors had OS of 63.6%, 29.6%, and 12.5%, respectively. Forty-six patients were dead at a median of 2 months (range 0.5–26 months). The causes of death were organ failure,[17] infections,[17] graft rejection,[4] acute GvHD,[1] and disease progression.[7] While most of these studies used ATG for *in vivo* T-cell depletion, Rizzieri *et al*. treated 49 patients with a nonmyeloablative regimen comprising fludarabine, cyclophosphamide, and alemtuzumab followed by the transplantation of G-CSF-mobilized unmanipulated PBSC.[47] TRM was 10.2% and 8% experienced severe GVHD. The 1-year survival rate was 31%.

Induction of tolerance

In vivo allodepletion

A promising approach is *in vivo* allodepletion using cyclophosphamide (CY) to induce tolerance. In these protocols, donor-derived lymphocytes are exposed to host antigens that induce the proliferation of antigen-specific lymphocytes. The theory is that by timely exposure to post-transplant CY, proliferating lymphocytes are killed whereas resting lymphocytes are spared.[48] In a study of 68 patients with hematological malignancies, CY (50 mg/kg) was given on day 3 or day 3 and 4 after non-myeloablative conditioning and transplantation of T-cell-replete haploidentical BM.[49] Graft failure occurred in 13% of the patients and the cumulative incidences of acute grade II-IV, grade III-IV GvHD, and chronic GvHD were 34%, 6%, and 22%, respectively. Actuarial overall and EFS at 2 years after transplantation were 36% and 26%, respectively.

Using non-inherited maternal antigen mismatched donors

Another possible method of inducing tolerance might be haploidentical transplantation from non-inherited maternal antigen (NIMA) mismatched donors. The tolerance induction is the result of in utero exposure to maternal antigens and the development of long lasting feto-maternal microchimerism.[50] Van Rood *et al*. showed that the incidence of grade ≥II acute GVHD following non-T-cell depleted haploidentical stem cells is related to haplotype inheritance.[51] In a subsequent study, these results were confirmed and NIMA mismatch in the GVHD direction was associated with a lower risk of severe grade III-IV acute GVHD.[52] In a long-term follow-up study of survivors after NIMA-mismatched haploidentical HSCT, immunosuppressive agents could be discontinued despite the frequent occurrence of moderate to severe chronic GVHD.[53] Another strategy to induce tolerance or anergy in host-reactive T-cells is the exposure of the graft *ex vivo* to host alloantigens while simultaneously blocking T-cell costimulatory signals.[54]

Optimizing NK alloreactivity

One of the most important developments from the studies using highly purified CD34+ stem cells in haploidentical HSCT was an improved understanding of NK alloreactivity.[55] In this concept, lysis of tumor cells by NK cells is influenced by mismatching of the killer immunoglobulin-like receptors (KIR) ligands between the donor NK cells and the recipients' target cells. KIR receptors are expressed on NK cells and recognize certain HLA alleles (ligands). Their engagement can induce inhibition or activation of the NK cells (for review, see[56]). It has been shown that mismatching of the inhibitory KIR ligands in GVHD direction can exert a graft-versus-leukemia effect in the absence of GVHD. This anti-leukemia effect is especially pronounced in adult patients with AML, but not in patients with ALL.[57] The probability of relapse at 5 years was 75% in patients receiving a haploidentical graft from a KIR ligand-matched donor and 0% after a KIR ligand-mismatched graft. While these studies used KIR ligands (ligand-ligand model; Figure 1) to predict NK alloreactivity, other studies used a direct determination of the donors' KIR and the patients corresponding ligands (receptor-ligand model; Figure 2).[58] By employing the receptor-ligand model, the important role of NK alloreactivity was demonstrated and pediatric patients with ALL had a lower probability of relapse after KIR receptor- ligand mismatched haploidentical transplantation with CD34+ positively selected cells.[59,60] Interestingly, the gender of the parental donor was an independent prognostic factor for survival and patients who were transplanted with CD34+ positive stem cells from NK alloreactive maternal donors had a better event free survival (EFS) than patients transplanted from NK alloreactive fathers. NK non-alloreactive maternal donors also conferred a

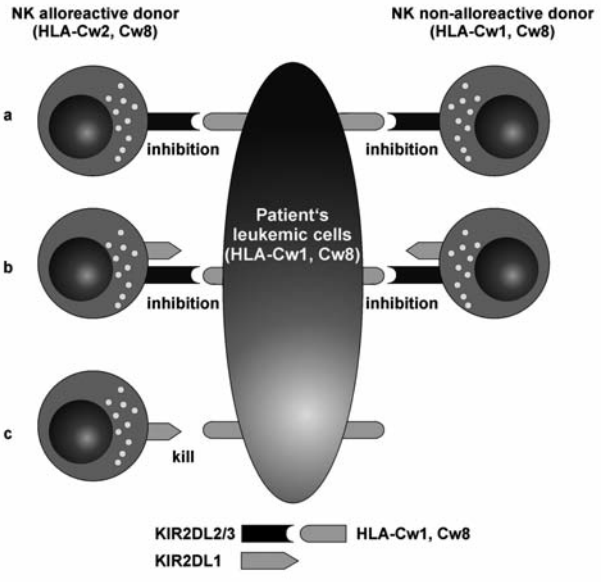

Figure 1. The **ligand-ligand model** is based on the assumption that all NK cells have at least one killer-inhibitory receptor (KIR) for self HLA class I alleles. In this example, (patient's HLA type: HLA-Cw1, Cw8), a donor mismatched at the HLA-Cw allele in GvHD direction (donor HLA-Cw2, patient HLA-Cw1) has NK cells in his repertoire, which express at least, the inhibitory receptor KIR2DL1 (receptor for self HLA-Cw2). These NK cells cannot be inhibited by the patient's HLA-Cw1 or Cw8 alleles, and the donor is NK alloreactive (depicted in c). Since all NK cells should have at least one KIR for self HLA class I alleles, NK cells only expressing KIR2DL1 would not be present in an HLA-matched donor's repertoire and a matched donor would be non-alloreactive.

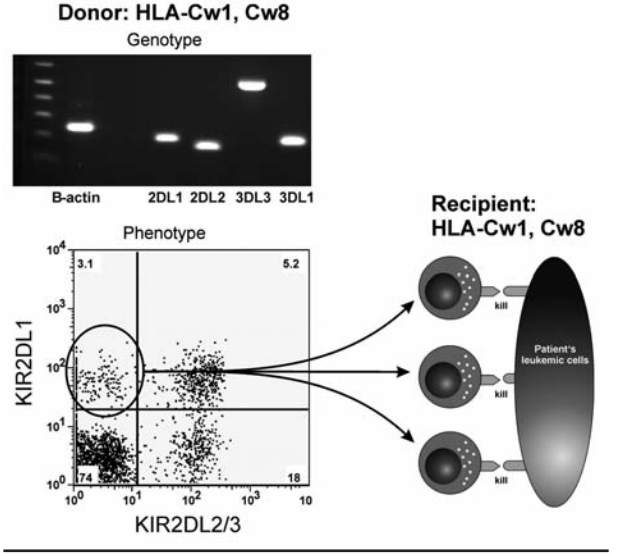

Figure 2. The **receptor-ligand model** is based on the direct analysis of the donor's KIR repertoire by genotyping or phenotyping. In this example, the donor is matched with the patient at the HLA-Cw locus. In contrast to the assumption of the ligand-ligand model, genotype analysis of the donor's KIR repertoire reveals that he has NK cells expressing KIR2DL1 and KIR2DL2/3. Phenotyping further reveals that he has NK cells that are single-positive for KIR2DL1. These single-positive NK cells cannot be inhibited by the patient's HLA-Cw1 or Cw8 alleles and are cytotoxic towards the leukemic blasts. Despite the HLA match and in contrast to the prediction of the ligand-ligand model, this donor is NK-alloreactive.

better outcome compared with NK non-alloreactive paternal donors.[61]

Less data are available on the role of donor-derived NK cell receptors and patient KIR ligands in CD3/19 depleted haploidentical transplantation. Pfeiffer *et al*. analyzed the reconstitution of NK cell receptor repertoire in pediatric patients transplanted with CD3/19-depleted PBSC after non-myeloablative conditioning.[62] A predominant expansion of CD158b+ NK cells was seen early post-transplant and patients who were homozygous for the corresponding inhibitory KIR ligands (HLA-C1 alleles) had a poorer outcome compared with patients heterozygous for HLA-C1 alleles. Similar findings were recently reported by Stern *et al*. after transplantation of CD34+ positively selected stem cells.[63] The role of NK alloreactivity in T-cell-replete haploidentical HSCT requires further study. The effects of alloreactive NK cells might be overridden by alloreactive T-lymphocytes resulting in GVHD and its concomitant immunosuppressive treatment.[64] While Huang *et al*. reported deleterious effects of KIR ligand incompatibility on clinical outcome, and KIR ligand mismatch was an independent risk factor for acute GVHD and relapse rate,[65] Symons *et al*. reported an improved survival with KIR gene mismatches and KIR haplotype B donors after non-myeloablative haploidentical BM transplantation followed by high-dose post-transplant cyclophosphamide to induce tolerance.[66] Recipients of inhibitory KIR gene mismatched BM or KIR haplotype AA recipients of BM from KIR Bx donors had a lower risk of relapse and nonrelapse mortality.

Re-building the immune system after haploidentical transplantation

Common to all approaches of allogeneic HSCT in both pediatric and adult patients is slow immune reconstitution.[67] Especially in haploidentical HSCT, poor T-cell reconstitution is the major cause of infections and contributes considerably to the TRM observed in unmanipulated or in *in vitro* T-cell depleted haplotype mismatched transplants.[68] In order to reconstitute the immune system more quickly, various strategies are investigated in clinical studies, among them the adoptive transfer of donor-derived virus-specific T-cells,[69,70] adoptive immunotherapy with allodepleted donor-T-cells,[71] the use of donor T-cells selectively depleted of alloreactive lymphocytes by photodepletion,[72] the use of donor pathogen-specific T-cells,[73] or the post-transplantation infusion of CD8-depleted donor lymphocytes.[74] A potential role of Thymosin α1 to harness immunity to pathogens after haploidentical HSCT has recently been described.[75] Another recent approach is the adoptive transfer of CD4+CD25+ regulatory T-cells (Treg) post-transplant. It has been shown previously in mouse models that these cells can preserve graft-versus-tumor activity while inhibiting GvH.[76] Di Ianni *et al*. infused donor-derived CD4+CD25+ Treg into 28 patients after haploidentical transplantation of CD34+ positively selected stem cells followed by large numbers of conventional T-cells in the absence of any post-transplant immunosuppression. The adoptive transfer of Treg prevented GVHD, promoted lymphoid reconstitution, improved immunity to opportunistic pathogens, and did not weaken the graft versus leukemia (GvL)

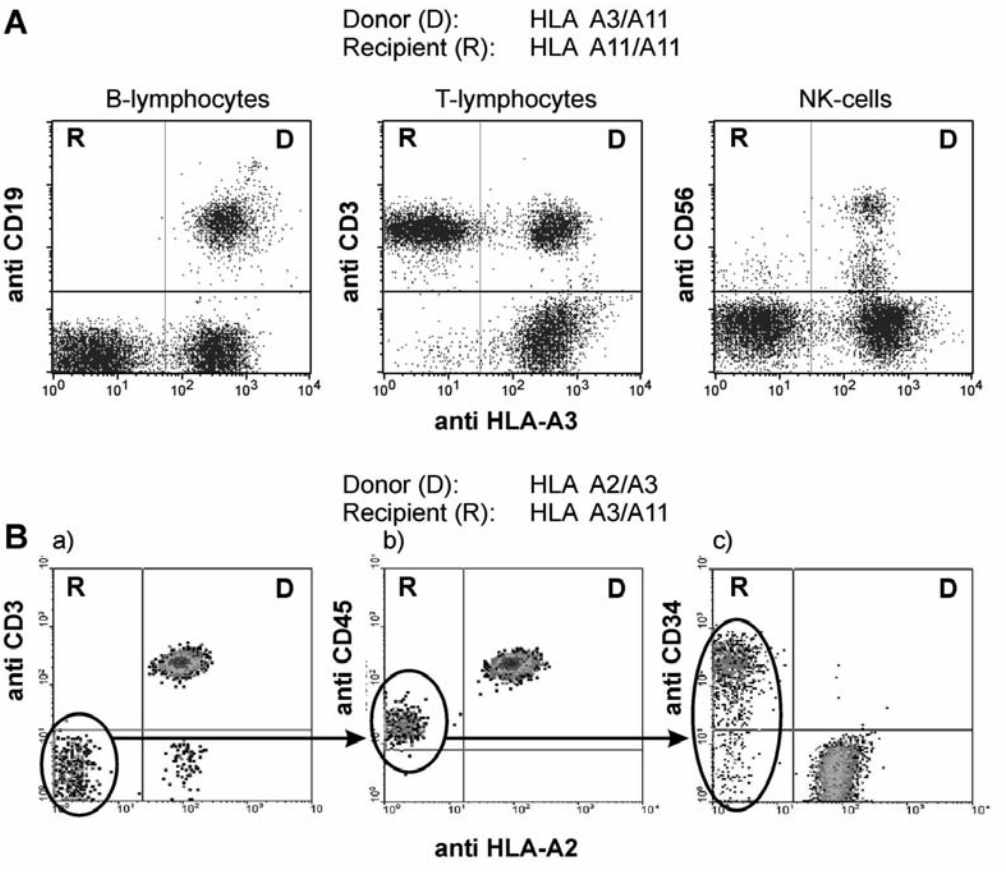

Figure 3. The HLA disparity in haploidentical transplantation facilitates the rapid and detailed flowcytometric analysis of the lymphocyte populations post-transplant. In A, an example is shown 3 months after transplantation: Whereas all B-lymphocytes are donor derived, approx. 50% of the T-lymphocytes are recipient-derived. Most NK cells are donor-derived, but there is a small recipient-derived CD56dim population. In B, the combination of flowcytometric chimerism analysis and flowcytometric determination of minimal residual disease is shown 5 months after transplant: While all CD3+ T-cells are donor-derived, there is a small population of recipient-derived HLA-A2-negative cells (a). Further analysis of this population reveals a CD45dim phenotype (b) and most of the CD45dim cells stain positive for CD34 (c), which is identical to the original phenotype of the patient's blasts.

effect. Surprisingly, none of the patients developed GVHD and immune recovery was improved.[77] It is currently unclear whether the *in vitro* T-cell depletion method has a positive impact on immune recovery. Federmann *et al*. reported faster immune reconstitution in adult patients after non-myeloablative conditioning followed by CD3/19 depleted haploidentical grafts,[35] and no lethal infections were seen in children after reduced intensity conditioning and transplantation of CD3/19-depleted PBSC.[78]

The thymic damage induced by a myeloablative regimen might also have an impact and immune reconstitution was faster in pediatric patients after reduced intensity conditioning and transplantation of CD3/19-depleted PBSC compared with patients treated with a TBI-based myeloablative regimen followed by CD3-depleted PBSC[79] Thymic function as assessed by TREC prior to allogeneic HSCT plays an important role for the subsequent thymus-dependent T-cell production and patients with low TREC copy numbers had a significantly slower T-cell reconstitution.[80] Whether the rapidly expanding non-alloreactive γδ+ T-lymphocytes after transplantation of αβ/CD19-depleted stem cells protect the patients from infections needs to be investigated in further clinical studies.

Attempts to prevent relapse

Besides the optimal donor choice, the challenge in haploidentical transplantation is the prevention of GVHD without losing the GvL effect. Delayed donor lymphocyte infusions, as often used in HLA-matched transplantation,[81] carry the risk of GVHD in any haploidentical HSCT and small T-cell inoculums can induce severe GVHD in the absence of immunosuppression.[82] However, T-lymphocytes play an important role in post-transplant immune surveillance for leukemic cells. This is supported by the loss of mismatched HLA antigens in leukemia after haploidentical HSCT.[83] In this report, in 5 of 17 patients with leukemia relapse after haploidentical HSCT and infusion of donor T-cells, mutant variants of the original leukemic cells were identified. In the mutant leukemic cells, the HLA haplotype that differed from the donor's haplotype had been lost due to acquired uniparental disomy of chromosome 6p. Therefore, donor-derived T-cells could not recognize the mutant leukemic cells, and this escape

mechanism might have been the reason for the relapse. The selective pressure mediated by alloreactive donor-derived T-cells strongly supports the use of T-cell adoptive immunotherapy. In another study, a similar tumor escape strategy was found in three pediatric patients after haploidentical HSCT.[84] An approach associated with a lesser risk of GVHD might be the adoptive transfer of NK cell-enriched donor lymphocyte infusions[85] or the infusion of NK cells activated *ex vivo* with interleukin 15.[86] A strategy to override the KIR-mediated NK cell inhibition could be the post-transplant *in vivo* application of monoclonal antibodies directed against targets expressed on leukemic blasts, such as CD19 or CD20.[87] A more recently described approach to treat relapse is the use of the CD19/CD3-bispecific T-cell engaging (BiTE) antibody Blinatumomab.[88] This antibody was used in pediatric patients who had relapsed after allogeneic HSCT. Following treatment with blinatumomab, all three patients showed complete morphological remission and reduction of minimal residual disease (MRD) below the limits of detection.[89] It is noteworthy that two of the patients were treated after haploidentical transplantation and although the engaged T-cells were donor-derived, no signs of GVHD were observed.

Strategies for the early detection of leukemic relapses will become more and more important. Due to the HLA-mismatch in haploidentical transplantation, donor or host cell-derived hematopoietic cells can easily be discerned with the use of fluorochrome-conjugated HLA antibodies and flow cytometry, which is used for routine chimerism analysis (Figure 3A).[90] In addition, the combined methods of MRD detection by flow cytometry[91] and detection of host-derived hematopoietic cells is highly sensitive and allows early intervention in the case of relapse (Figure 3B).

Conclusions

Advances in haploidentical HSCT over the past years have raised significant interest in this approach. It is still an evolving field, and a lot of further improvements can be envisioned over the next years. Due to the continuous availability of the donor, post-transplant strategies to improve immune reconstitution and to prevent disease recurrence can be designed. Given the current promising clinical results in children and adults, haploidentical transplantation should no longer be regarded as a last resort treatment for hopeless patients, but should be offered to patients with an indication for allogeneic transplant who do not have a matched sibling or a matched unrelated donor identified within a reasonable time frame.

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Role of anti-idiotypes in the neutralization of inhibitor antibodies in hemophilia

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Administration of factor VIII (FVIII) to hemophilia A patients elicits an immune response that includes Abs inhibiting FVIII cofactor activity (referred to as inhibitors). The prevalence of inhibitors varies from one study to another, but a consensus value of plus or minus 25% is generally accepted. Presently, much knowledge has been acquired on the specificity and the mechanisms by which such Abs exert their inhibitory activity. Three lines of evidence have converged to clarify these questions: (1) the identification of FVIII binding sites for von Willebrand factor (VWF), phospholipids, FIX, FX, and APC; (2) the elucidation of the three-dimensional structure of FVIII domains by crystal formation and/or computer modeling; and (3) the production of the first human monoclonal Abs to FVIII. Unfortunately, the mechanism by which these anti-Factor VIII (FVIII) antibodies are produced by FVIII infusion in hemophilia A patient remains unclear. Even if a few risk factors for inhibitor formation have been identified, such as race, type of mutation, and more recently polymorphism in the IL-10 genes,[1] the reasons as to why some patients make inhibitors are largely unknown.

It is well established that tolerance to self-protein is first induced at an early stage by clonal deletion of self-reactive B and T cells in the bone marrow and the thymus, respectively. However, not all self-reactive lymphocytes are eliminated by central deletion. Auto-reactive B cells are a common feature in peripheral blood, as well as intermediate-affinity self-reactive T cells. Clearly, some cells escape such deletion mechanisms and end up in the periphery. Tolerance to FVIII may be viewed as the result of a subtle equilibrium between anti-FVIII and corresponding anti-anti-FVIII so called "anti-Idiotypic antibodies", which are second-generation antibodies (Ab2s) directed towards the variable part of pathogenic Abs (Ab1s) and which have the potential to neutralize Ab1 FVIII-inhibiting activity.

Previous studies have demonstrated that administration of high-doses intravenous gammaglobulins in the case of auto-immune diseases, such as autoantibodies to FVIII, could modulate the immune response up to a curative effect for patients.[2,3] This effect was shown to be associated with the presence of anti-idiotypic Abs in pool of immunoglobulins.

To confirm these observations and understand the role of anti-Idiotypes in the neutralization of inhibitor Abs in hemophilia, immune response was evaluated at clonal level. Human anti-FVIII moAbs and corresponding anti-Ids were generated. It has been demonstrated that a mixture of different anti-Ids completely neutralized the anti-FVIII activity in most of the plasmas not only from hemophilia A patients (allo-antibodies) but also from acquired hemophilia (auto-antibodies). Further studies at B cells level also demonstrated that anti-Id Abs bind to anti-FVIII human B cell line producing the corresponding anti-FVIII Ab and could therefore potentially also modulate memory B cell function.

Anti-idiotypic antibodies neutralize the anti-FVIII immune response

To understand the mechanisms by which anti-FVIII antibodies neutralized FVIII activity and how inhibitors could be neutralized by anti-idiotypic antibodies, we decided to evaluate the FVIII immune response at clonal level.

Accordingly, we derived human mabs that react with high affinity to the FVIII C1, C2, and A2 domains and are representative of most of the specific inhibitors observed in hemophilia A patients.[4]

First of all, mouse anti-Id mabs against the human inhibitors were generated, and we provided the demonstration that an anti-Id mab directed towards a C2-specific inhibitor restored FVIII activity in the presence of the inhibitor, both *in vitro* and *in vivo*.[5] Furthermore, we demonstrated that FVIII inhibition obtained by a mixture of two anti-FVIII mabs (anti-C2 and anti-A2) was neutralized up to 100% when a mixture of the corresponding anti-Ids was added to the assay, establishing the proof of concept that combination of anti-Id mabs was able to neutralize the anti-FVIII immune response of hemophilia A patients. Based on this observation, we went on to demonstrate that a mixture of the different anti-Ids (neutralizing the corresponding anti-C2, -A2, -C1 antibodies) completely neutralized the cumulative anti-FVIII activity obtained in a functional assay by a mixture of monoclonal anti-FVIII

antibodies. We next evaluated whether such a mixture of anti-Ids (anti-anti-A2,-C1,-C2) had the ability to neutralize the inhibitory properties of polyclonal antibodies obtained from hemophilia A patients.

All these findings were confirmed when the inhibiting capacity of the anti-Idiotypic mixture was applied to a larger number of samples. To this end, 30 samples of hemophilia A patients with allo-antibodies and 14 patients with acquired hemophilia were evaluated. Out of the 30 samples from hemophilia A patients, anti-FVIII activity was inhibited at least to 80% in 58% of the cases and the anti-FVIII activity was neutralized by at least 10% in 23% of the cases. With regard to the plasmas of acquired hemophilia patients, FVIII activity in plasma was recovered at least by 80% or 10% in 27% or 54% of the cases respectively, indicating that in both groups, the coagulation cascade could be restored in more than 80% of the cases after addition of the anti-idiotypic antibody mixture, indicating that the panel of monoclonal Abs (and corresponding anti-Ids) were representative of the immune response of a majority of hemophilia patients.

The neutralization of circulating inhibitors by formation of complexes with specific anti-idiotypic antibodies[6,7] should be followed by rapid elimination of such complexes. Interestingly, anti-idiotype administration can be made by subcutaneous injections, which might be advantageous under some clinical circumstances. The fact that most inhibitors are IgG4 Abs renders it unlikely that activation of complement would occur. Altogether, neutralization of circulating inhibitors should be easy and with no side effects.

Anti-idiotypic antibodies as immunomodulators

Neutralization of circulating inhibitors is only one aspect of the therapeutic potential of anti-idiotypic antibodies (in emergency situations, such as prior to surgery or to increase FVIII half-life upon infusion in hemophilia patients with inhibitor). A second aspect is the induction of a long lasting immunomodulation. It is, therefore, of major interest to evaluate whether anti-id Abs carry the potential to modulate signaling of memory B cells bearing the corresponding antigen-specific receptor (BCR for B cell receptor). This modulation could lead to significant alteration of the memory B cell pool function, and thereby induce long-term antigen-specific tolerance.

That the memory B cell compartment can be efficiently altered following the binding of anti-idiotypic Abs to the B cell receptor (BCR) is strongly supported by our current knowledge of BCR-dependent B cell activation and by experimental data. However, in contrast to the neutralization of circulating Abs, which has been amply demonstrated in human diseases (e.g., the neutralization of IgE Abs by administration of anti-IgE Abs in allergy), no direct evidence of memory B cell specific modulation has been shown in man. Worth noticing, high doses of FVIII inhibit the restimulation of FVIII-specific memory B cells and their differentiation into Ab-secreting plasma cells *in vitro* and *in vivo* in a murine model of hemophilia A.[8] This indicates that anti-idiotypic Abs could potentially also modulate memory B cell function.

In vitro prediction of the effects of anti-idiotypic antibodies on B cells

The main role of the B cell receptor (BCR) is to transmit signals regulating B cell fate. The specificity of the BCR and that of the anti-idiotypic antibodies ensures that any effect at the level of B cells will be exquisitely specific for that BCR, thereby preventing any non-specific effect on the immune system.

We have demonstrated that anti-id Ab bind to anti-FVIII human B cell line producing the corresponding anti-FVIII Ab. Preliminary studies on immortalized B cells demonstrated that anti-Ids Abs specifically bound to B cells producing the corresponding anti-FVIII Ab. This specific binding is followed by capping of the complexes. To avoid interference, which could be due to the immortalization process of the B cells, B cell lines transfected with constructs containing the inhibitor Ab of interest (Bo2C11)[9] had to be developed for analyzing the modulation of B cells signaling and activation. On the other hand, it should be understood that the population of B cells, including cells sharing specificity, is heterogeneous in terms of maturation state and differentiation. With this in mind, we devoted time to engineering two mouse B cell lines expressing the human BCR corresponding to Bo2C11 at their surface. The first cell line, CH12, is in the early phase of differentiation. By contrast, the second cell line, derived from WEHI cells, is a well-differentiated B cell. Furthermore, the use of such transfected cell lines was also justified by the fact that human antibody-producing lymphoblastoid cells are known to have a disruption of the molecular mechanism linking surface recognition and early kinase recruitment. The first results show specific binding of anti-idiotypic antibodies, but when we analyzed the expression of the human BCR at the surface of the mouse B cell lines by FACS, we observed some discrepancy. Indeed, labeling with an anti-human Fc antibody gave 60% positive cells. But if we analyzed the same population with the corresponding anti-idiotypic Ab 14C12 labeled with a fluorochrome (FITC), we only detected 2 to 12% of the cells. To prevent cross-bridging of BCR at cell surface inducing early signaling, which initiates internalization of the human BCR, monovalent scFv fragments of 14C12 were produced, marked with FITC, and used as a flag to detect specific BCR on the B cell surface. In a next step, we performed experiments in which we inhibited the binding of murine 14C12 to Bo2C11 by addition of scFv. We clearly demonstrated that the scFv was able to inhibit binding of the murine 14C12 in a dose dependent manner, indicating the correct folding of the recombinant scFv14C12 and its capacity to bind to Bo2C11. A further step will be the use of transfected mouse B cell lines to study the early recruitment of kinases, such as Lyn and Syk. Furthermore, particular attention will be devoted to pathways leading to cell apoptosis, including the Fas-FasL pathway and caspase activation, as this may represent the hoped-for effect resulting from anti-idiotypic binding to BCR.

Before any further investigation and any first clinical application, we have to "humanize" the monoclonal anti-Id Abs and evaluate their capacity to exert the same function as native mouse monoclonal abs.

To this end, the mouse monoclonal anti-Id antibody

14C12 was humanized by grafting VH and VL sequences on a human IgG1 Fc chain (14C12 hu), and all assays (non functional, such as binding to insolubilized FVIII, as well as functional) indicated that the capacity to inhibit the corresponding anti-FVIII Ab (Bo2C11) was identical to the mouse monoclonal anti-Ids 14C12.

Assays performed with 14C12 hu showed that the B cell lysis is induced in a dose dependent manner by increasing doses of the chimeric anti-idiotypic antibody.

Accordingly, the amount of anti-idiotypic Abs required to neutralize inhibitors in hemophilia A plasma is comparable with the amount use for the treatment of other diseases by administration of antibodies (e.g., anti-CD20, etc.).

In vivo prediction of the effects of anti-idiotypic antibodies on B cells

In the meantime, to understand and evaluate the mechanism of action *in vivo* of anti-Id Ab on B cells, we have generated transgenic mice by insertion of a B cell antigenic receptor (BCR) of human origin and specific to the FVIII C2 domain (B02C11). A first vector was constructed to encode an artificial antibody made of the Bo2C11 scFv linked to its heavy-chain constant and transmembrane (TM) domains. This was assembled and cloned into a second vector containing an intronic E and 3' regulatory enhancers with a pVH promoter. Transgenesis was carried out by microinjection of the linearized V2-2C11scfv-FC-TM construct into the male pronucleus of inbred C57BL/6J zygotes. Genotyping was performed by PCR across 2C11scfv-Fc-TM cDNA by using specific primers. Transgenic mice BCR-2C11 mice express a significant proportion of transgenic receptors with an apparent physiological distribution, show normal growth and fertility, and a total B cell number similar to that of wild type C57BL/6J mouse. FACS analysis using either labeled donkey IgG to human IgG or an anti-idiotypic antibody to B02C11 showed that 1–3% of peripheral B cells and 15–40% of spleen and bone marrow B cells carried the transgene. FACS analysis show that IV injection of recFVIII or anti-Id Ab increased the number of B Tg BCR-2C11 in the spleen and bone marrow demonstrating the functionality of this model. The transgenic mice should now be immunized with FVIII to understand the mechanism by which anti-FVIII antibodies are produced and how the transgenic B cells are affected in terms of function, phenotype, localization in the body, and fate after administration of anti-Ids antibodies, and finally to set up a protocol of treatment by immunomodulation. To avoid any interference in the interpretation of the mechanism due to the presence of circulation mouse FVIII, we have generated a transgenic-FVIII knocked-out model by cross-breeding the transgene BCR 2C11 with C57Bl6 FVIII knocked-out mice (exon 16). Offspring were selected by genotyping and functional FVIII assay. As for the transgene BCR 2C11, the mouse colony showed normal growth and fertility, and the first BCR analysis demonstrated the functionality of the BO2C11 receptor. Therefore, this model appears as a model of choice for the evaluation of B cell reactivity towards FVIII and clarifies the mechanism by which PUP's hemophiacs develop inhibitors. It will also serve for evaluating cytotoxicity of the corresponding anti-idiotypic antibody on the BCR Tg cells, as well as to determine whether NK cells are recruited. This should lead to establish a first protocol for the suppression and prevention of inhibitor in hemophilia patients.

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Von Willebrand disease

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A B S T R A C T

Von Willebrand Disease (VWD) is a bleeding disorder characterized by reduced plasma von Willebrand factor (VWF) levels (VWD types 1 and 3) or functionally abnormal VWF (VWD type 2). Type 1 VWD is the most frequent form and leads to mild or moderate bleeding tendencies. Low plasma levels in type 1 VWD patients are the result from mutations in the *VWF* gene, leading to decreased synthesis, impaired secretion, increased clearance of VWF, or a combination of these conditions. Several studies have shown that genetic changes within the *VWF* gene are common and are highly penetrant in the more severe type 1 VWD cases. However, in approximately 30 to 40% of the index cases, no (causative) mutations were found, suggesting that other factors outside the *VWF* gene could determine VWF plasma levels. Factors, such as ABO blood group, platelet $\alpha_2\beta_1$ polymorphisms, age, and hormonal alterations, have major influences on VWF levels and/or bleeding phenotype. This illustrates that VWD is a complex multifactorial disease, with inter-relating genetic and environmental components contributing to the variable phenotype of the disease. Despite the growing understanding of the pathophysiology of VWD, the diagnosis is often difficult because of the many factors influencing VWF levels.

Introduction

Von Willebrand factor (VWF) is a glycoprotein circulating in plasma as large multimers. When activated upon vascular damage, VWF serves as an adhesion molecule for platelets thereby initiating platelet plug formation. VWF is also the carrier protein of coagulation factor VIII (FVIII). VWF synthesis is restricted to endothelial cells and megakaryocytes.[1,2] Upon translocation to the endoplasmic reticulum (ER), VWF dimerizes through the formation of C-terminal disulfide bonds at the CK-domains of the VWF monomers.[3] In the ER, N-linked glycosylation is initiated. The dimers are transported to the Golgi apparatus, where the N-linked glycosylation is completed and O-linked sugars are added.[4] In the trans Golgi network, dimers form multimers via N-terminal disulphide bonds at the D'D3 domain and the propeptide is proteolytically removed.[5]

Part of the synthesized VWF multimers is secreted constitutively into the plasma, where it has a variable half-life of about 12 hours. The remaining VWF is stored in cell-specific organelles: the Weibel-Palade bodies (WPB) in endothelial cells or α-granules in megakaryocytes. The highest molecular weight VWF multimers are stored and thus are released at sites of vascular damage in response to secretion stimuli like thrombin, stress, vasopressin, or its synthetic analogue desmopressin (DDAVP).

After secretion into the plasma, the large VWF multimers are proteolytically cleaved by ADAMTS13 (*A D*esintegrin *a*nd *M*etalloproteinase with *T*hrombospondin motifs). The ADAMTS13 cleavage site in the A2 domain is accessible only after partial unfolding of VWF, which is most likely induced by the shear stress exerted on VWF after binding to the endothelial surface.[6] The mechanisms involved in clearance of VWF are not yet fully understood. Recently, it was shown that the Ashwell receptor on hepatocytes is capable of binding VWF that lacks its sialic acid group.[7] It has been shown in mice that macrophages contribute to the cellular uptake of VWF, which was confirmed *in vitro* for primary human macrophages, but until now it is unclear which receptors are involved.[8]

Von Willebrand disease

A decreased concentration or an abnormal function of the VWF protein is responsible for von Willebrand Disease (VWD), the most common inherited bleeding disorder with an estimated prevalence ranging from 3–4 per 100.000 to 1.3% of the population.[9,10] Prevalences based on the number of patients registered at specialized centers are much lower than estimates based on population screenings. Patients registered at hemostasis centers probably have a more symptomatic phenotype, while people identified during screening only have a mild form of the disease.[11,12] VWD is a heterogeneous disorder, which resulted in an initial description of more than 20 subtypes. The current classification merged these to a total of six subgroups (Table 1).[13] A reduced concentration of structurally normal VWF is classified as type 1 VWD. Qualitatively abnormal vari-

Table 1. VWD Classification.

VWD Subtype	Description
Type 1	Partial quantitative deficiency of VWF. Multimers may be abnormal, but the proportion of large multimers is not significantly decreased. Typically autosomal dominant in inheritance although diagnosis is complicated by reduced penetrance and variable expressivity.
Type 2A	Qualitative VWF defect resulting in a reduction of VWF-dependent platelet adhesion. Associated with absence of the largest multimers. Generally autosomal dominant.
Type 2B	Qualitative VWF defect resulting in increased VWF-dependent platelet adhesion. Associated with (usually) reduced high molecular weight multimers and often reduced platelet counts. Inheritance is autosomal dominant.
Type 2M	Qualitative VWF defect associated with specific defects in platelet/VWF interaction but with a normal range of multimers. Inheritance is autosomal dominant.
Type 2N	Qualitative VWF defect resulting from defective VWF binding to FVIII and consequently low levels of circulating FVIII. Inheritance is autosomal recessive.
Type 3	Clinically severe quantitative disorder resulting from a markedly reduced or absent platelet and plasma VWF (less than 5U/dL). Consequently, FVIII activity is also reduced. Inheritance is autosomal recessive.

From the ISTH-SSC VWF Online Database[16] and the latest classification update.[13]

ants of VWF are classified as type 2 VWD (with subgroups 2A, 2B, 2M, and 2N). Complete deficiency of the VWF protein is classified as type 3 VWD.[13] For an update on type 3 VWD, see Leebeek and De Wee.[14]

Treatment of VWD is based on increasing functional plasma VWF levels. This may be achieved by infusion of exogenous VWF concentrate or by releasing endogenous VWF, stored in WPBs, via administration of DDAVP. Therefore, treatment with DDAVP is only useful in type 1 VWD and in some cases of type 2 VWD. Responsiveness to DDAVP varies per person, but is rather stable intra-individually. Normally, it will give a two to four-fold rise in VWF and FVIII levels. Clinical effectiveness to prevent or control bleeding symptoms depends therefore (largely) on the plasma VWF levels achieved after administration, which in turn depends primarily on the basal levels in the patient. DDAVP is mainly effective in patients with baseline VWF ristocetin cofactor activity (VWF:RCo) and FVIII coagulant activity levels higher than 10U/dL.[15]

Type 1 Von Willebrand disease

While type 3 is rare (1 in 1 million), type 1 VWD is the most common form of the disease (approximately 50–75% of all VWD cases). VWD type 1 is characterized by reductions in VWF antigen (VWF:Ag), VWF:RCo, as well as FVIII, resulting in mild to moderate bleeding tendency. The distribution pattern of the VWF multimers is normal. Diagnosis of type 1 VWD can be difficult, especially in cases with mild symptoms, as it is hard to distinguish them from healthy individuals who have VWF levels at the lower end of the normal distribution.

Low plasma levels in these VWD patients result from mutations, leading to decreased synthesis, impaired secretion, increased clearance, or a combination of these conditions. Unlike type 3 patients, who are usually homozygous or compound heterozygous for *VWF* gene mutations in both alleles, type 1 patients usually have a single mutated allele. Since type 1 VWD is a quantitative defect, one would expect that these patients are carriers of type 3 mutations, and that the normal allele accounts for the reduced, but functionally normal VWF levels found in the plasma. However, mutations identified in type 1 VWD are predominantly missense mutations and only 15% of the mutations lead to null alleles.[16] This is in sharp contrast to type 3 VWD, where approximately 85% of the mutations are predicted to result in null alleles. The majority of type 1 VWD patients thus do not appear to be just carriers of type 3 mutations.[17] This difference in the genetic basis between the two quantitative defects is further supported by the fact that the average VWF level in obligatory type 3 carriers is significantly higher compared with obligatory type 1 carriers. This is also reflected by differences in bleeding symptoms: 40% of the type 3 obligatory carriers have at least one bleeding symptom, compared with 89% of the type 1 obligatory carriers.[18]

Three multicenter studies in Europe,[19] Canada,[20] and the UK[21] have recently investigated the molecular pathogenesis of type 1 VWD in over 300 patients. Despite the difference in recruitment criteria between the studies, the seven most common mutations found in Index Cases (IC) were both identified in Europe and Canada (Table 2). These studies further showed that genetic changes within the *VWF* gene are common and are highly penetrant in the more severe type 1 VWD cases. In the European "*M*olecular and *C*linical *M*arkers for the *D*iagnosis and *M*anagement of Type *1 VWD* (MCMDM-1VWD)" study, no mutation was found in 30% (45 out of 150) of the IC. Similar results were found in studies performed in the UK (47%) and Canada (37%).[19–21] The lack of finding a mutation in approximately 35–40% of the IC indicate that other (environmental) factors outside the *VWF* gene could also influence VWF levels in patients diagnosed with VWD type 1. In the next paragraphs, several phenotypes and genotypes of patients diagnosed with type 1 VWD will be discussed to understand the large variability seen in phenotypes of patients with type 1 VWD.

Multimer patterns

Although one of the criteria for diagnosis of type 1 VWD is having a normal multimer pattern, several IC in the multicenter studies showed abnormal multimers in the plasma. In the UK and Canadian studies, individuals with abnormal multimers were excluded. In the MCMDM-1VWD study, 38% (57 out of 150) of the IC had abnormal multimer patterns and may formally not

Table 2. Most common missense mutations found in the three multicenter studies.

Candidate Mutation	UK study (32 IC) n (%)	Canadian study (123 IC) n (%)	European study (150 IC) n (%)	Total (305 IC) n (%)
Y1584C	8 (25)	18 (14.6)	13 (8.7)	39 (12.8)
R1205H	4 (12.5)	3 (2.4)	10 (6.7)	17 (5.6)
R924Q	3 (9.4)	8 (6.5)	4 (2.7)	15 (4.9)
R854Q	–	3 (2.4)	5 (3.3)	8 (2.6)
R2464C	–	3 (2.4)	3 (2)	6 (2)
P1266L	–	4 (3.3)	1 (0.7)	5 (1.6)
P2063S	–	3 (2.4)	1 (0.7)	4 (1.3)
R1315C	–	–	4 (2.7)	4 (1.3)
R1374C	–	–	4 (2.7)	4 (1.3)
V1279I	–	4 (3.3)	–	4 (1.3)
C1130F	–	–	3 (2)	3 (1)
C1130R	–	–	3 (2)	3 (1)
M740I	–	–	3 (2)	3 (1)
N1231T	–	3 (2.4)	–	3 (1)
R1325C	–	3 (2.4)	–	3 (1)
V1229G	–	3 (2.4)	–	3 (1)
C2304Y	–	–	2 (1.3)	2 (0.7)
P1413L	–	1 (0.8)	1 (0.7)	2 (0.7)
G1415D	–	–	2 (1.3)	2 (0.7)
R1374H	–	–	2 (1.3)	2 (0.7)
R1668S	–	2 (1.6)	–	2 (0.7)
Y1146C	–	1 (0.8)	1 (0.7)	2 (0.7)

Listed mutations were found in at least two or more IC, unless the mutation was also found in another study. In bold, the mutations found in at least two out of the three studies. Note that the mutations that were only found in the European Study are all associated with an abnormal multimer pattern (Figure 1). IC with these mutations could therefore have been excluded from the two other studies.

fit into the accepted criteria for type 1 VWD. However, when evaluated against the 2006 classification criteria[13] that state that the proportion of high molecular weight (HMW) multimers should not be decreased significantly, 22 of 57 IC (39%) with abnormal multimers could be reclassified as type 2, whereas 35 of 57 (61%) still fitted classification as type 1 VWD.[22] Furthermore, those abnormal multimers reflect only very subtle abnormalities and are different from the abnormal multimer pattern characteristic for type 2A or 2B VWD. Interestingly, in all those IC with abnormal multimers, mutations were found.[19,23] In contrast, in only 55% of the IC with a normal multimer profile a mutation was identified. All mutations found in the group of abnormal multimers are located carboxyl to the D' domain, except for a single occurrence of the R854Q mutation. None were found in the propeptide region (Figure 1). Three mutations were found in both the normal and abnormal multimer groups (R854Q, R1205H, and C2304Y), indicating that a specific group of mutations is responsible for the abnormal multimer profile (Figure 1).[19] In the Canadian study, recent analysis of multimer patterns using the same technique as in the European study identified abnormal multimers in 39% of the IC, similar to the European study.[24] Several of those mutations leading to abnormal multimer profiles were expressed in COS-7 cells. Mutations located in the D4-CK domain (L2207P, C2257S, G2441C, and C2477Y) all showed marked intracellular retention and impaired secretion of VWF. Also, major loss of the HMW multimers and anodic shifts of multimeric bands was observed in single transfections of the mutants. Cotransfections with wild-type VWF (wt-VWF), mimicking heterozygosity, largely corrected the decrease and anodic shifts.[25] The R2464C and Q2520P mutations, which showed smeary multimer patterns with faster running oligomer bands in patients plasma samples,[22] showed abnormal anodic migration in single transfections. This was shifted towards (nearly) normal in cotransfections with wt-VWF.[25] Also, other mutations, which showed a small loss or a relative decrease of the largest multimers in patients carrying those mutations,[22] have been investigated in expression studies. For example, C1130F/R showed impaired secretion, intracellular retention, and abnormal multimer pattern in cotransfections with wt-VWF.[26,27] In multimer patterns from other patients, the oligomers showed barely visible outer sub-bands with smears around the central band. This pattern is indicative of reduced proteolytic processing by ADAMTS13. Although the differences in multimers are minor, the group of abnormal multimers is clearly distinguishable from the group with normal multimers: the average VWF:Ag level is much lower,[19] linkage to *VWF* is much stronger,[28] and the chance of finding a mutation is much higher in the group with abnormal multimers (100%) than normal multimers (55%).[19,23]

Impaired synthesis and secretion

Decreased levels of VWF can be due to several mechanisms. Decreased synthesis of the VWF protein is the cause for reduced plasma levels in patients carrying a mutation leading to a null allele. In the European cohort, the average VWF levels in eight IC who had a single mutation predicted to lead to a non-expressed allele,

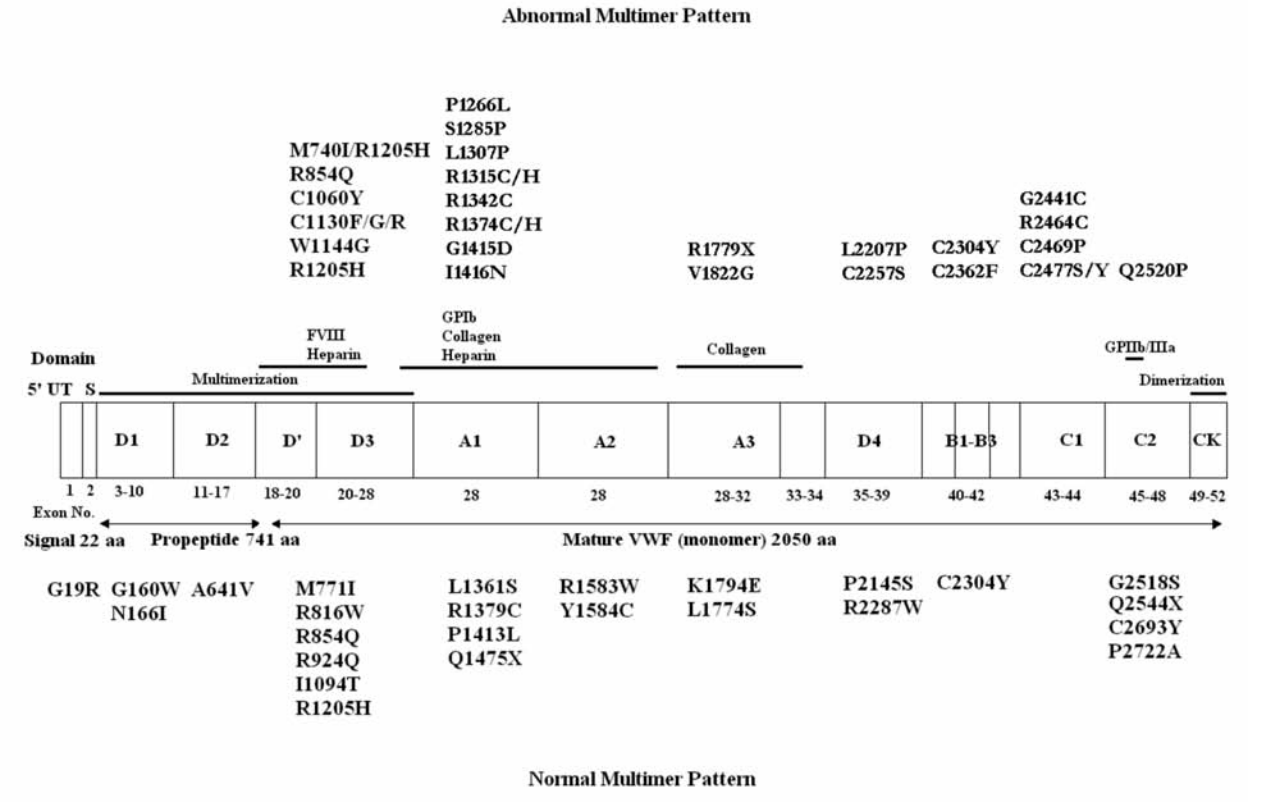

Figure 1. Missense mutations found in index cases with normal and abnormal multimer patterns. The mutations listed are placed at their specific position in the VWF protein. This figure is largely based on the results of the MCMDM-1VWD study, with some additions and deletions due to new insights.[19]

was 39 IU/dL.[19] Impaired secretion and increased intracellular retention of synthesized VWF protein seems to be the major mechanism involved in patients with missense mutations. We have expressed 14 mutations found in the MCMDM-1VWD study to evaluate their contribution to the phenotype observed in the patients. We have established that seven mutants, located throughout the *VWF* gene, contribute to the phenotype on the basis of increased intracellular retention and impaired secretion of VWF compared with wt-VWF, while four mutants are probably causative mutations based on the mild reduction of secreted VWF.[25] Patients with mutations G160W, N166I, or M771I showed a normal VWF propeptide (pp)/VWF:Ag ratio and normal multimer structure. However the VWFpp levels were below the normal range. Reduced levels of VWFpp and VWF:Ag with normal multimer structure suggest that these mutations are responsible for reduced synthesis, rather than increased intracellular retention.[29] Several mutations that were expressed are involved in the loss or gain of a cysteine residue. Four out of five (C2257S, C2304Y, G2441C, and C2477Y) caused marked intracellular retention and loss of HMW multimers in combination with faster migration of multimeric bands. Upon cotransfection with wt-VWF, the defect was largely restored. Tjernberg *et al.*[30] have described similar results for another mutation (C2362F) involving cysteine residues, located in the same region. Although cysteines in these regions are not directly involved in the dimerization and multimerization process of VWF, the mutations do result in slightly abnormal multimer patterns. This is probably due to the fact that the unpaired cysteines lead to changes in the three-dimensional structure.[26,31] Impaired secretion of VWF may also be due to a defect in the WPB formation. Indeed Michaux *et al.* showed that three type 1 VWD mutations had a delay in the formation of WPB and showed a reduction in the length and number of WPB.[32]

Accelerated clearance

Another mechanism that could explain the low VWF plasma levels in type 1 VWD is increased clearance of VWF. Some clearly causative mutations show significantly reduced VWF levels upon expression; however, in cotransfections with wt-VWF, the expression seems normal. This led to the hypothesis that mutations may be involved in faster clearance of the protein. The first report on increased clearance described the rapid disappearance of VWF upon DDAVP infusion in seven patients carrying the combined M740I/R1205H mutations (Type Vicenza).[33] Haberichter *et al.*[29] identified seven patients with reduced VWF survival predicted by a markedly increased VWFpp/VWF:Ag ratio. In all these seven patients, mutations were identified that have been previously reported with reduced VWF survival, including R1205H, C1130G/F/R, and W1144G. The

majority of the cases with decreased VWF survival is characterized by significantly reduced steady-state VWF:Ag levels, usually below 30 IU/dl.[34,35] The half-life of VWF:Ag is also markedly reduced after DDAVP infusion.

The possible contribution of ADAMTS13 proteolysis to the rapid clearance has been postulated. Indeed one mutation, the Y1584C, seems to be linked with increased susceptibility of VWF to ADAMTS13 proteolysis.[36] But detailed analysis of mutations C1130F and C1149R revealed that increased clearance in these cases was not due to increased proteolysis by ADAMTS13.[34] Sialic acid residues on the VWF glycans are known to promote ADAMTS13-mediated proteolysis and also seem to play a role in the clearance of the protein. Asialo-VWF is cleared more rapidly than sialylated VWF in animal models.[37] The Ashwell receptor, an asialoglycoprotein receptor on hepatocytes, is able to bind asialo-VWF, thereby removing it from the circulation. Recent data suggest that the receptor might also be able to bind sialylated glycoproteins,[38] but whether this is the case for VWF remains unknown. The VWFpp/VWF:Ag ratio measured in healthy blood group O subjects indicate that they have a faster VWF clearance than non-O subjects.[39] This suggests that changes in carbohydrate structure of VWF may affect clearance and therefore VWF levels. For the clinical practice, increased clearance might be an important phenotype to be considered. Insight into the survival of VWF after DDAVP infusion is relevant for the treatment of type 1 VWD patients. If the half-life of endogenous circulating VWF after DDAVP treatment is too short, it might be preferable to treat the patient with a VWF concentrate instead of DDAVP infusion.

Factors outside the *VWF* gene

The lack of finding a mutation in approximately 35–40% of the IC in the multicenter studies indicates that also other (environmental) factors outside the *VWF* gene could influence VWF levels in patients diagnosed with VWD type 1. Also the variability of responses to DDAVP treatment between patients with the same mutation suggests that other factors contribute to the VWF levels.[40] This notion is also reflected in the 2006 VWD classification that no longer restricts the diagnosis VWD to mutations in the *VWF* gene.[13]

Blood groups

It has been shown that 66% of the variation in VWF levels is genetically determined and that the ABO blood group locus accounts for 30% of that variation.[41] VWF is one of the few plasma proteins that contain the ABO blood group sugars. The ABH antigens are only attached to the N-linked oligosaccharide chains of VWF. Almost 30 years ago, it was recognized that the ABO blood groups are associated with VWF plasma levels,[42] and this has been confirmed in several other studies.[43] Individuals with blood group O have approximately 25% lower VWF plasma levels compared with non-O individuals. Furthermore, blood group O is overrepresented among VWD type 1 patients. The mechanisms how ABO blood groups are responsible for VWF levels remain unknown. A direct mechanism seems likely as individuals with the Bombay blood group phenotype, who lack expression of the ABH antigens, even have lower plasma levels than individuals with blood group O.[44] Furthermore, the VWF levels correlate with the amounts of A and B antigens present on the VWF molecule.[45,46] The ABH antigens may have an effect on biosynthesis, secretion, clearance of VWF, or a combination thereof. The ABH antigens on VWF do not seem to play a role in synthesis and secretion as the plasma propeptide levels are independent of the ABO blood group.[47] Platelet VWF levels are independent of ABO blood group, which is probably explained by the fact that platelet VWF does not express the ABH antigens.[48] Another study showed that the rise in VWF level after DDAVP treatment appears to be similar between patients with type 1 VWD with different blood groups, suggesting that secretion efficiency is not affected by the blood group antigens.[49] Nossent *et al*.[47] estimated that the half-life of VWF in controls with blood group O is almost 2 hours shorter compared with non-O blood group controls, and it was shown that in healthy individuals, the VWF half-life after DDAVP administration was shorter in blood group O individuals.[39] Furthermore, the ratio between propeptide and mature VWF, which are released in an equimolar ratio, is increased in individuals that have blood group O compared with non-O individuals.[35,47] These observations suggest that the ABH antigens on VWF determine its clearance rate, where blood group O-VWF might be cleared faster compared with non-O VWF. However, this supposition is not yet challenged in a direct comparison, nor have the molecular mechanisms been elucidated. One *VWF* mutation seems to be linked with the ABO blood group. In the UK study, the Y1584C mutation was found together with blood group O in 95% of the VWD cases,[21] and in another study, 11 out of the 12 IC had blood group O,[50] a higher proportion than the overall 77% prevalence of group O reported among type 1 VWD.[51]

Age

In preterm neonates, the VWF levels are moderately low, but at birth, the levels rise above adult norms. Stress in the neonate during the delivery might be the cause of this rise, since it is known that stress or exercise can induce significant release of VWF.[52] Several other reports have shown that with age, the VWF levels slowly increase, with a rate of approximately 10 IU/dl per decade.[51,53] This increase during life has an influence on the diagnosis in relation to age. Diagnosis of VWD at a young age might be questioned later in life.

Genetic modifiers

Several studies have shown that locus heterogeneity may play a role in the severity of the VWD type 1 phenotype. Among type 1 VWD patients, the frequency of the integrin $\alpha_2\beta_1$ 807C allele, which is associated with low receptor density, was found significantly higher than in the normal population.[54] Low density of the integrin $\alpha_2\beta_1$ might result in less efficient binding of platelets to collagen and may explain the variability between bleeding symptoms in patients with the same VWF levels. Indeed Kunicki *et al*.[55] found that this haplotype was

associated with an increased bleeding score. The integrin α_{IIb} haplotype 1 was also associated with an increased bleeding score.[55] Further evidence of locus heterogeneity was provided by Daly *et al*.[56] who identified a heterozygous mutation in the $P2Y_{12}$ ADP receptor gene in a patient with mild type 1 VWD and a *VWF* defect. Platelets from this patient showed reduced and reversible aggregation in response to ADP. So the defect in the $P2Y_{12}$ gene could contribute to the bleeding tendency in type 1 VWD patients.[56] We have found a polymorphism in the arginine vasopressin 2 receptor (V2R) gene, which was associated with higher VWF propeptide, VWF, and FVIII levels.[57] Some modifier genes for plasma VWF levels have been identified in mice, but human homologues remain to be identified.[58,59]

Hormonal influences

Data obtained from several studies on the relationship between menstrual cycle and VWF level are contradictory. One study showed no change in VWF:Ag and VWF:RCo levels during the normal menstrual cycle.[60] A similar study, however, found lower levels for both parameters in the follicular phase,[53] while another study showed significantly lower levels of VWF:Ag during menses compared with the follicular phase.[61] Women investigated in these studies were healthy women; no data are available about the possible change in VWF levels during the menstrual cycle in VWD type 1 patients. The use of oral contraceptives causes a small rise in levels of both VWF:Ag en VWF:RCo, but again no data are available for women who have VWD.[62] During pregnancy, the level of VWF increases three to five-fold in women without VWD, and in most women with VWD type 1, the levels will reach normal range at the end of pregnancy. Castaman *et al*.[63] studied 23 women with VWD during pregnancy. Women with R854Q, R1374H, V1665E, V1822G, and C2362F mutations showed complete normalization of VWF levels during their pregnancy. Women with R1205H and C1130F mutations had only a slight increase of VWF during pregnancy.[63] This correction during pregnancy might have clinical consequences for the therapy, although postpartum hemorrhage may occur due to a rapid fall of VWF after delivery.[64]

Conclusions

In the large multicenter studies on type 1 VWD, many mutations were found, located throughout the entire *VWF* gene. The majority of these mutations are missense mutations and only a few IC had mutations that were predicted to lead to null alleles. An interesting finding in the two large European and Canadian cohorts was the fact that although VWD type 1 is a quantitative defect, some patients showed slightly abnormal multimer patterns. This observation asks for reconsideration of the criteria for VWD type 1. The mechanisms by which these mutations cause the observed clinical manifestations are slowly revealed. For some mutations, it has been shown in expression studies that they cause impaired secretion by intracellular retention or degradation. This seems to be the more general mechanism for the reduced levels found in the plasma of type 1 VWD patients with missense mutations. However, several mutations were found to (also) have an association with increased clearance, which may have therapeutic implications.

Besides mutations in VWF, other factors influence VWF levels or bleeding phenotype. The ABO blood group is a major determinant of plasma levels. Blood group O individuals have approximately 25% lower levels and are overrepresented among patients with type 1 VWD. Other environmental factors, such as age, stress, and hormonal influences also alter plasma levels, making the diagnosis of type 1 VWD even more complicated. Recently, some polymorphisms in platelet integrins were found to be associated with increased severity bleeding scores and a polymorphism in the V2R is associated with VWF levels. All these genetic variants, along with genetic modifiers that have yet to be identified, show that VWD type 1 is a multifactorial disorder, where all these factors contribute to the highly variable genotype and phenotype of this bleeding disorder. With all the knowledge now available on the variety of mutations found in type 1 VWD, the question arises whether we should screen patients with possible VWD for *VWF* gene mutations (for a recent debate see Peake[65] and Favaloro[66]). When laboratory testing shows abnormal multimer patterns, screening for mutations seems rather pointless, because a mutation will be found in 100% of the cases, as evidenced in the MCMDM-1VWD study. Finding a mutation in these cases will not add any information that will change the diagnosis or treatment of the patient. One could argue that screening may be useful in cases where the mutation leads to accelerated clearance, for example, the R1205H and C1130F mutations, since this might change the therapy in case of bleedings. But genetic testing is time-consuming and costly, while more accurate information on clearance will be available from a DDAVP test infusion when blood samples are taken at later time points. And, as shown in the multicenter studies, genetic testing will fail to find a causative mutation in approximately one-third of the patients. As discussed earlier, several other factors beside a *VWF* gene mutation can influence plasma levels and ultimately the clinical manifestation. So this only leaves a very small group of patients with type 1 VWD where screening for a mutation might be more informative. For clinical purposes, therefore, genetic testing in type 1 VWD seems for now unwarranted.

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New treatments in hemophilia

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A B S T R A C T

There are several challenges in the treatment of hemophilia, and prophylaxis is costly and performed using frequent intravenous injections. This is cumbersome for the patients, and venous access is difficult to achieve, especially in small children, where ports sometimes need to be implanted. The development of an inhibitor jeopardizes the health of the patient, and the treatment of acute bleeds is still not satisfactorily in all patients. Prophylaxis in inhibitor patients is also a possibility that needs to be better addressed. To improve future treatment, new products are being developed, some of which already are in clinical trials. There are several approaches to prolong half-lives, such as PEGylation and Fc fusion for FVIII, and FIX and albumin fusion for FVII and FIX. Increased activity has been demonstrated in preclinical trials for FIX and in a human trial with FVII, where the molecules have gained increased activity by manipulation of the molecular composition. Additional approaches, including gene therapy, are also being studied. Results so far are promising, and there is great hope that hemophilia treatment in the near future will make substantial progress.

Introduction

Hemophilia A and B are hereditary X-chromosomal recessive disorders caused by deficiency or absence of coagulation factors VIII (FVIII) or IX (FIX), respectively. The disorders are classified according to the coagulation factor activity (FVIII:C or FIX:C, respectively) present in blood with three categories comprised of severe (<1% of normal activity), moderate (1–5%), or mild (>5–<40%).[1] Although these categories define overall bleeding manifestations, clinical phenotype may vary within each group. Hemophilia A is four-fold more common than hemophilia B, with about 35% affected with the severe form of the disease, 15% moderate, and 55% mild, according to data from Sweden.[2] Mild hemophilia may go unrecognized based upon the level of deficiency and the stressors the affected individual may have experienced to unmask the disorder, for example, the proportion of mild hemophilia represented in different countries or registries may vary.[2,3] The incidence and prevalence of hemophilia are commonly reported as 1 in 5,000 males or 1 in 10,000 of the general population. Treatment of hemophilia is based on replacing the missing factor with concentrates containing FVIII or FIX. Mild hemophilia A can usually be treated with desmopressin.[4] Long-term prophylaxis is becoming widely used in severe forms of hemophilia.[5]

Current treatment

To understand the driving force behind development of new treatments in hemophilia, it is important to have knowledge about current treatments.

Prophylaxis

Prophylaxis is becoming more commonly utilized in countries that can support the associated medical costs and in those who give priority to hemophilia care. Prophylactic treatment began on a broader scale in Sweden during the 1950s, with a report on 25 years of follow-up encompassing 60 patients published in 1992.[6] In this cohort, the results seemed very promising but only historical controls were available for comparison. The Netherlands also have a long history of use of prophylactic regimens.[7-9] The Dutch strategy has differed from the Swedish, most notably with a later start (at older age with a history of several joint bleeds), lower dosing, and longer dose intervals, resulting in less costly regimens due to direct concentrate consumption.[10] Whereas the Swedish regimen has been more fixed, aiming at a trough level of 1% or greater, the Dutch regimen has been more tailored to the individual bleeding phenotype. The trend in treatment in Europe has been towards primary prophylaxis.[11] It appears that early start is important as it appears to be an independent predictor of future joint disease.[12] The challenge with early initiation at the age of 1–2 years remains one of venous access, often requiring the use of a central venous catheter.[13-16] Several authors have recommended beginning therapy with once weekly infusion as a method to decrease use of these devices. Issues with venous access and substantial variability of clinical phenotype observed in severe hemophilia[17-19] with the resultant wide variation of age at first hemarthroses, are raised as contributing to overtreatment of or unnecessary invasive procedures in some patients whose prophylaxis is started early before the first joint

bleed. These issues are especially important from an economical and societal perspective. In clinical practice, most prophylactic regimens are tailored to the need of the individual patient. Methods to develop individual tailored regimens have included individual pharmacokinetic data and computer simulated dose level and interval to achieve a predetermined trough level.[20,21] In both hemophilia A and B, this has been accomplished with verification of theoretical data through measurement of actual FVIII/IX levels. Decreasing intervals between prophylactic doses from 2–3 infusions weekly to every other day theoretically reduces average FVIII consumption by 43% with maintained or increased trough FVIII levels, while daily dosing would reduce mean FVIII usage by 82%. In Figure 1, the pharmacokinetic profiles of typical calculations in a patient are shown. A modified dosage regimen with infusions every other day, were implemented in a group of Swedish patients with obtained data supporting the pharmacokinetic models.[21] The feasibility of this method to maintain desired trough levels with an associated decrease in FVIII consumption was confirmed. Therefore, coagulation factor dosing based on pharmacokinetic principles results in more cost-effective utilization of expensive medical resources.

Inhibitors

Inhibitors develop in approximately 30% of patients with severe hemophilia A and are less common, approximately 5% in those with severe hemophilia B;[22] a broad range of inhibitor incidence/prevalence has been reported among different studies. A variety of factors may impact inhibitor development, including but not limited to type of hemophilia, level of deficiency, specific genetic mutation, race, immune response genes, and environmental influences. Among environmental issues are included the type of clotting factor replacement therapy utilized, such as plasma derived versus recombinant, intermediate versus high purity products, treatment regimen (prophylaxis vs. on-demand), age at start of treatment, and so on.[23-25]

During recent years, there has been a vigorous discussion as to whether recombinant products are more immunogenic than plasma-derived intermediate purity products especially those with intact von Willebrand factor (VWF).[26-30] Despite this active debate representing quite different opinions, present studies reflect a low scientific evidence level as no prospective randomized study has ever been performed addressing this issue. Based upon this, a recent European consensus report stated that there is no difference in risk of inhibitor development between different concentrate types. Hopefully, the question regarding type of concentrate and rate of inhibitor development will be finally settled by the SIPPET study, where classes of products are compared in a randomized prospective fashion.[31]

Issues with current treatments

The main issues can be referred to prophylaxis and to inhibitor risk and treatment:

- Factor concentrates are distributed by intravenous injections and, as with prophylaxis, this may become a burden that negatively impacts quality of life. As prophylaxis should be started early,[12] small children often are exposed and are in need of ports to get venous access. This means a surgical procedure early in life with its risks. Also, surgical procedures have been suggested to be a risk factor for inhibitor development.[32] Novel products with longer biological half-life have a potential to increase convenience and reduce the need of port implantations. Such products are now in clinical trials.

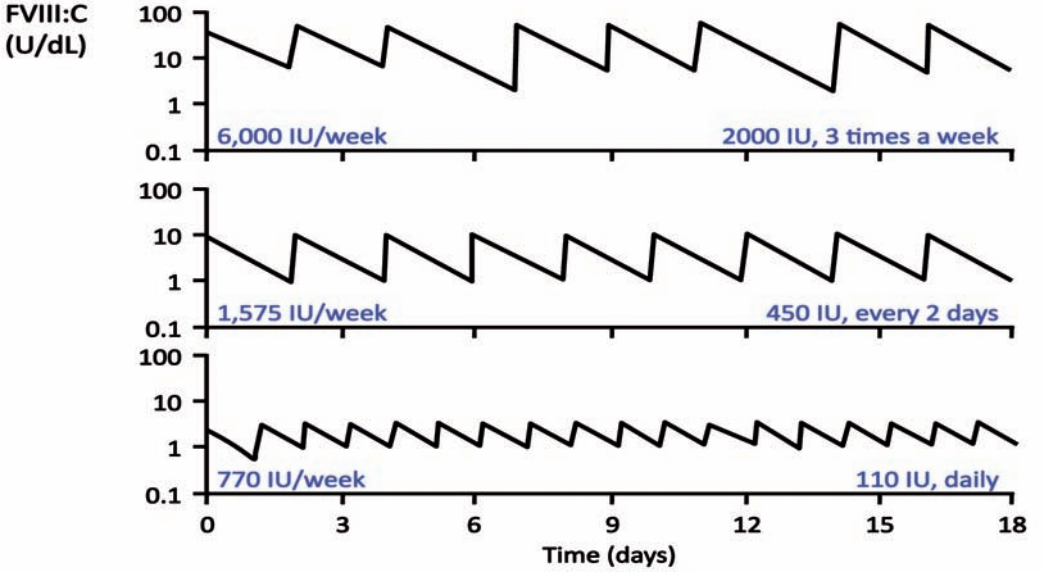

Figure 1. Computer modeling of pharmacokinetic dosing where PK parameters for the patient are known. Dosing schedules were modeled to have similar trough levels. Shortening the intervals will dramatically reduce factor consumption, but also peak levels. The clinical impact of the different schedules remains to be evaluated. The standard dose for this patient is 2000 units 3 times per week (Adopted from ref.[21]).

- Inhibitor risk is strongly associated to genetic factors,[22] especially the disease causative mutation.[33] This means that treatment with FVIII in a FVIII deficient patient having a so called high-risk mutation renders a substantial risk of developing an inhibitor. A logic rationale would be to treat such individuals with products not recognized as FVIII by the immune system.
- Patients having high inhibitor titers cannot be treated using replacement therapy. Therefore, bypass products have been used since decades. The currently available products are recombinant activated factor VII (FVIIa) and activated prothrombin complex concentrate (aPCC). These drugs are very different in terms of content and mode of action, but seem to have rather similar overall efficacy with regard to treatment of acute bleeds[34] with efficacy rates in the order of 80–90%,[35] leaving a number of patients without effective treatment. Obviously there is a need for more efficacious products that can promote hemostasis in a more secure way when an inhibitor is present.

Novel products to treat hemophilia

The techniques to develop products are summarized in Table 1, and use in humans indicated. Study identifications given in the text have been obtained from www.clinicaltrials.gov.

PEGylation is an established method for prolonging the half-life of protein products and it has successfully been used in several therapeutic proteins.[36,37] The underlying mechanism of the effect of the PEGylation is not completely understood. The volume of PEGylated proteins is increased, leading to reduced renal clearance, and shielding effects may diminish receptor mediated clearance, susceptibility to proteolytic degradation and reduction in immunogenicity.[38] A long-acting factor VIII (FVIII) as a replacement therapy for hemophilia A would significantly improve treatment options for patients with hemophilia A. Variants have been engineered with introduced surface-exposed cysteines to which a polyethylene glycol (PEG) polymer was specifically conjugated.[39] Screening of variant expression level, PEGylation yield, and functional assays identified several conjugates retaining full *in vitro* coagulation activity and VWF binding. PEGylated FVIII variants exhibited improved pharmacokinetics in hemophilic mice and rabbits. In addition, pharmacokinetic studies in VWF knockout mice indicated that larger molecular weight PEG may substitute for VWF in protecting PEGylated FVIII from clearance *in vivo*. In bleeding models of hemophilic mice, PEGylated FVIII not only exhibited prolonged efficacy consistent with the improved pharmacokinetics but also showed efficacy in stopping acute bleeds comparable with that of unmodified rFVIII.

Clinical trials with PEGylated FVIII and FIX have been initiated but so far, virtually no clinical data in humans have been published in detail. An exception is initial trials with PEGylated liposomes.[39-42]

Liposomes can be efficacious vehicles for medicines, and surface modification by PEGylation can prolong liposome circulation time. When reconstituted with PEGylated liposomes (PEGLips), recombinant FVIII binds noncovalently but with high affinity to the external liposome surface. This preparation showed prolongation of FVIII half-life and increased protection from bleeding in preclinical models. This product (KG-Lip) has been further evaluated in a large clinical trial, the Liplong study, where once-a-week treatment with the product was compared with Kogenate FS Bayer (Ingerslev, personal communication, 2010). A total of 139 patients were enrolled. The study was stopped because the KG-Lip study product failed to meet the endpoints and was inferior to the comparative product given three times per week.

PEG has also been selectively attached to N-glycans of the activation peptide of FIX, and a prolonged half-life was shown in cynomolgus monkeys and mice with

Table 1.

Techniques used to alter function or increase production	Product	Results in humans published
PEGylation	FVIII FIX	No. Clinical trial ongoing No. Clinical trial ongoing
PEGylated liposomes	FVIII	Yes, refs.[38,39]
Polysialic acid	FVIII	No
Albumin fusion	FVIIa FIX	No No
Fc fusion	FVIII FIX	No. Clinical trial ongoing No. Clinical trial ongoing
Increased catalytic activity	FVIIa	Yes, ref.[52]
Gene therapy	FVIII FIX	Yes, reviewed in[53]
Read-through of premature stop codons	Hemophilia A or B	No. Clinical trial ongoing
Transgenic animals	FVIII FIX	No
Chloroplast transgenic tobacco plants	FIX	No

restored function.[43,44] A phase I trial has been completed with this compound (NCT00956345, Novo Nordisk).

Polysialic acid

Polysialic acid (PSA) is an anionic moiety that adds multiple negative charges to the protein thereby changing its surface charge and binding capabilities. PSA is thought to interfere with receptor-mediated clearance processes of FVIII as a result of these changes.[45] The compound is under preclinical evaluation.

Albumin fusion

Albumin has a long half-life which exceeds 20 hours.[46] As albumin is a product with a long safety record and does not seem to be immunogenic, it would be an option for extension of clotting factor half-life using genetic fusion. Weimer *et al.*[47] reported the generation of a recombinant FVIIa molecule with an extended half-life based on genetic fusion to human albumin. The recombinant FVII albumin fusion protein (rVII-FP) was expressed in mammalian cells and upon activation, displayed a FVII activity close to that of wild type FVIIa. Pharmacokinetic studies in rats demonstrated that the half-life of the activated recombinant FVII albumin fusion protein (rVIIa-FP) was extended six- to seven-fold compared with wild type rFVIIa. The *in-vitro* and *in-vivo* efficacy was evaluated and was found to be comparable with a commercially available rFVIIa (NovoSeven, Novo Nordisk). The results of this study demonstrated that it is feasible to develop a half-life extended FVIIa molecule with haemostatic properties very similar to the wild-type factor. Albumin has also been fused to FIX and a phase I trial has started (NCT01233440,CSL Behring) but no results are yet available.

Fc fusion

The neonatal Fc receptor (FcRn) is a MHC class I like molecule that functions to protect IgG and albumin from catabolism, mediates transport of IgG across epithelial cells, and is involved in antigen presentation by professional antigen presenting cells. Its function is evident in early life in the transport of IgG from mother to fetus and neonate for passive immunity, and later in the development of adaptive immunity and other functions throughout life. The unique ability of this receptor to prolong the half-life of IgG and albumin has guided engineering of novel therapeutics.[48] Peters *et al.*[49] have summarized studies where Fc has been fused to FIX. Taken together, these studies showed the enhanced pharmacodynamic and pharmacokinetic properties of the rFIXFc fusion protein and provided the basis for evaluating rFIXFc in patients with hemophilia B.

A recombinant fusion protein (rFIXFc) containing a single FIX molecule attached to the Fc region of immunoglobulin G was administered intravenously and found to have an extended half-life, compared with recombinant FIX (rFIX) in normal mice, rats, monkeys, and FIX-deficient mice and dogs. The half-life of rFIXFc was approximately three- to four-fold longer than that of rFIX in all species. In contrast, in mice in which the neonatal Fc receptor (FcRn) was deleted, the half-life of rFIXFc was similar to rFIX, confirming the increased circulatory time was due to protection of the rFIXFc via the Fc/FcRn interaction. Whole blood clotting time in FIX-deficient mice was corrected through 144 hours for rFIXFc, compared with 72 hours for rFIX; similar results were observed in FIX-deficient dogs. The fusion of Fc and FVIII has also been obtained with similar beneficial results although full publications are still not present. The phase I trial has been completed for FIX

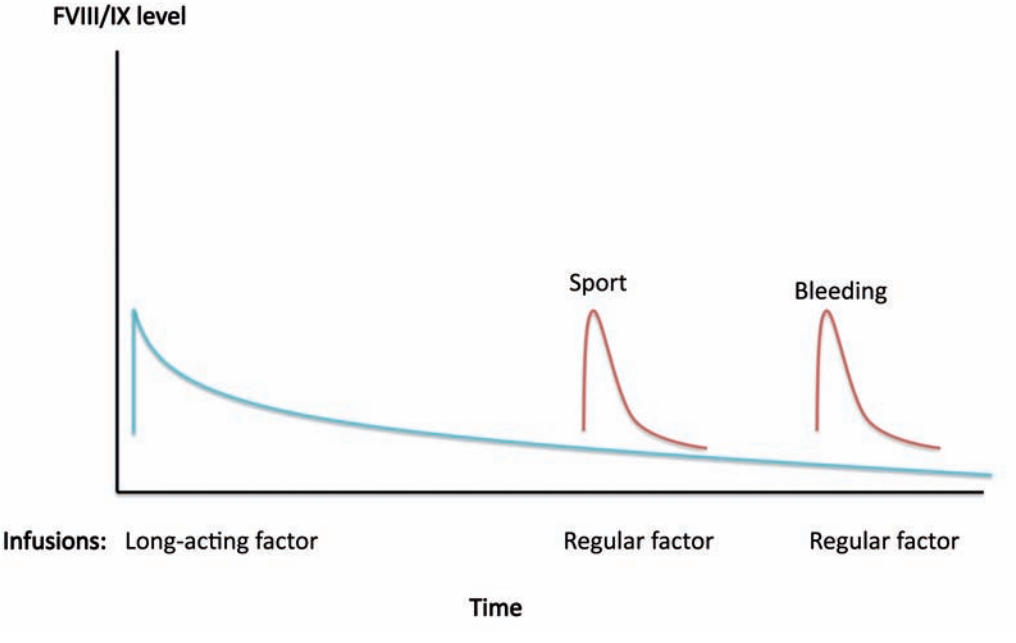

Figure 2. Hypothetical plasma coagulation levels during treatment with long-acting clotting factor concentrates. Extradoses of regular concentrate are given in anticipation of bleeds or in case of a bleeding event.

(NCT00716716, Biogen Idec) and recruitment in the FVIII phase I trial seems completed (NCT01027377, Biogen Idec). Clinical trials (phase II/III) in patients with hemophilia A and B have started and are recruiting patients.

Increased activity of molecules

It has been possible to increase the catalytic activity of FIX using different techniques: replacing the EGF-like domain of FIX with that of FVII[50] or changing residue 338 in human FIX from arginine to alanine.[51] Intensifying the catalytic activity has an obvious potential to construct a drug, which is more efficacious for treatment of hemophilia B but so far the concept has not proceeded into trials in humans. For FVII, the progress has come further, although much information is only available in abstract form, and clinical trials are ongoing or planned. Bayer Healthcare, as well as NovoNordisk, has developed FVIIa molecules with increased activity compared with the currently available product (Novoseven, NovoNordisk). The fast acting rFVIIa analog (NN1731, NovoNordisk) has three amino-acid substitutions that stabilize the molecule in its active conformation in the absence of tissue factor. Preclinical data indicate that compared with rFVIIa, the analog has a more rapid onset of action, forms a stronger clot that is more resistant to fibrinolysis, and exhibits an improved therapeutic window.[52-54] The analog was compared with other compounds in a severe tail-bleeding model in hemophilia A mice, and demonstrated significantly greater efficacy than rFVIIa, plasma-derived activated prothrombin complex concentrate, and FVIII (pd-aPCC, FEIBA or FVIII (Refacto). Assessment of the blood loss over time showed that NN1731 significantly and dose-dependently reduced the blood loss in the first 5-minute observation period, whereas the effect of rFVIIa, FVIII and pd-aPCC first became evident 5–10 minutes after injury.[54] The product has been administered to healthy subjects.[55] Eight subjects were randomized to either NN1731 (n = 6) or placebo (n = 2) in each tier. No thromboembolic or serious adverse events were reported and no antibody formation towards NN1731 was detected. NN1731 was demonstrated to be pharmacologically active based on coagulation-related parameters (prothrombin fragment 1+2, activated partial thromboplastin time, and prothrombin time).

Results of clinical trials need to be awaited until the future of these products can be evaluated. There is a need for more efficacious products to treat bleeds in inhibitor patients and perhaps also for prophylaxis. The safety aspects are important and the potential of an increased risk of thromboembolism needs to be carefully evaluated.

Other approaches

Five gene transfer phase I clinical trials have been conducted using either direct *in vivo* gene delivery with viral vectors or *ex vivo* plasmid transfections and reimplantation of gene-engineered cells.[56] Although there was evidence of gene transfer and therapeutic effects in some of these trials, stable expression of therapeutic factor VIII or FIX levels has not yet been obtained. Further improvements of the vectors and a better understanding of the immune consequences of gene transfer are warranted, as new trials are being initiated. As some of the mutations causing hemophilia are nonsense, changes in small molecules have been developed that can read-through premature stop codons. Ataluren (PTC124) is an orally delivered, investigational drug that acts to overcome the effects of the premature stop codon, potentially enabling the production of functional FVIII/FIX (NCT00947193, PTC Therapeutics). Another approach is to develop pharmaceuticals using transgenic animals and plants. Both FVIII and FIX have been produced in transgenic pigs as bioreactors[57,58] and FIX in chlorplast transgenic tobacco plants.[59]

Concluding remarks

The possibility to improve prophylaxis with long-acting products is obvious. A concern that could be raised is that with once a week dosing the factor levels in plasma, although measurable, will be rather low during a substantial number of hours. Given the broad variation in clinical phenotype, some patients will bleed with FVIII/IX levels, which exceed those obtained with the new products during a substantial time each week.[60] This fear is even more relevant if the patient is physically active, doing sports, and so on. Therefore, extra doses of concentrate may be needed prior to activity (as exemplified in Figure 2). This could be an extra dose with a product with shorter half-life and the treatment idea approaches that for insulin treatment.

The advent of more potent by-pass products not only opens up the possibility of treating acute bleeds better, but also to implement efficacious prophylaxis to inhibitor patient. As inhibitors develop, early in life institution of effective prophylaxis could hypothetically be a reality prior to start of joint disease, as is the case in non-inhibitor children, that is, as primary prophylaxis.

Development of an inhibitor is a major concern and threat to the health of the person with hemophilia. If reliable scores for inhibitor risk could be developed, new products could hypothetically be used to avoid challenge of the immune system in such patients. An example would be to give biosuperior FVIIa to patients with hemophilia A and a very high risk score for developing an inhibitor. The future will tell if such approaches will become a reality.

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Mechanisms of leukemia cell trafficking, homing, and tissue retention in chronic lymphocytic leukemia

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A B S T R A C T

Leukemia cell trafficking and homing is a complex, highly regulated process with emerging clinical and therapeutic relevance. Chemokine receptors and adhesion molecules expressed on Chronic Lymphocytic Leukemia (CLL) cells navigate leukemia cell traffic between secondary lymphoid organs, blood, and the bone marrow, and position and retain leukemia cells within marrow and lymphoid tissue sub compartments. In response to signals from the leukemia microenvironment, such as B cell receptor engagement, CLL cells also secrete chemokines, presumably to attract T cells and other immune cells for cognate interactions. CXCR4 is the most prominent chemokine receptor in CLL and targeted in a first clinical trial. Specific inhibitors of the spleen tyrosine kinase (Syk), Bruton's tyrosine kinase (Btk), and the PI3 kinase isoform delta are in early clinical development in CLL, and characteristically cause a "compartment shift" of CLL cells from the tissues into the peripheral blood. Clinically, this is associated with a transient surge in lymphocyte counts and lymph node shrinkage during the first weeks of treatment. These remarkable effects are thought to be due to inactivation of chemokine receptors and adhesion molecules, thereby antagonizing tissue retention and causing CLL cell mobilization. These findings emphasize that CLL cell trafficking and homing has become a highly dynamic, therapeutically relevant research field.

Tissue microenvironments: fatal attraction between CLL and stromal cells

Chronic Lymphocytic Leukemia (CLL) cells relentlessly accumulate *in vivo*, but rapidly undergo spontaneous apoptosis *in vitro,* implying that their survival and expansion depend upon external signals from the tissue microenvironments.[1–3] This is similar to normal B-cells, which also are selected and expanded within the bone marrow (BM) and secondary lymphatic tissues in response to external signals,[4,5] transmitted by accessory cells, such as T cells, antigen (Ag)-presenting cells (APC), and mesenchymal cells collectively referred to as "stromal cells". Initial studies in CLL demonstrated the protective effect of unselected BM stromal cells,[6,7] and subsequent studies defined that mesenchymal marrow stromal cells (MSC),[7–9] monocyte-derived nurselike cells (NLC),[8] and follicular dendritic cells (FDC)[10] can protect CLL cells from spontaneous and drug-induced apoptosis.[9]

In vitro[8,11] and *in vivo*[12] leukemia cells are attracted to stromal cells, and the protective effects of stromal cells requires the close proximity between CLL and the stromal counterparts.[7–9,11] The high affinity of CLL cells for stromal cells is exemplified by a striking *in vitro* phenomenon termed pseudoemperipolesis.[11] Pseudoemperipolesis describes the spontaneous migration of a fraction of CLL cells beneath MSC, which occurs within a few hours of co-culture. In phase contrast microscopy, pseudoemperipolesis is characterized by the dark appearance of lymphocytes that migrated into the same focal plane as the stromal cells. Generally, the term pseudoemperipolesis is used to describe symbiotic complexes of leukemia cells with their stromal cell component.[13,14] During this cell interaction, leukemia cells migrate beneath the adherent cells or are trapped by membrane projections, but do not become internalized by the stromal cells.

Pseudofollicular structures, called proliferation centers or pseudofollicles, are a hallmark feature in CLL histopathology, and thought to be the main areas of leukemia cell proliferation. Proliferation centers are clusters of larger, oftentimes dividing CLL cells intermingled with accessory cells.[2,15,16] The cellular composition of proliferation centers remains incompletely defined but likely includes APC, mesenchymal stromal cells,[17] and numerous, mostly $CD4^+$ T-cells,[18–20] suggesting that some of the cellular interactions required for the expansion of Ag-specific normal B-cells within germinal centers (GC) may also be functional in CLL.

At this point, we can conclude that in the tissue compartments (BM, secondary lymphoid tissues) CLL cells engage in complex cellular and molecular interactions with accessory cells that collectively are referred to as the leukemia microenvironment. These interactions, which still remain incompletely understood, shape the unique microanatomy in CLL and are responsible for survival, expansion, and protection of the CLL cells from cytotoxic drugs. These findings also imply that mobilization of CLL cells from

the tissues into the blood would remove CLL cells from supportive stromal cells and make them more vulnerable to conventional drugs. This approach is particularly attractive in CLL, given that conventional, chemotherapy- and antibody-based therapies are not curative, presumably because residual CLL cells survive in protective tissue niches and ultimately pave the way to relapses.

Basic mechanism of normal B cell and CLL cell migration and adhesion

Normal B cell trafficking and function largely depends upon interactions between B cells and accessory stromal cells.[21,22] For example, stromal cells in secondary lymphatic tissues constitutively express chemokines, such as CXCL12 and CXCL13 that provide guidance for B cell positioning within distinct lymph node compartments.[21,23–25] According to the multistep paradigm, initially proposed by T. Springer,[26] lymphocyte trafficking and homing require the cooperation between chemokine receptors and adhesion molecules, such as integrins, CD44, and L-selectins, which are expressed on normal and malignant lymphocytes. Lymphocytes actively enter and home within tissue microenvironments, such as the secondary lymphatic tissues, where stromal cell networks provide guidance cues by secreting chemokines, establishing chemokine gradients, and expressing ligands for lymphocyte adhesion molecules (Figure 1). Coordinated lymphocyte entry, migration, and territoriality are essential during immune surveillance and induction of specific immune responses.[23,27–29] In B cell lymphomas/leukemias, the neoplastic B cells largely retain the capacity of their normal counterparts for trafficking and homing, as demonstrated in CLL and B cell acute lymphoblastic leukemia (ALL), both *in vitro*[11,30] and *in vivo*.[12]

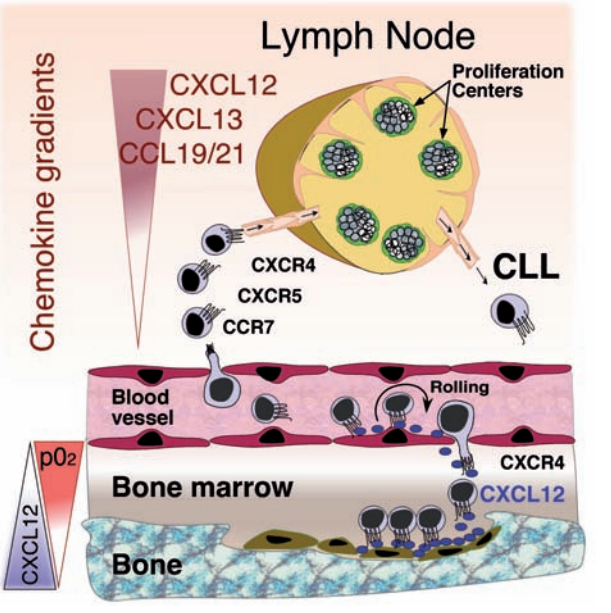

Figure 1. CLL cell trafficking between tissues and the peripheral blood. This diagram represents a model of CLL cell trafficking between the different tissue compartments and the blood. Gradients of lymphatic tissue chemokines (CXCL12, CXCL13, CCL19, CCL21), which are constitutively expressed by stromal cells, direct CLL cell migration, and localization within the secondary lymphatic tissues, acting through cognate chemokine receptors expressed on the CLL cells (CXCR4, CXCR5, CCR7). These interactions are likely involved in organization of the specific micro-architecture in CLL, which is characterized by proliferation centers, also called pseudofollicles. Gradients of CXCL12, which is the dominant chemokine in the bone marrow, direct CLL cell migration from the blood into the marrow. During this process, CLL cells attach to adhesion molecules and chemokines presented on the luminal side of the endothelium, causing adhesion, rolling, and transendothelial migration. Bone marrow stromal cells express CXCL12, which is in part, dependent on the oxygen partial pressure (pO_2), and direct CLL cell homing and retention in the marrow (adapted after[107]).

Overview: chemokines and their receptors

The term chemokines initially was coined in 1992 as a short form of "chemotactic cytokines". Currently, the human chemokine system includes more than 40 chemokines and 18 chemokine receptors.[31] Chemokines are small secreted proteins that are released either constitutively or in response to stimulation, and cause migration of cells towards a gradient of the chemokine (chemotaxis). The two main subfamilies of chemokines, CXC and CC chemokines, are distinguished based upon two conserved cysteine residues, which either are separated by an intervening amino acid or adjacent, accounting for CXC or CC chemokines, respectively.[31] Chemokines bind to chemokine receptors, which belong to the large family of seven transmembrane domain G-protein-coupled cell surface receptors (GPCRs). Following activation, the intracellular domains cause dissociation of G-proteins, which are composed of three distinct subunits (α, β, γ heterotrimers). This leads to formation of the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG), resulting in cytoplasmatic calcium mobilization, and activation of multiple downstream signaling cascades, such as the phosphatidylinositol 3-kinase (PI3K)/Akt and the Ras/mitogen-activated protein-kinase (MAPK, also called ERK 1/2) signaling pathways.

T and B lymphocytes express receptors for various chemokines, and their expression and function is modulated during lymphocyte differentiation and activation.[32] Circulating lymphocytes interact transiently and reversibly with vascular endothelium through adhesion molecules (selectins, integrins) in a process called rolling. Chemokines displayed on the luminal endothelial surface activate chemokine receptors on the rolling cells, which triggers integrin activation.[26] This results in the arrest, firm adhesion, and transendothelial migration into tissues, where chemokine gradients guide localization and retention of the cells.[33] These steps are collectively referred to as "homing", and are essential for normal development of the organism, organization, and function of the immune system and for tissue replacement.

Chemokine receptors on CLL cells

CXCR4 (CD184)

CXCR4 is expressed at high levels on the surface of peripheral blood CLL cells,[11,34–36] and mediates CLL cell

chemotaxis, migration across vascular endothelium, actin polymerization, and migration beneath and underneath CXCL12-secreting MSC.[11,34,37,38] CXCL12 also has a pro-survival effect on CLL cells[8,39,40] which is not surprising, given that CXCL12 initially was characterized as pre-B-cell growth-stimulating factor (PBSF).[41]

CXCR4 surface expression is regulated by its ligand CXCL12 (previously called stromal cell-derived factor-1/SDF-1) via receptor endocytosis.[11] This characteristic can be used to distinguish tissue (lymphatic tissue- and marrow-derived) from blood CLL cells, which express low or high CXCR4 levels, respectively.[11,42,43] Proliferating, Ki-67$^+$ CLL cells from marrow and lymphatic tissue displayed significantly lower levels of CXCR4 and CXCR5 than non-proliferating CLL cells.[44] *In vivo* deuterium (^{2}H) labeling of CLL cells revealed that patients with higher CXCR4 expression on their CLL cells had delayed appearance of newly produced CD38$^+$ cells in the blood, and increased risk for lymphoid organ infiltration and poor outcome.[45] These ^{2}H studies also revealed intraclonal heterogeneity of CXCR4 expression, with an enrichment of CLL cells expressing lower CXCR4 surface levels in the CD38$^+$/CD5^{bright} fraction, along with increased ^{2}H incorporation.[45] These *in vivo* data indicate that lower blood CXCR4 surface levels label a fraction of CLL cells that has recently exited the tissues into the blood.

B cell antigen receptor (BCR) signaling results in down modulation of CXCR4,[46,47] along with enhanced chemotaxis towards CXCL12 and CXCL13, at least in our hands.[46] This may explain why ZAP-70$^+$ CLL cells display increased chemotaxis and survival in response to CXCL12 when compared with ZAP-70-negative CLL cells,[40] given that ZAP-70 expression is associated with a higher responsiveness to BCR stimulation.[48] CD38$^+$ CLL cells also display higher levels of chemotaxis,[49] and CD38 activation enhanced chemotaxis towards CXCL12, whereas a blocking anti-CD38 mAbs inhibited chemotaxis.[50] CXCR4 signaling in CLL cells is pertussis toxin-sensitive and induces calcium mobilization, activation of PI3 kinases,[11] p44/42 MAP kinases,[8] and serine phosphorylation of signal transducer and activator of transcription 3 (STAT3).[51] CXCR4 signaling can be inhibited by isoform-selective PI3 kinase inhibitors,[52] including CAL-101,[53] and inhibitors of Syk,[46] and Btk,[54] leading to impaired CLL cell migration. CXCR4 also can be specifically be blocked by CXCR4 antagonists (reviewed in[55]). We reported that CXCR4 antagonists inhibit CLL cell activation by CXCL12, and reverse, at least in parts, stomal cell-mediated drug resistance.[51] These data are the basis for an ongoing clinical trial in relapsed CLL patients, in which patients are treated with the combination of the anti-CD20 mAb rituximab and plerixafor, a small molecule CXCR4 antagonist. Preliminary data from this trial indicate a plerixafor dose-dependent mobilization of CLL cells from the tissues to the blood.[56]

Other chemokine receptors in CLL (CXCR3, CXCR5, CCR7)

CXCR3 (CD182) is the receptor for the CXC chemokines CXCL9, 10, and 11. These interferon-gamma (IFNγ)-induced chemokines are secreted at sites of inflammation and function in a paracrine or autocrine fashion.[57] CXCR3 is expressed on subsets of normal B and T cells.[58] CXCR3 is consistently expressed on CLL and splenic marginal zone lymphoma B cells, but not on normal CD5$^+$ B cells, and more inconsistently on neoplastic B cells from patients with other B cell lymphomas.[59,60] CXCR3 expression levels on CLL cells are variable, and low CXCR3 expression was strongly associated with advanced stages (Rai III/IV), diffuse marrow infiltration, other risk factors, and poor survival.[61]

CXCR5 (CD 185) is the receptor for CXCL13, a chemokine that regulates lymphocyte homing and positioning within lymph follicles.[62] CXCR5 is expressed by mature B cells, a small subset of T cells, and skin-derived dendritic cells (reviewed in[63]). CXCR5 gene deleted mice display defective formation of primary follicles and germinal centers in the spleen and Payer's patches, and lack inguinal lymph nodes.[64] Subsequently, the ligand for CXCR5 was identified and termed B cell-attracting chemokine 1 (BCA-1)[65] and now is designated CXCL13. CXCL13 is constitutively secreted by stromal cells in B cell areas of secondary lymphoid tissues (follicles), where B cells encounter antigen and differentiate.[62,66] CXCR5 induces recruitment of circulating naïve B cell to follicles[62,66] and is responsible for the microanatomic positioning within the germinal center (GC).[22,24,29,67] In addition, it has been suggested that the primordial function of CXCL13 may be the recruitment of primitive B cells to body cavities for T-independent responses, prior to its involvement in the complex lymphocyte positioning during T-dependent antibody responses.[68] CLL cells express high levels of CXCR5.[30,35,38,60,69] CXCR5 expression levels are similar on CLL B cells and normal, CD5$^+$ B cells, and higher when compared with normal, CD5 negative B cells, T_{FH} cells, or neoplastic B cells from other B cell neoplasias.[30] Stimulation of CLL cells with CXCL13 induces actin polymerization, CXCR5 endocytosis, chemotaxis,[38] and prolonged activation of MAPK (ERK 1/2). In CLL, CXCR5 signals through Gi proteins, PI3-kinases, and p44/42 MAPK pathway.[30] CXCL13 mRNA and protein is expressed by NLC *in vitro* and *in vivo*.[30] These data suggest that CXCR5 plays a role in CLL cell positioning and cognate interactions between CLL and CXCL13-secreting stromal cells, such as NLC in lymphoid tissues.

The CCR7 (CD197) receptor has two ligands, CCL19 and CCL21. CCL19 and CCL21 are constitutively expressed by reticular cells, high endothelial venules (HEVs), and dendritic cells (DC) and play a role in lymph-node homing of naïve and regulatory T cells and DC.[70] Moreover the CCR7-CCL19/CCL21 axis is involved in organizing the architecture and function of the thymus. CCR7 is expressed by DCs, thymocytes during defined stages of their development, naive B and T cells, regulatory and a subpopulation of central memory T cells. CCR7 is also expressed by various neoplastic cells, and CCR7 expression correlates with lymph node metastasis in solid tumors,[71] including malignant melanoma, colorectal, and prostate cancer. In sharp contrast to CXCR5-deficient mice, which show reduced peritoneal B-1 and B-2 B cells, CCR7 deficiency results in a massive accumulation of T cells and B-2 B cells in the peritoneal and pleural cavities, caused by an impaired egress of CCR7-deficient lymphocytes from body cavities.[72] CLL cells express CCR7 and migrate across vascular endothelium in response to CCL19 and CCL21.[37,69] Moreover, expression levels of CCR7 corre-

lated with lymphadenopathy[37,69] and expression of ZAP-70 and CD38.[40] CCL19- and CCL21-induced migration and actin polymerization of ZAP-70+/CD38+ CLL cells was higher when compared with CLL cells lacking ZAP-70 and CD38.[40] Moreover, CCL21 significantly increased B-CLL metalloproteinase-9 (MMP-9) production in MAP kinase- (ERK1/2-) dependent fashion,[73] suggesting cross talk between these pathways during trafficking and tissue homing. CCR7 signaling for chemotaxis in response to CCL19 and CCL21 involves PI3 kinases and the Rho kinase.[74] Anti-CCR7 mAbs recently were shown to cause complement-dependent cytotoxicity against CLL cells and therefore were proposed as a potential therapeutic.[75] Overall, these data support the concept that CCR7 plays an important role in trafficking and homing of CLL cells to the lymphatic tissues.

Chemokines secreted by CLL cells: CCL3, CCL4, and CCL22

CCL3 and CCL4 are chemoattractants for monocytes and lymphocytes.[76] CCL3 expression in normal B cells is induced by BCR triggering and CD40 ligand,[77–79] and repressed by Bcl-6.[80] We and others previously demonstrated that activated CLL cells express and secrete CCL3/4.[81–83] CLL cells secrete CCL3/4 in response to BCR stimulation and in co-culture with NLC.[81] This BCR- and NLC-dependent induction of CCL3/4 is sensitive to inhibition of BCR-signaling, using for example, a Syk inhibitor.[46,81] CLL patients display elevated CCL3/4 plasma levels[81] and plasma levels of CCL3 were strongly associated with established prognostic markers and time to treatment. A multivariable analysis revealed that CCL3, advanced clinical stage, poor risk cytogenetics, and CD38 expression were independent prognostic markers in a cohort of 351 CLL patients.[84] The function of CCL3/4 in CLL remains poorly defined, but based upon the function of B cell-derived CCL3/4 in normal immune responses, increased CCL3/4 secretion by CLL cells may induce trafficking and homing of accessory cells, particularly of T cells and monocytes to CLL cells in the tissue microenvironments.[81,85]

Table 1. Chemokine receptors (top) and inducible chemokines (bottom) expressed by activated CLL cells.

Receptor	Other Names	Ligands	Expression in CLL	Known functions
CXCR3	CD182, GPR9	CXCL9, CXCL10, CXCL11	Low/intermediate, consistently expressed[59,60]	Th1 response, angiostasis, leukocyte recruitment, inflammation, integrin activation, cytoskeletal changes, and chemotactic migration
CXCR4	CD184, Fusin, HM89, LCR1, LESTR	CXCL12	High,[11,34] downregulated by CXCL12 (receptor endocytosis)[11] and by BCR triggering[46]	Organogenesis, lymphopoiesis, hematopoiesis, cell migration and survival, angiogenesis
CXCR5	CD185, BLR1, MDR15	CXCL13	High[30]	B-cell migration, Th2 response, organogenesis (cooperative with the CCR7 receptor)
CCR7	CD197	CCL19, CCL21	Intermediate to high, correlates with lymphadenopathy[37,69]	T cell development in the thymus, lymph-node homing of naive and regulatory T cells and dendritic cells
Chemokine	**Other Names**	**Receptor**	**Expression in CLL**	**Presumed Functions**
CCL3	MIP-1α; SCYA3; GOS19-1; LD78ALPHA	CCR1 and CCR5	After BCR-triggering and NLC co-culture,[81] higher in ZAP-70+ CLL, Syk-dependent[46]	Inflammation, recruitment and activation of polymorphonuclear leukocytes, activated B cells: recruitment of T cells for T-B cell interactions
CCL4	MIP-1β, SCYA4	CCR5	Same as for CCL3	Same as for CCL3
CCL22	DC/B-CK, MDC, MDC(1-69), MGC34554, SCYA22	CCR4	After CD40 ligation[18]	Recruitment of regulatory T cells (T_{reg})

Table 2. Expression and function of selected CLL adhesion molecules.

Adhesion molecule	Other Names	Ligands	Expression in CLL	Known functions
VLA-4 (Very Late Activation Antigen-4)	Integrin alpha (4) beta (1), CD49d-CD29	VCAM-1, CS1 protion of fibronectin	Variable levels, correlation with CXCR4[104] and prognosis[95,96]	Involved in both cell-cell and cell-matrix (ECM) adhesion; plays a role in inflammation, hematopoietic cell homing and immune function, and morphogenesis/organogenesis
CD44		hyaluronic acid, osteopontin, collagens, and matrix metalloproteinases (MMPs)	Variable, poor prognosis linked to higher expression,[105] CD44v constitute docking molecules on CLL cells for MMP-9[106]	Lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis. CD44 transcripts undergo complex splicing that results in functionally distinct CD44 isoforms
L-selectin	**CD62L**	GlyCAM-1 (on high endothelial venules of lymph nodes), CD34 (on endothelial cells), MadCAM-1 (on endothelial cells of gut-associated lymphoid tissue)	High	Homing receptor for leukocytes to enter secondary lymphoid tissues via high endothelial venules

Regulatory T cells (T_{reg}), identified by expression of the transcription factor FoxP3, typically express the chemokine receptor CCR4 and migrate towards the ligands for CCR4, called CCL22 and CCL17. It was proposed that CCL17 and/or CCL22 secretion could be responsible for an accumulation of FoxP3$^+$ T_{reg} cells in the in the tumor microenvironment, which might suppress local immune responses and favor tumor progression in diseases, such as breast cancer or Hodgkin's disease.[86,87] CLL cells obtained from the tissues, but not from the blood express CCL22 and variable levels of CCL17 mRNA. After CD40 ligation, CCL22 and CCL17 mRNA became induced in blood CLL cells, and CCL22 protein was released into CLL cell supernatants, which in turn attracted CCR4$^+$ T cells. Conceivably, by attracting T cells and other immune cells, CLL cell-derived chemokines foster the co-evolution of CLL cells and their supportive microenvironment, actively creating a favorable microenvironment in which CLL cells interact with T cells and other accessory cells that deliver survival- and proliferation-signals.

VLA-4 (CD49d) adhesion molecules in CLL

Integrins are a superfamily of heterodimeric glycoproteins, consisting of various α (1 through 11) and β (1 through 6) subunits, whose function is to mediate cell-cell and cell-matrix adhesion in various cell types. The term "integrin" was first proposed in 1986 to describe membrane complexes involved in the transmembrane association between fibronectin as part of the extracellular matrix (ECM) and the actin cytoskeleton.[88] Integrins are categorized into subfamilies with members sharing a common β subunit pairing with a unique α subunit. β_1 integrins are very late activation antigens (VLA) that have the same β_1 subunit but various α chains (α 1 through 6).[89] The $\alpha_4\beta_1$ integrin VLA-4 (CD49d) is a receptor for fibronectin (FN) and vascular cell adhesion molecule-1 (VCAM-1/CD106, Figure 2). VLA-4 is expressed on lymphocytes, monocytes, and most other hematopoietic cells (except for neutrophils); VLA-4 is involved in both cell-cell and cell-extracellular matrix adhesion and plays a role in lymphocyte trafficking and homing as part of immune surveillance,[32] trafficking, and homing of other hematopoietic cells, and inflammation. Integrins are highly versatile adhesion molecules; their adhesiveness can rapidly be regulated by the cells on which they are expressed, for example, by chemokine receptor activation.[26] VLA-4 mediates lymphocyte adhesion to the VCAM1, also known as CD106, which is expressed on cytokine-activated endothelium. VCAM1 mediates leukocyte-endothelial cell adhesion, and may play a role in the development of artherosclerosis and rheumatoid arthritis. VLA-4 also binds fibronectin, an ECM component expressed on MSC,[90] by interacting with at least three fibronectin sites, CS-1 and REDV in the IIICS region, and H1 in the HepII region.[91] VLA-4 plays a particularly important role for interactions between normal and malignant hematopoietic cells and the marrow microenvironment. Ryan *et al*.[92] and Dittel *et al*.[93] demonstrated that VLA-4 and VCAM-I are involved in the adhesion of human B cell precursors to MSC. Matsunaga and colleagues demonstrated that VLA-4 mediates drug resistance, and anti-VLA-4 mAbs induce long-term disease-free survival in a mouse model of acute myelogenous leukemia (AML).[90] VLA-4 integrins cooperate with chemokine receptors in CLL cell adhesion to stromal cells.[11,94] Moreover, VLA-4 expression on CLL cells has prognostic impact,[95,96] indicating the relevance of these interactions *in vivo*. Collectively, these studies indicate that VLA-4 integrins play a key role for adhesion of CLL and other leukemia cells to stromal cells and ECM, and provide a rationale to further explore and target this molecule in CLL.

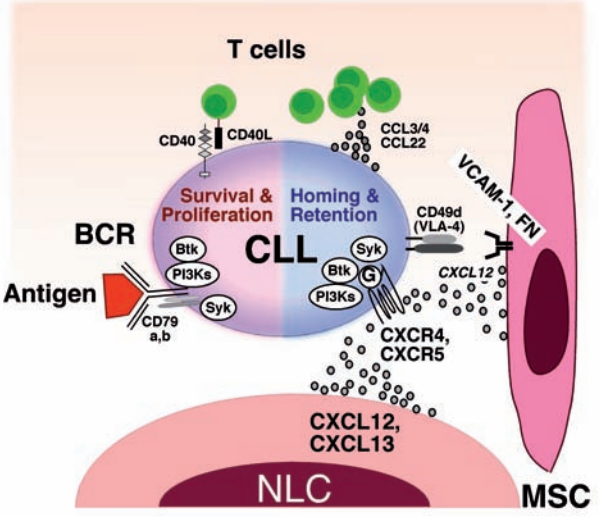

Figure 2. Molecular interactions in the CLL microenvironment. This diagram depicts molecular interactions between CLL and stromal cells in the marrow and/or lymphoid tissue microenvironments that are considered important for a) CLL cell survival and proliferation (left hand side) or b) CLL cell homing and retention in the tissues (right hand side, adapted after[16]). Contact between CLL cells and NLC or MSC is established and maintained by chemokine receptors and adhesion molecules expressed on CLL cells. NLCs express the chemokines CXCL12 and CXCL13, whereas MSCs predominantly express CXCL12. NLCs and MSCs attract CLL cells via the G protein-coupled chemokine receptors CXCR4 and CXCR5, which are expressed at high levels on CLL cells. Integrins, particularly VLA-4 integrins (CD49d), expressed on the surface of CLL cells cooperate with chemokine receptors in establishing cell-cell adhesion through respective ligands on the stromal cells (VCAM-1 and fibronectin/FN). The precise role of Syk, Btk, and PI3 kinases (PI3Ks) for signaling of chemokine receptors and adhesion molecules in CLL is defined in ongoing studies. However, the clinical responses to small molecule antagonists to each of these kinases indicate an important role of these kinases for CLL tissue homing and retention, likely involving chemokine receptor- and integrin-signaling, as indicated in the diagram.
Self and/or environmental antigens (Ag) are considered a key factor in activation and expansion of the CLL clone. The nature and source of Ag and its mode of presentation to CLL cells are largely unknown and currently the focus of intensive research. Stimulation of the BCR complex (BCR and CD79a,b) induces downstream signaling by recruitment and activation of Syk, Btk, and PI3Ks. Finally, BCR stimulation and co-culture with NLCs also induce CLL cells to secrete high levels of chemokines (CCL3, CCL4) which are potent T cell–attracting chemokines. Through this mechanism, CLL cells can actively recruit T cells for cognate T-cell interactions with CLL cells. CD40L$^+$ T cells are preferentially found in CLL proliferation centers[18] and can interact with CLL cells via CD40.

Therapeutic targeting of chemokines and their receptors in chronic lymphocytic leukemia

The CXCR4-CXCL12 axis

CXCL12 is constitutively secreted by marrow stromal cells (MSC), and induces leukemia cell trafficking and homing to the marrow microenvironment *in vitro*[11] and *in vivo*[12] via CXCR4 receptors, which are expressed at high levels on circulating CLL cells. In the marrow microenvironment, CXCL12 retains leukemia cells in MSC niches. CXCR4 antagonists, such as Plerixafor (AMD3100) and T140 analogs, can disrupt adhesive CLL-stroma interactions[51] and mobilize CLL cells from their protective tissue microenvironments to the blood, making them more accessible to conventional drugs. Therefore, targeting the CXCR4-CXCL12 axis is a novel, attractive therapeutic approach that is currently explored in a first clinical trial in CLL patients.[56] Initially, CXCR4 antagonists were developed for treatment of HIV, where CXCR4 functions as a co-receptor for virus entry into T cells. Subsequently, CXCR4 antagonists were noticed to induce leukocytosis, and currently are used clinically for mobilization of hematopoietic progenitors in the context of autologous stem cell mobilization in myeloma and lymphoma patients. The ongoing CLL trial combines plerixafor with rituximab, and the first preliminary data indicate a plerixafor dose-dependent CLL cell mobilization to the blood, as well as safety of this drug combination.[56] Future studies in CLL using this approach of leukemia cell mobilization and sensitization could combine a CXCR4 antagonist with established CLL drugs, such as antibodies, established cytotoxic agents, or combinations of antibodies and cytotoxic agents (chemoimmunotherapy). An alternative approach would be the use of a CXCR4 antagonist in the setting of residual disease (MRD), where these agents could help to mobilize and then eliminate residual CLL cells from tissue sites.

VLA-4 (CD49d)

VLA-4 can be targeted by agents that either block binding or signaling of VLA-4. Monoclonal antibodies (mAbs), such as Tysabri®, and small molecule VLA-4 antagonists effectively block VLA-4 binding, whereas Syk and other kinase inhibitors may inhibit signaling of VLA-4 and other integrins.[97] The development of VLA-4 as a therapeutic target to disrupt cancer-microenvironment interactions is in its infancy, but the anti–VLA-4 antibody natalizumab (Tysabri®, Biogen IDEC), approved for the treatment of multiple sclerosis, and small molecule integrin inhibitors are available and could be developed for targeting CLL-stroma interactions.

B cell kinase inhibitors (Syk, Btk, PI3 kinase delta inhibitors)

Orally bio-available inhibitors of kinases downstream of the BCR are currently tested in first clinical trials in CLL patients and are generating excitement because of the promising early response data and the benign side effect profiles.[98–100] These new targeted agents are the Syk inhibitor fostamatinib disodium,[98] the Bruton's tyrosine kinase (Btk) inhibitor PCI-32765,[101] and the PI3K-delta inhibitor CAL-101.[102] Characteristically, these kinase inhibitors induce rapid lymph node shrinkage along with a transient lymphocytosis during the first weeks of treatment, which presumably is due to mobilization of CLL cells from the tissues into the blood. Inhibition of signaling through CXCR4 and potentially other chemokine receptors and adhesion molecules[46,52,103] seems to be the basis for this remarkable phenomenon (Figure 2). Future research on these agents will need to address the question whether CLL cell mobilization is the key effect of these agents, leading to a compartment shift of tissue-resident CLL cells into the peripheral blood (as discussed before), causing lymphocytosis and resolution of lymphadenopathy during the first week(s) of treatment. Subsequently, CLL cells may simply die from neglect, i.e., lack of tissue-derived survival- and growth-signals. *In vitro* data, however, suggest that these agents also effectively block BCR-derived survival- and growth-signals, implying dual effects on CLL cells.

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Updating treatment of chronic lymphocytic leukemia

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A B S T R A C T

Over the past decade there have been tremendous advances in the treatment of chronic lymphocytic leukemia (CLL), with the goal of therapy no longer being just to palliate symptoms, but now to achieve complete remission and improve survival. Front line chemo-immunotherapy approaches now offer prolonged survival. However, novel treatment approaches are required for those patients who do not respond or relapse following this therapy, as well as for those who are too frail to tolerate these approaches. Numerous novel agents are being developed and their role in the frontline treatment of frail patients or those who relapse after previous treatment is being explored in ongoing clinical trials.

Introduction

The clinical course of chronic lymphocytic leukemia (CLL) is extremely heterogeneous. Some patients will live for decades and never require treatment, while others require immediate treatment[1]. A major focus of research has been to try to identify those clinical and biological factors that influence the clinical course to help determine whether patients will have indolent disease or rapid progression, and which patients will respond best to which treatment[2]. High risk features predictive of disease progression include deletion (del) 17p and del 11q, IgV_H unmutated status, use of the *IGHV3-21* gene segment, and expression of either ZAP70 or CD38. It remains a major challenge to understand how these biomarkers can be used in clinical practice and whether the detection of "high-risk" features will alter treatment offered. Therefore, assessment of the impact of these biomarkers remains a vital component of research studies.

Treatment of CLL

The results of clinical trials in previously untreated CLL have demonstrated major advances over the last decade. The best outcomes have been reported with chemo-immunotherapy, the most active combination described being the three drug regimen of fludarabine, cyclophosphamide, and rituximab (FCR). Phase II clinical studies at the MD Anderson Cancer Center evaluated FCR in previously untreated,[3-4] as well as in treated patients[5]. In a series of 300 previously untreated patients, overall response rate (ORR) was 95%, with 72% achieving complete remission (CR) CR, 7% complete remission with incomplete bone marrow recovery (Cri), 10% nodular partial response (PR) and 6% PR and at a 6 year median follow-up, overall survival (OS) was 77% and progression free survival (PFS) 51%.[4] Importantly, these findings were confirmed in a phase III clinical trial demonstrating the advantage of chemo-immunotherapy. The German CLL Study Group (GCLLSG) CLL8 randomized clinical trial demonstrated a significant improvement in response rates, duration of response, and overall survival with FCR compared with FC.[6] Although this clearly represents a major advance, many patients with CLL are not suitable candidates for FCR therapy and for this population, new approaches are needed.

Notwithstanding, the improved results with first line therapy in fit patients with CLL, the disease remains incurable, and CLL patients are destined to relapse after primary treatment. The management of relapsed CLL patients is dependent upon age, performance status, previous therapy, response, and duration of response to therapy. The goal of therapy, whether palliative or aggressive, must also be weighed into the decision when deciding on the next line of treatment. With many potential treatments available in clinical trials, the sequence of treatments and the timing of procedures, such as stem cell transplantation (SCT), remain questions being addressed in clinical trials.

With improvement in therapy, as some groups respond better to newer treatment combinations, it is likely that the prognostic significance of some of these parameters will change. Of particular significance is the detection of cases with del 17p. These patients have poor outcomes using even optimal chemo-immunotherapy approaches, and novel agents are required for this group of patients. Presently, alemtuzumab alone or in combination is a reasonable option for patients with del 17p or p53 mutations, since this agent has been shown to have efficacy in this patient population.[7]

Novel agents in clinical trials

Bendamustine is a potent alkylating agent widely used in Germany and approved for use in CLL in the USA in 2008 based on a

randomized trial demonstrating improved response rates and duration of response with bendamustine compared with chlorambucil in previously untreated patients with CLL.[8] Although this agent has structural similarity to purine analogs, it does not demonstrate purine-analog based activity.[9] Recent data from the German CLL Study Group suggest that bendamustine in combination with rituximab (BR) produces higher response rates than those seen with bendamustine alone.[10] This combination produced a CR rate of 33% and an ORR of 89%. Although CR rates appear somewhat inferior in this phase II trial compared with those seen with FCR, ORR appear comparable, and an ongoing multicenter randomized trial is assessing is comparing the combination of BR to FCR in previously untreated CLL patients.

There is no established role for maintenance therapy in CLL. Alemtuzumab has been assessed in this setting with some intriguing results,[11] but enthusiasm has been tempered by the high toxicity observed. Ongoing clinical trials are examining maintenance therapy with alternative schedules of alemtuzumab, rituximab, ofatumumab, or lenalidomide.

Ofatumumab is a humanized monoclonal antibody targeting CD20 that binds to a different epitope than rituximab. This agent is approved by the FDA for use in fludarabine and alemtuzumab refractory CLL based upon the results of a trial in heavily pretreated patients. The OR rate was 58% with a median PFS of 6 months.[12] The significant activity of this agent in the refractory population has raised the question of whether combining it with chemotherapy will provide more potent efficacy than current rituximab and chemotherapy based regimens. Results with the combination of FC and ofatumumab has been examined in trial of 61 patients randomized to receive either 500 mg or 1000 mg; ORR were comparable, but the CR rate with 1000 mg was 50% versus 32% with 500 mg.[13]

There are a large number of ongoing clinical trials exploring new agents in the treatment of CLL, including novel monoclonal antibodies (GA101, lumiliximab, lucatumumab), BH3 mimetics (obatoclax, ABT-263), cyclin-dependent kinase inhibitors (flavopiridol, SNS-032), Lyn-kinase inhibitors (dasatinib, bafetinib), hypomethylating agents (azacytidine, decitabine), histone deacetylase inhibitors (parobinastat), purine analogues (8-chloroadenosine, forodesine), and small modular immunopharmaceuticals (TRU-016), In addition, agents that have no direct cytotoxicity to CLL cells have also been proven to be effective in this disease, presumably by significant modulation of the microenvironment, most notably lenalidomide.

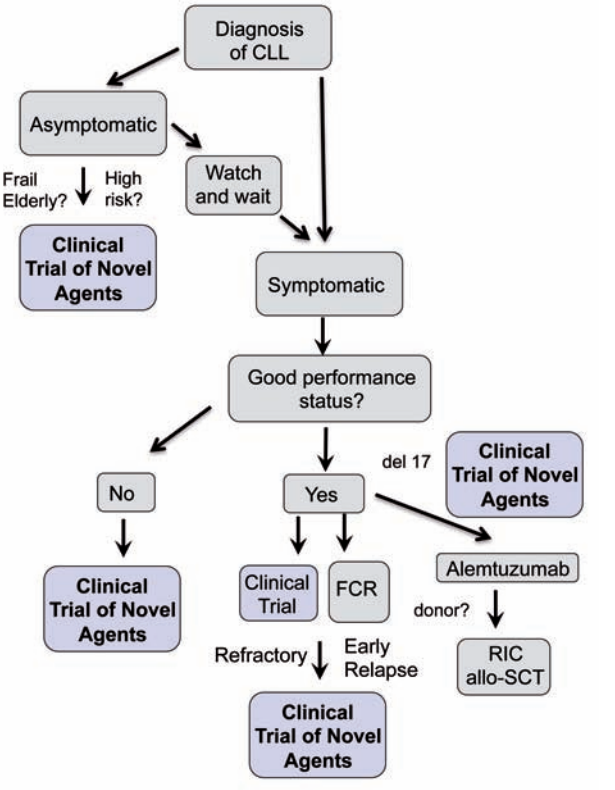

Figure 1. Role of novel agents in CLL. Asymptomatic patients should be treated with a watch and wait approach until they are symptomatic, unless they are enrolled in clinical trials assessing impact of prognostic features on early treatment. Treatment of choice is FCR or a suitable clinical trial. Patients who are not fit for consideration for FCR should be considered for clinical trials investigating novel approaches for these patients. Patients with del17p or p53 mutations should be considered for clinical trial and offered RIC allo-SCT.

Targeting B cell receptor BCR signaling in CLL

The BCR consists of a surface immunoglobulin (Ig) molecule non-convalently associated with the Ig-α/Ig-β (CD79a/CD79b) heterodimer. Engagement of the BCR by antigen induces phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic domains of Ig-α and Ig-β in normal B-cells. This phosphorylation is mediated primarily by the Src family kinase Lyn, and results in recruitment and activation of the tyrosine kinase Syk. Activated Syk forms a membrane associated complex with other tyrosine kinases, including Lyn and the Tec kinase, Bruton's tyrosine kinase (Btk), and adapter molecules, such as B-cell linker protein (BLNK). This complex mediates activation of downstream signaling pathways, including phosphatidyl 3-kinase (PI3K), and phospholipase Cγ2 (PLCγ2). PI3K generates the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3), which recruits the kinase Akt. PLCγ2 activation leads to the release of intracellular calcium and subsequent activation of protein kinase C (PKC). These events lead in turn to activation of mitogen-activated protein kinases (MAPKs) including extracellular signal regulated kinase (ERK), c-JUN NH_2-terminal kinase (JNK), and p38 MAPK. Activation of PKC also increases expression of nuclear factor-κB (NFκB), while the rise in the intracellular calcium concentration causes activation nuclear factor of activated T-cells (NF-AT). These transcription factors are fundamental in determining B-cell fate.

Following the success of tyrosine kinases inhibitors in CML, there has been interest in developing these agents for use in CLL. Dasatinib is a tyrosine kinase inhibitor used in the management of CML and Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL). In addition to inhibition of Bcr-Abl, dasatinib has been found to inhibit Src family kinases, such as Lyn, which are often dysregulated and constitutively activated in

CLL B-cells.[14] Dasatinib also induces apoptosis of CLL B-cells *in vitro*, with cells that have unmutated IgVH genes appearing more sensitive, and this agent is also able to overcome CD40-mediated drug resistance, and to sensitize the tumor cells to chlorambucil and fludarabine *in vitro*.[15] A phase II study of dasatinib in mostly high risk patients with relapsed or refractory CLL, showed partial responses in 20%, although in two-thirds of cases. there was also significant myelosuppression.[16]

The importance of Syk in BCR-signaling has made it an attractive target for the development of novel therapies in CLL. Syk is constitutively activated in CLL B-cells, and its inhibition results in a reduction of BCR-induced Akt activation and Mcl-1 upregulation.[17] The Syk inhibitor fostamatinib was demonstrated to decrease BCR-signaling, selectively reduce tumor cell proliferation, and prolong animal survival mice in the Eμ-TCL1 transgenic mouse model.[18] A phase I/II study of fostamatinib in a variety of B-cell lymphoproliferative disorders, showed the highest response rate in CLL, with 55% of patients achieving partial remission (PR). IgVH mutation analysis was not reported in this study, although clinical responses were observed in patients with and without ZAP-70 expression.[19] Similarly, there has been interest in developing inhibitors of Btk as a therapeutic tool in CLL. Treatment of primary CLL B-cells with the Btk inhibitor PCI-32765 results in significant apoptosis that is caspase dependent. There was no correlation between response to PCI-32765 and prognostic factors, such as IgVH gene mutation status, suggesting potential efficacy in high risk patients. This agent also appears to have effects on the microenvironment, by blocking the protective effect of factors, such as CD40 ligand (CD40L), tumor necrosis factor-α (TNF-α), IL-6, and B-cell activating factor (BAFF).[20] An ongoing phase I clinical trial of PCI-32765 in relapsed or refractory CLL has shown it to be relatively well tolerated, with overall response rates of 64% in an interim analysis.[21]

Inhibitors of PI3K are also under investigation as potential treatments for CLL. The PI3K pathway has been shown to play a pivotal role in CLL B-cell growth, with sustained activation being critical for their survival.[22] Constitutive activation of this pathway in the malignant cells prevented the downregulation of (B-cell lymphoma-xL) Bcl-xL and the caspase inhibitor proteins FLICE-inhibitory protein ligand (FLIPL) and X-linked inhibitor of apoptosis protein (XIAP). CAL-101 is a small-molecule inhibitor of the p110δ isoform of PI3K, which is generally restricted to cells of hematopoietic origin. It has been demonstrated to promote caspase dependent apoptosis in primary CLL cells independently of common prognostic markers.[23] CAL-101 has also been shown to overcome the anti-apoptotic effects of CD40L, TNF-α, and fibronectin, and to inhibit the protective effects of stromal cells.[24] There was also pre-clinical evidence of an effect on T-cells, with decreased production of various inflammatory and anti-apoptotic cytokines by activated T-cells. A phase I study of the use of single agent CAL-101 in patients with relapsed or refractory CLL has shown it to be well tolerated, with partial remissions being observed in 33% of patients. Interestingly, it was noted to be particularly efficacious in reducing lymphadenopathy, with over 90% of patients showing a lymph node response. An *increase* in the circulating lymphocytosis in the first two cycles was observed in the majority (60%) of patients, which, with the improvement in the lymphadenopathy, suggests a drug-mediated shift of lymphocytes from the nodes to the peripheral blood.[25] Early dose finding studies of CAL-101 in combination with rituximab or bendamustine look encouraging, and other therapeutic combinations with drugs, such as the immuno-modulatory agent, lenalidomide, are of potential interest.[26]

Role of stem cell transplantation

A major challenge remains the decision as to which other patients should be considered for allogeneic SCT and when in their disease, course choice is usually reduced intensity conditioning allogeneic SCT is most frequently used.[27] The European Bone Marrow Transplant (EBMT) guidelines outline indications for SCT in CLL.[28] In addition to those with p53 abnormalities, allogeneic SCT is also recommended for younger patients with CLL who fail to respond to, or relapse within 2 years of first line chemoimmunotherapy.

Impact of performance status on treatment

Most patients with CLL are elderly, but age alone is not a contraindication to the use of FCR[29] and the limiting factors for its use are impaired renal function, poor performance status, and comorbidities. There was no upper age limit in the GCLLSG CLL8 trial (FCR versus FC), but patients had to meet eligibility criteria including creatinine clearance greater than or equal to 70 cc/minute and good physical fitness as assessed by a Cumulative Illness Rating Scale (CIRS) less than or equal to 6. There was no difference in side effects when comparing patients younger or older than 70 years old in that trial. Thus, FCR can certainly be utilized in older, fit patients with CLL. In the GCLLSG CLL5 trial in older patients, fludarabine resulted in a significantly higher OR rate and CR rate than chlorambucil; there was no difference in PFS or OS.[30] The best treatment for elderly unfit patients or those with complex comorbidities remains to be determined. Ongoing clinical trials are assessing the addition of monoclonal antibodies, including rituximab, ofatumumab, or GA101 to chlorambucil compared with chlorambucil alone. Other agents being assessed in clinical trials in this patient population include bendamustine alone and in combination with rituximab, lenalidomide, ABT263, PCI-32765, and CAL-101 alone or with rituximab.

Richter's transformation

Richter's transformation to high-grade non-Hodgkin's lymphoma occurs in approximately 5–10% of patients with CLL.[31] The large cells of RS either arise through a transformation of the original CLL clone by the acquisition of new genetic abnormalities, or, less frequently, represent a new secondary neoplasm. The clinical outcome of the disease is generally poor with median survival of months from transformation, but prognosis is better when transformation occurs in previously untreated patients. Treatment is with regimens that are effective in high-grade NHL and although numerous regimens have been proposed, there is no consensus on the best therapeutic approach for RS patients and novel agents are urgently required.

Conclusions

Chemoimmunotherapy has had a dramatic impact on the outcome of patients with CLL, has improved CR rates, eradicates minimal residual disease, and has resulted in improved survival. Remissions with frontline therapy last for years and data are now emerging that more effective frontline regimens are associated with improved overall survival in CLL. Studies are also assessing whether specific treatments are indicated for groups of patients with specific cytogenetic abnormalities. However, not all patients can tolerate aggressive chemo-immunotherapy regimens, and patients continue to relapse so new drugs are needed to effect cure in these patient populations. The encouraging array of ongoing clinical trials and investigational drugs, many of which have novel mechanisms of action, engender great optimism that cure of CLL may be accomplished within the next 20 years.

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Treatment of chronic lymphocytic leukemia in the elderly patients

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A B S T R A C T

Although elderly patients above the age of 70 years represent the largest group of CLL patients, they are heavily underrepresented in clinical trials. A careful assessment of the physical fitness and comorbidity should be performed before initiation of therapy. The burden of comorbidity has significant influence on the outcome of treatment in elderly cancer patients. Therefore, not only age, but also the incidence and burden of comorbidity influence the choice of treatment strategy for every patient. In trials of the German CLL study group, the cumulative illness rating scale (CIRS) has proven to be a reliable tool for measuring comorbidity and to distinguish between physically fit and non-fit patients. While chlorambucil seems to be the current standard for treating non-fit patients, chemoimmunotherapies, or bendamustine, alone or in combination with antibodies, are currently investigated within clinical trials for this patient group. Because fully dosed combination therapies might cause increased toxicity rates in relapse situation, dose-reduced combination therapies should be considered in this situation. An adequate supportive treatment is necessary for the prevention of toxicities, as well as for the improvement of health-related quality of life.

Introduction

The incidence of CLL is markedly increased in patients older than 65 years, with a median age at diagnosis of 72 years.[1] About three-quarters of all patients with CLL are older than 65 years. In sharp contrast, the age of patients enrolled to pivotal trials evaluating the first-line treatment of CLL has ranged between 58 and 66 years.[2-6] Patients treated within trials on (immuno)chemotherapy were even younger with 57 to 63 years on average.[7-10] Thus, elderly patients are frequently underrepresented in clinical trials. In contrast to that, recently published data show that age is one of the most important prognostic factors in CLL.[11] Therefore, it is unclear whether this patient group equally benefits from the introduction of new chemoimmunotherapy regimens and new drugs in a similar way to younger patients. One of the major problems in the treatment of elderly CLL patients is the increased incidence of comorbidities. This review highlights typical clinical problems and current treatment approaches in elderly CLL patients.

Clinical aspects to be considered before treatment of elderly patients

There are several clinical aspects to be considered during the treatment of elderly patients. Geriatric assessment of physical fitness and comorbidity might be a helpful tool for the evaluation of older cancer patients. The geriatric assessment should help to determine the functional and cognitive status and to assess depression, nutritional status, and all associated diseases. This assessment allows to identify "vulnerable" patients with different degrees of impairment. An individualized approach must be followed to provide optimal treatment.[12]

Older patients show stronger cognitive impairments, including verbal learning, word fluency, and memory after start of chemotherapy than younger cancer patients.[13] Patients with cognitive impairment may have problems remembering signs and symptoms of cancer therapy, as well as appointments.

An impairment of activity in daily living (ADL) increases the risk of mortality[14] and chemotherapy-associated toxicities.[15] Social support also has a significant impact on the outcome of elderly patients after chemotherapy. A study showed that in geriatric patients receiving outpatient chemotherapy social support was found to be crucial in coping with fatigue.[16]

Most importantly, the associated diseases, which increase with the patients' age, have a significant impact on the tolerability and outcome of chemotherapy. A negative correlation between the burden of concomitant illness and the survival of cancer patients has been demonstrated for many malignancies.[17] Thus, the burden of comorbidity has been shown to have a significant impact on the outcome of CLL. An analysis of 554 patients with advanced CLL who received chlorambucil, fludarabine, or fludarabine plus cyclophosphamide within the CLL4 and CLL5 trial of the GCLLSG revealed a trend towards inferior survival in CLL patients suffering from concomitant diseases.[18] Survival was significantly impaired in CLL patients

with multiple comorbidities (≥ 2) or with severe comorbidity (Charlson score ≥ 2). Interestingly, the impact of both, the numbers and the severity of concomitant diseases on survival were independent of the patients' age. These data suggest that multiple and severe comorbidities are independent predictors of survival in CLL. Another analysis evaluating 373 unselected patients who presented between 1995 and 2006 with newly diagnosed CLL at the Mayo Clinic College of Medicine in Rochester provided different results.[19] In a multivariate analysis, Rai stage and age only were found as significant predictors of overall survival, while concomitant diseases were not associated with impaired survival time. Since both studies considered very different patient populations, definitive conclusions on how comorbidity affects outcome of CLL patients cannot be drawn yet.

Indeed, there are multiple ways that comorbidity may affect the risk of death:

- An accompanying illness may contribute to CLL unrelated deaths (e.g., a coronary heart disease may lead to fatal cardiac infarction). Both toxicity to chemotherapy and symptoms of disease progression may, however, aggravate a preexisting comorbid condition with subsequent lethality (e.g., a fatal cardiac infarction may result from tissue hypoxia due to anemia). Such a fatal event would be also primarily judged to be CLL unrelated, but actually can be linked to the underlying leukemic disease.
- Comorbidities (e.g., renal impairment, hepatic dysfunction) may facilitate toxicity to treatment, thereby eventually increasing the rate of treatment related deaths.
- Comorbid conditions may predispose to earlier progression of the leukemic disease (e.g., by pre-emptive withdrawal of treatment) and finally result in a higher rate of CLL-related deaths.

Overall, published data indicate that fatal outcome in CLL patients with concomitant diseases cannot be attributed to one single cause. Comorbidity seems to be associated with increased rates of both CLL-related and unrelated deaths. In comparison with their otherwise healthy counterparts, comorbid CLL patients seem to be more vulnerable to die from both toxicity and disease progression.

Treatment initiation

The criteria for treatment initiation are the same in elderly and younger patients with CLL. Comorbid conditions and medical fitness should be carefully assessed in the elderly by history taking and physical examination, since these parameters should influence the treatment decision more than age. The Cumulative Illness Rating Scale (CIRS)[20] seems a reliable tool for the evaluation of the burden of comorbidity in elderly cancer patients.[21]

Radiological examinations for staging of the disease before initiating the therapy can be done in elderly CLL patients, but should be limited to the clinical need. Computed tomography (CT) scans are only recommended within clinical trials.[22] Outside clinical trial, physical examination, and eventually ultrasound or chest X-ray are sufficient for staging and evaluation of progressive disease. Radiological examinations, which might result in complications, should be avoided in elderly and comorbid patients whenever possible (e.g., aggravation of renal insufficiency due to contrast medium use for CT scan in patients with previously impaired renal function).

Therapeutic strategies in the elderly

As outlined above, not only the numeric age, but also the incidence and burden of comorbidity should influence the choice of treatment strategy for each patient individually. Elderly patients with no relevant burden of concomitant diseases and normal renal function should be treated with the same regimen as younger patients. A separate analysis of the CLL8 trial of the GCLLSG has shown, that response rates in patients above the age of 65 years were the same as in younger patients without increasing incidence of toxicities.[10] In patients with moderate and severe comorbidity administration of aggressive treatments seems not possible because of non-acceptable high toxicities.

Frontline therapy

The alkylating agent chlorambucil is probably the most commonly used drug in elderly and comorbid patients with CLL. There are several advantages using this drug (easy to administer, well tolerated especially in lower doses), but it results only in a minority of patients into good remission results. Therefore, other drugs have also been used in this patient population (Table 1).

Purine analogues (alone or in combination)

Monochemotherapy with fludarabine has been evaluated in two large randomized trials, including elderly patients. A trial of the GCLLSG (CLL5 trial) including patients with mild to moderate comorbidity compared chlorambucil with fludarabine. Fludarabine treatment was well tolerated by the elderly, thus indicating that fludarabine can be safely administered to patients with a low burden of concomitant diseases. Though response rates with fludarabine were comparable to those in younger patients, no significant differences in progression-free or overall survival were assessed when fludarabine was compared to chlorambucil.[23]

The LRF CLL4 trial by Catovsky *et al*. compared the efficacy and toxicity of six courses of fludarabine plus cyclophosphamide (FC) with six courses of fludarabine alone and twelve courses of chlorambucil. 30% of 777 patients included were 70 years of age or older.[5] In this trial, complete and overall response rates were better with FC than with fludarabine, which were in turn better than with chlorambucil, and PFS at 5 years was significantly better with FC than with fludarabine or chlorambucil. Importantly, the trial also showed that FC was the best combination for all ages, including patients older than 70 years. However, all patients included in this study were very fit elderly patients. Hence, there is no clear evidence if elderly patients with significant comorbid burden benefit from full-dosed purine-analogue based combination therapies as well. Moreover, data from both trials show that full-dosed fludarabine

Table 1. Treatment regimen evaluated in patients with higher age and/or reduced physical fitness.

Regimen	Dosing	Comment	Reference
CLB	0,4 mg-0,8 mg/kg BW po d1 q 15d; 10 mg/m²/d d1-7 q28d up to 12 months	Very well tolerated, not inferior to F in PFS.	23 5
F	25 mg/m²/d iv d1-5 q 28d for 6 cycles	Well tolerated, no more toxicities than younger pts. Better RR than with Clb, but no better PFS or OS.	23
FCR	F 25 mg/m²/d iv d1-3 C 250 mg/m²/d iv d1-3 R 375 mg/m² iv d1 cycle1 R 500 mg/m² iv d1 cycle 2-6,q 28d	Well tolerated in elderly with normal renal function and no relevant comorbidities. Superior to FC. To toxic in elderly with comorbidities and pretreatment. Data on dose reduced FCR pending.	10,25,26
PCR	P 2 mg/m² iv d1 C 600 mg/m² iv d1 R 375 mg/m² iv d1 q 28d for 6 cycles	Well tolerated by elderly and patients with impaired renal function. Similar efficacy in elderly and younger patients.	27
B	100 mg/m²/d iv d1+2 q28d for 6 cycles	Good tolerance and RR in multicenter trial.	32
BR	B 70 mg/m² iv d1+2 R 375 mg/m² iv d1 cycle1 R 500 mg/m² iv d1 cycle 2-6, q 28d B 90 mg/m² iv d1+2 R 375 mg/m² iv d1 cycle1 R 500 mg/m² iv d1 cycle 2-6,q 28d	In relapsed elderly patients good tolerability and efficacy. In first line therapy also good tolerability and efficacy. No detailed data about elderly or comorbid patients yet.	49 33
R	R 375 mg/m² weekly for 4 weeks HDMP 1 g/m²/d d1-5	Good response rates and low toxicity in first line and relapse. Only data from phase II single center studies.	50 35
L	L 5 mg po for 56d, than increase in 5 mg steps to max. 25 mg	For elderly patients only data from phase II single center study.	44

BW = bodyweight, pts = patients, RR = response rates, PFS = progression-free survival, OS = overall survival, CLB = chlorambucil, F = fludarabine, C = cyclophosphamide, R = rituximab, P = pentostatin, B = bendamustine, HDMP = high dose methylprednisolone, L = lenalidomide.

monotherapy contributes not to a significant clinical improvement for elderly patients. A dose reduced fludarabine regimen was evaluated within a phase III trial of the GCLLG (CLL9 trial). The results from this trial, which are not yet released, will show if dose reduced fludarabine could be a treatment option in this patient group.

There are some reports on the combination of (dose-reduced) FC with or without antibodies in previously untreated elderly patients. Forconi *et al*. reported on 26 elderly patients with a median age of 71 years, who received low-dose FC.[24] Response rates with this regimen were promising in pretreated and untreated patients (92% overall response, 46% complete response), while non-hematological toxicity was mild. The overall median event-free survival time of 48 months shows that this regimen is promising in the group of elderly patients.

Ferrajoli *et al*. evaluated full-dose FC alone or in combination with rituximab (FCR) in a single centre cohort of 125 elderly patients with treatment naïve or relapsed CLL.[25] Though response rates were favorable, the rate of life threatening adverse events was unacceptably high (severe CTC grade 3 or 4 myelotoxicity in 65–82%, severe infections in 11–48%) resulting in a high incidence of early treatment discontinuation. The CLL8 trial of the GCLLSG evaluated the FCR and the FC regimen in a randomized fashion and has also enrolled 30% patients older than 65 years with no or mild comorbidities.[10] As mentioned above, response rates and toxicity rates were comparable with those in younger patients. Therefore, full-dosed FCR should only be administered to physically fit elderly patients with no relevant comorbidity burden and normal renal function. A currently ongoing multicentre study investigates the tolerability and efficacy of a dose-reduced FCR regimen in patients above the age of 65 years. First results on safety have shown that this regimen is well tolerated, when fludarabine and cyclophosphamide are administered in this dose reduced setting.[26]

So far it is not clear if purine analogues combination therapies based on pentostatin are less toxic than fludarabine based therapies. A single center trial evaluated the combination of pentostatin, cyclophosphamide, and rituximab (PCR) in previously untreated CLL patients.[27] Eighteen of 64 included patients were above the age of 70. Although more treatment delays had to be performed in the elderly patients no differences in efficacy were assessed (83% overall response rate in the elderly patients).[27]

Alkylating agents

Based on the results of the LRF CLL4 trial, chlorambucil plus rituximab were given to 100 patients with advanced CLL and a median age of 70 years. Chlorambucil was administered on days 1 to 7 at a dose of 10 mg/m²/day and rituximab on day 1 of cycle 1 with 375 mg/m² followed by 500 mg/m² for cycles 2–6. The second interim analysis, which was done after treat-

ment was finished in all 100 patients, showed that the most common SAEs were infections (12 SAEs).[28] The OR rate was 80%, which in a matched pair analysis was 14% higher than patients receiving chlorambucil alone within the LRF CLL4 trial. The CR rate was 12% with the combination therapy in comparison to 6% with chlorambucil alone. With a median time to progression of 23.9 months, the addition of rituximab to chlorambucil seems effective and feasible for untreated patients with CLL who cannot tolerate a more intensive regimen.[28]

Recently, a randomized international trial (GCLLSG CLL11 trial) was initiated comparing chlorambucil alone versus chlorambucil plus rituximab versus chlorambucil plus GA101, an anti-CD20-antibody of the third generation. A total of 780 patients with advanced CLL and concurrent moderate or severe comorbidity will be included in this trial. Main endpoint is the difference in progression-free survival between all three arms. A multicentre phase II study currently evaluates chlorambucil plus rituximab followed by a maintenance therapy with rituximab in elderly patients above the age of 60 years.[29]

Due to the promising results of bendamustine alone or in combination with rituximab in follicular lymphoma,[30,31] this combination is an attractive treatment option in CLL as well. Bendamustine therapy was assessed in comparison to chlorambucil in a randomized multicenter study including 319 previously untreated patients with stage Binet B and Binet C CLL. The patients' median age was 64 years, including 25% of patients aged 70 years or older.[32] Overall response rates (68% vs. 31%) and CR rates (31% vs. 2%) were significantly higher with a median PFS of 22 months in the bendamustine arm versus 8 months with chlorambucil. in comparison to the UK LRF CLL4 trial,[5] the chlorambucil arm showed an exceptionally low efficacy in this trial which results most probably from the different dosing regimens applied.[32] The combination of bendamustine plus rituximab was evaluated in first line setting of CLL within a phase II trial.[33] Among 117 included patients with advanced stage CLL 30 patients (26%) were 70 years or older. A detailed analysis of the response and toxicity data of the elderly included patients is not published. The response rate among all patients was 88%, CR was reported in 23%.[33]

Antibodies alone

As outlined above, antibodies in combination with conventional chemotherapy have been investigated in several clinical trials, including a significant proportion of elderly patients. Antibodies alone might also represent an attractive treatment option in the elderly, because tolerability of these regimens was very good. A single center trial investigated rituximab in combination with GM-CSF as frontline therapy of CLL patients with 70 years of age or older.[34] Thirty-two patients were enrolled to receive one or two cycles of 8 week treatment schedule consisting of rituximab and GM-CSF. The overall response rate was 72%, with two patients achieving a CR.[34] The toxicity rate with this regimen was minimal. Rituximab combined with high dose methylprednisolone was shown to achieve high overall response rate (up to 96%) when administered in first line therapy of CLL.[35] This combination was evaluated by another single center study in 28 patients with a median age of 65 years. Only a few CTC grade three or four events were reported. Therefore, this combination might be an alternative treatment option especially in patients with a very high comorbidity burden and low tolerance of toxicities. Ofatumumab and GA101 are two novel anti-CD20 antibodies, which are expected to be more active in CLL than rituximab.[36,37] Both antibodies are currently investigated in combination with chlorambucil within large randomized trials.

The anti-CD52 antibody alemtuzumab is approved for first line and relapse treatment with CLL. Especially in patients with very high risk and/or refractory disease, this antibody has shown significant efficacy.[38-40] No detailed data about the tolerance of alemtuzumab in comorbid and elderly patients are available. Due to the extensive T-cell suppression this antibody should be restricted to elderly CLL patients with very high risk disease (del(17p) and/or refractoriness).

Newer drugs

The immunmodulatory drug (IMiD) lenalidomide is a new compound with remarkable response rates in the relapsed setting and first line therapy.[41-43] The drug was evaluated within a single center trial for patients above the age of 65 years.[44] The median age of all 43 previously untreated patients was 72 years. The overall response rate among 35 evaluable patients was 54%. Though tumor lysis syndrome is a frequently observed complication, especially with initially high doses, toxicity rates in this trial were low due to the slow dose increase.[44]

However, more trials are needed to evaluate the role of this drug alone or in combination within multicenter trials. There are diverse more substances (e.g. dasatinib, flavopiridol, ABT 263) with promising toxicity and efficacy profiles, which may hopefully be evaluated in elderly or comorbid CLL patients and broaden the spectrum of treatment options in these patients within the near future.

Relapse treatment

Several dose-reduced regimens have been proposed for elderly and comorbid CLL patients in relapsed or refractory situation. A dose-reduced regimen with fludarabine alone showed mild toxicity rates and favorable response rates in relapsed CLL.[45] In contrast, fludarabine-based combination regimen in relapse treatment of elderly CLL patients might be associated with higher toxicity rates. A phase II trial evaluated fludarabine-based regimen combining fludarabine, cyclophosphamide and mitoxantrone in 32 previously treated elderly patients.[46] All included patients were older than 65 years. However, these treatment regimens were associated with an excessive toxicity rate in the elderly: 22 out of 32 included patients developed neutropenic fever or severe bacterial infection. Moreover, only 10 patients completed six courses of treatment, because of poor compliance due to the toxicity.[46] Another phase II trial evaluating a dose-reduced combination of fludarabine and cyclophosphamide in relapse setting showed better results. Twenty-eight elderly patients received four courses of fludarabine (25 mg/m² i.v. d1–4) plus cyclophosphamide (150 mg/m² i.v. d1–4) repeated every 4 weeks. Due to an overall response rate of 89% and a

low hematological toxicity rate, this regimen seems very promising for relapse treatment of the elderly.[47] Similar data were obtained by the same group evaluating the FC regimen with a different dosing regimen using 15 mg/m^2 of fludarabine and 200 mg/m^2 of cyclophosphamide for 4 consecutive days.[48] Again clinical toxicity in 20 elderly patients with refractory CLL was mild and the response rates were favorable. However, multicentre trials will have to show if dose-reduced FC combination with or without antibodies are feasible in elderly CLL patients with reduced physical fitness and relevant comorbidity burden.

Another phase II trial evaluated the combination of bendamustine plus rituximab in relapsed setting.[49] Bendamustine was administered in a dose of 70 mg/m^2 for 2 days combined with rituximab 375 mg/m^2 on cycle 1 and 500 mg/m^2 on cycles two to six. Twenty nine of the 78 patients (37%) were 70 years or older. The overall response rate among all patients was 59%, including 9% CRs.[49] Response rates in patients above the age of 70 years were comparable to those with younger age. Though data on comorbidity burden using the CIRS were not assessed within this trial, the combination bendamustine and rituximab appears to be a good relapse treatment option in elderly CLL patients.

However, in relapse situation bone marrow recovery may be significantly reduced in comorbid patients of higher age. Therefore, treatment regimens with very mild myelotoxicity are warranted for this group. More recently, the anti CD20-antibody ofatumumab has been approved for treatment of refractory CLL based on the interim analysis of a trial, including 138 patients refractory to fludarabine and alemtuzumab (FA-ref; n=59) or refractory to fludarabine with bulky lymphadenopathy (BF-ref; n=79).[37] The ORR was 58% for the FA-ref group and 47% for the BF-ref group and median time to progression was 5.7 and 5.9 months, respectively. Beside some usually mild infusion-related adverse events at the first application of ofatumumab, the main toxicities were infections and neutropenia.[37] High dose methylprednisolone (HDMP, 1 g/m^2 for five days) in combination with rituximab (375 mg/m^2 weekly for 4 weeks) has also proven to be effective in patients with advanced, fludarabine resistant CLL showing an overall response rates of 93% and a complete remission rate of 36% in a small trial including 14 patients.[50] The median time-to-progression was 15 months and the median time-to-next treatment was 22 months. Treatment was well tolerated and serious adverse events were rare.[50]

However, if the relapse or progression occurs at least 12 months after a monotherapy or 24 months after chemoimmunotherapy, first line regimen might be repeated in the relapse setting.[51]

Disturbances of the immune system

Most of the patients develop a severe immune defect during the course of their disease. The immune defects are both quantitative and qualitative and are associated with an impaired humoral and cellular immune response. More than 70% of CLL patients develop severe hypogammaglobulinemia,[52] which correlates with an increased risk of microbiological infections. The use of prophylactic intravenous immunoglobulin may reduce the incidence of less severe infections,[53] but does not have an impact on overall survival.[54] Because even a low-dose treatment with intravenous immunoglobulin is not a cost effective way to prevent infection in CLL patients, only selected patients with a very high risk of bacterial infection should receive immunoglobulin substitution.[55]

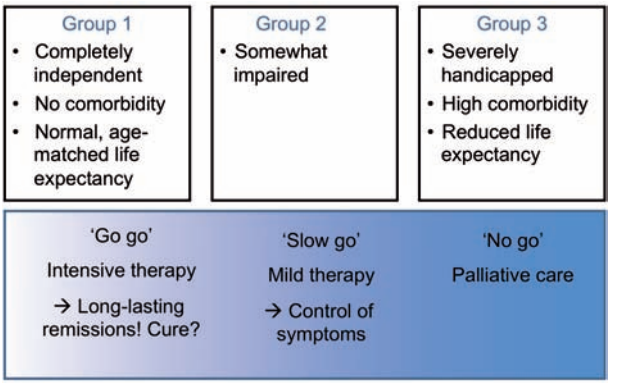

Figure 1. Classification of treatment goal by geriatric assessment.[74]

Regarding the use of antibiotic prophylaxis no standard guidelines for CLL patients independent from their physical fitness or age exist. There is no clear evidence to use routine application of anti-infectives in first line therapy of younger patients[56] with the exception of pneumocystis jiroveci prophylaxis during prolonged neutropenia and CMV prophylaxis during alemtuzumab administration. However, some advocate the use of antiviral prophylaxis in elderly CLL patients, because high rates of infectious complications may occur especially with purine analogue-based combination therapies.[46] Pneumococcal and seasonal influenza vaccines are generally used in CLL patients, though response to vaccination may be suboptimal. Elderly CLL patients have probably lower response rates to vaccination as demonstrated by one study showing a negative correlation between higher age and response to haemophilus vaccine.[57] Therefore, vaccination in early stage CLL should be considered, because adequate antibody response was more frequent in patients with lower stage CLL.[58]

Besides infectious complications autoimmune diseases may occur as an expression of the immune defect as well. Special attention should be paid to the appearance of autoimmune hemolytic anemia (AIHA) and autoimmune thrombocytopenia (AITP) that occur in 4–11% of CLL patients.[59-61] Prognosis of CLL patients with AIHA has been significantly improved during the past decades, which is mainly due to improved diagnostic procedures and to more treatment options in relapse of autoimmune cytopenia.[61,62] Though there are no special recommendations for treatment of autoimmune cytopenias in elderly patients, the first line treatment of choice in these patients should be the use of corticosteroids such as in younger patients. Besides high dose immunoglobulines, cyclophosphamide, cyclosporine or other immune suppressive medication, the antibody rituximab represents an alternative treatment option in the relapse situation of AIHA or AITP.[63]

Table 2. Treatment recommendation for patients with reduced physical fitness (SLOW GO).

Binet Stage	**Prognostic factors**	**First line treatment**
A, asymptomatic B	not relevant	None, except in clinical trials
C, symptomatic B	all, except 17p-	CLB
	17p-	No standard; try alemtuzumab (plus steroids); clinical trials
	Prognosis	**Second line treatment**
Early PD (< 1–2 years) = refractory disease	all, except 17p-	No standard; try dose reduced FC or FCR, BR, PCR
	17p-	Alemtuzumab; within clinical trials: lenalidomide
Late PD (> 1 -2 years)	all	repeat first line

PD = progressive disease, CLB = chlorambucil, F = fludarabine, C = cyclophosphamide, R = rituximab, P = pentostatin, B = bendamustine.

Supportive care and quality of life

Treatment strategies in elderly CLL patients should rather aim for symptom control than for induction of high complete remission rates. Therefore, besides supportive care, improvement of health-related quality of life should play an important role in this group of patients. However, so far only a minority of clinical trials evaluated health-related quality of life in elderly CLL patients.

One of the major problems in the treatment of elderly patients is the impaired capacity of bone marrow recovery. The role of hematopoietic growth factor administration in patients with CLL is not clear, because data from randomized trials are missing. In patients with solid tumors erythropoietin (ESP), administration may have a negative impact on survival.[64,65] Therefore, hematopoietic growth factors (G-CSFs, ESPs) should be used only in accordance with the current guidelines in CLL patients.[66,67]

Another problem in the elderly is an increased rate of impaired renal function, which can be frequently detected also in patients with normal serum creatinine, when the creatinine clearance is calculated according to the Cockcroft formula.[68] Tumor lysis syndrome was an uncommon complication in CLL so far.[69] However, with the introduction of more effective chemoimmunotherapy regimens, tumor lysis syndrome might become more frequent, especially in those patients with an impaired renal function. Along with hydration and urinary alkalinization, allopurinol should be used in the elderly to accompany chemotherapy. The administration of rasburicase in patients with high uric acid serum levels might be indicated.[70]

Though larger studies are pending so far, health-related quality of life is a major issue in the elderly. Possible changes of quality of life should be considered before treatment initiation. On the one hand, quality of life decreases very fast in elderly patients as soon as they become more symptomatic.[71,72] On the other hand, health-related quality of life might decrease due to the toxicities of chemotherapeutic regimen. Moreover, socio - demographic factors, such as marital status or employment play a major role for quality of life.[73] Therefore, the treatment decisions in elderly are carefully to be made in each patient individually considering also the patients' physical condition and his social environment.

Conclusion

Treatment decisions in elderly CLL patients need special consideration due to the fact that aggressive treatment regimens are mostly poor tolerated in this group of patients. On the other hand, treatment strategies in the elderly focus rather on symptom control than on the induction of high complete remission rates. Due to the relevance of the burden of comorbidity in cancer patients, it appears reasonable to distinguish rather between physically fit and non-fit patients for treatment decisions than between old and young patients (Figure 1). By using tools as the CIRS score three different patient groups can be distinguished:

1. Medically fit patients with no or mild co-morbidity and a normal life expectancy. These patients should be treated intensively with chemoimmunotherapy, irrespectively of their chronological age (principle of action: 'GO GO'). However, whenever possible, treatment of such patients should be performed in the context of a clinical trial.
2. Medically less-fit patients with multiple or severe comorbidities and an unknown life expectancy. Practitioners should try to enroll such patients in a clinical trial designed for comorbid CLL patients. Outside clinical trials, the treatment should be carefully adapted to the comorbidity burden and a higher risk of both toxicity and disease progression must be kept in mind (principle of action: 'SLOW GO').
3. Medically frail patients with fatal comorbidities and a very short life expectancy. These patients will not benefit from any CLL treatment and therefore should not receive any chemotherapeutic drugs (principle of action: 'NO GO').

For the elderly or 'SLOW GO' group of patients, chlorambucil still remains the standard treatment of choice, until randomized settings have shown that chlorambucil plus antibody is significantly more effective (Table 2). Within clinical trials, purine analogue-based regimens

(FC, FCR, and PCR) with reduced dosages are evaluated. Once therapy has started, further dose reductions or pre-emptive interruption of the treatment should be avoided. A sophisticated comedication for concomitant diseases is essential to minimize the risk of fatal interaction between the leukemia and additional health problems. Hopefully, current research efforts will rapidly result in diagnostic tools for a more reliable identification of 'SLOW GO' patients and provide evidence-based therapeutic strategies for this large group of elderly and comorbid CLL patients that has not been properly addressed by CLL researchers so far.

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Mechanisms of resistance in chronic myeloid leukemia stem cells

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A B S T R A C T

Chronic myeloid leukemia (CML) has emerged as a paradigm for cancer stem cells (CSCs); including leukemic stem cells (LSCs). Arising from a chromosomal translocation within an hemopoietic stem cell (HSC), which generates the Philadelphia (Ph) chromosome, the resulting oncogenic protein, BCR-ABL1, drives the disease. Targeted therapy against this constitutively active tyrosine kinase with tyrosine kinase inhibitors (TKIs) has revolutionized the treatment of CML. While high rates of response have been achieved, BCR-ABL1 gene transcripts can still be detected in the majority of patients, suggesting residual disease. This disease persistence has been attributed to a small pool of drug-resistant CML LSCs. Consequently, in recent years, the major focus of CML research has been to identify and target the molecular mechanisms that confer drug-resistance on this LSC pool. In this review, we provide a comprehensive overview of what is currently known about the mechanisms that mediate survival of CML LSCs and the novel therapies emerging as a consequence to target these pathways.

Introduction

Over recent years, the historical view that malignant tumors comprise a uniform population of cells, each able to recapitulate the neoplastic phenotype, has been challenged. In many cancers, the existence of small populations of tumor cells, which are refractory to therapy, has long been appreciated, leading to the cancer stem cell (CSC) hypothesis, which proposes that tumors have a hierarchical structure of differentiation akin to normal tissues.[1] Only the CSCs have the ability to self-renew and perpetuate the tumor, and are therefore considered the source of disease relapse. CSCs have been identified, and at some level characterized in solid tumors, including breast, brain, and prostate cancer.[1] In the hemopoietic system CSCs, referred to as leukemic stem cells (LSCs), were first demonstrated in acute myeloid leukemia (AML) by Dick *et al.* in 1994, who isolated a subpopulation of CD34+CD38- cells able to induce AML on serial transplantation in mice.[2] In this study, the LSCs comprised a tiny fraction of the leukemic bulk, only around 1 in 250,000 of the malignant cells. LSC in chronic myeloid leukemia (CML) were identified in 1999,[3] and this disease has become a paradigm of the CSC hypothesis. CML arises from a balanced translocation between the long arms of chromosomes 9 and 22 within a hemopoietic stem cell (HSC), which forms a constitutively active tyrosine kinase, BCR-ABL1.[4] Moreover, BCR-ABL1 expression within murine HSCs consistently leads to a myeloproliferative disorder in murine model systems.[5]

CML is also credited as being at the forefront of targeted cancer therapy, following the advent of imatinib mesylate, a small-molecule that inhibits BCR-ABL1 activity by binding directly to the kinase domain.[6] Preclinical studies demonstrated imatinib to exert anti-proliferative and pro-apoptotic effects on CML cells *in vitro* and *in vivo*,[6] supporting the hypothesis that the malignant cells in CML are *kinase-addicted*. The pivotal phase III clinical trial of imatinib in CML, the IRIS study, demonstrated imatinib to induce complete cytogenetic response in just under 87% of patients.[7] Despite these promising results, it is now appreciated that in most patients, detectable levels of minimal residual disease (MRD) persist on therapy,[7] and molecular relapse is frequent on drug discontinuation.[8] Elegant mathematical models of imatinib in CML demonstrate a biphasic early response, with rapid elimination of differentiated cells followed by slower depletion of proliferating progenitors, but appear unable to predict whether tyrosine kinase inhibitors (TKIs) are likely to eliminate the LSC compartment.[9,10] Fluorescence-activated cell sorting (FACS) analysis of chronic phase CML patient samples revealed that whilst the majority of malignant stem cells were actively recruited within the cell cycle, a subpopulation of quiescent (G0) cells could be consistently isolated.[3] Our laboratory has shown that these quiescent BCR-ABL1+ve stem cells are resistant to the apoptotic effects of imatinib.[11] Moreover, we found the TKI to exert an antiproliferative effect on the quiescent LSC compartment, which may further contribute to the persistence of MRD. In support of this hypothesis, LSCs have been shown to accumulate within the bone marrow during TKI treatment in a CML mouse model.[12] As LSCs are believed

to be the source of additional genetic aberrations leading to therapy resistance and progression to blast crisis, understanding the molecular mechanisms governing CML LSC survival and drug resistance has emerged as the most prescient area of investigation currently facing CML researchers. This review provides a comprehensive overview of current understanding and clinical advances in the field.

Mutations

Genetic aberrations in a number of genes can lead to therapy resistance and disease progression. More than 50 different mutations associated with the kinase domain of BCR-ABL1 that confer variable levels of resistance specifically to TKI therapy[13–15] have now been identified in clinical samples. As a result, second generation TKIs have been developed, which can target the majority of mutated forms of BCR-ABL1; however, some mutations, such as T315I, are resistant even to these agents. These mutations are likely the result of defects that arise in the cell's DNA repair mechanisms due to BCR-ABL1 expression,[16] rendering the cells genetically unstable. However, whether BCR-ABL1 mutations have any major impact on the resistance and survival of LSCs is unclear. Even LSCs, which harbor wild type BCR-ABL1, have been shown to be resistant to TKIs;[11] therefore, mutations may be important for acquired drug resistance and, in some cases, progression of the disease to blast crisis, but not for disease persistence.

BCR-ABL1 kinase addiction

Another possible explanation for LSCs being more resistant to TKIs than progenitor cells is that they are less sensitive to the drugs. Primitive CML cells have been shown to have higher levels of BCR-ABL1 than progenitors, which could result in decreased sensitivity to TKIs.[17,18] Also, it has been shown that CML stem cells have increased expression of certain drug efflux pumps, which could affect their response to treatment.[18] Whether these pumps are actually involved in the transport of TKIs is unclear.[19]

Recent evidence from our laboratory and others has called into question whether CML LSCs require BCR-ABL1 kinase activity for survival at all. Corbin *et al*. have shown that while BCR-ABL1 activity can be efficiently inhibited by imatinib in both quiescent stem and progenitor CML cells, this activity is not required for survival of the cells, that is, they are not 'kinase addicted'.[20] Data from our own laboratory further demonstrates that a primitive subset of CD34+ CML cells, as judged by long-term culture-initiating cell (LTC-IC) assay, can survive complete inhibition of BCR-ABL1 for 12 days in growth factor-free medium. In addition, using an inducible transgenic mouse model of CML, they have shown that LSC retain their transplantation and leukemogenic potential following complete reversion of BCR-ABL1.[21] If the CML stem cells do not require BCR-ABL1 activity for survival, this begs the question: how do they survive and how can we target them to eradicate CML?

Quiescence

The quiescent status of a proportion of CML stem cells may play an important role in survival of these cells, even in the absence of BCR-ABL1 activity. Indeed in some instances, TKIs may actually help to promote the quiescence and survival of the stem cells by activating pathways involved in this process. As a result, a major focus of CML research in recent years has been to identify pathways, which can be manipulated in order to induce quiescent CML LSCs into cycle so they can be targeted by TKIs.

FOXO

Work from our own laboratory and Naka *et al.* suggests that the FOXO family of transcription factors may play a part in protecting CML stem cells. FOXO transcription factors are involved in the induction of cell cycle arrest and apoptosis[22] and are negatively regulated by BCR-ABL1 through the PI3 kinase/AKT pathway.[23,24] They have also been shown to play a critical role in the maintenance of normal HSCs.[25] In bulk CML cells, FOXO3a is normally inactive and localized to the cytoplasm; however, inhibition of BCR-ABL1 by TKIs has been shown to activate FOXO, inducing cell cycle arrest and allowing the progenitor population to survive in the presence of TKIs.[26] In addition, it has been shown that FOXO3a is still active in the nucleus in a proportion of CML stem cells even in the absence of TKI.[26,27] Interestingly, Corbin and colleagues noted that pAKT levels were lower in a proportion of the CML stem cells compared with the progenitors and that the residual activity was not inhibited further by imatinib.[20] This would correlate with the presence of nuclear FOXO3a in this population. We have seen a similar reduction in downstream signaling from BCR-ABL1 in the most primitive CML CD34+CD38-CD90+ stem cells. In our hands, pAKT, pSTAT5, and Bcl2 levels were all reduced compared with CD34+CD38+ progenitors, but pCrkl levels were unaffected (Pellicano *et al.,* submitted to Blood, March 2011). These results suggest that BCR-ABL1 downstream signaling pathways are differentially regulated in CML stem cells compared with progenitors, that is, while signaling to pCrkl is intact, other signaling pathways are inhibited. FOXO transcription factors, and FOXO3a in particular, maintain normal HSCs through regulating reactive oxygen species (ROS) levels.[27] Given that LSCs are subject to hypoxic conditions within the stem cell niche, and to increased ROS and cytokine levels mediated by BCR-ABL1, it is possible that the signals positively regulating FOXO activity are over-riding the negative signals from BCR-ABL1. Indeed, TGF-beta signaling was shown to play a role in mediating the nuclear localization of FOXO in these primitive CML cells.[27] This being the case, BCR-ABL1 inhibition by TKIs would have little effect on this quiescent population and may even promote its survival. Indeed, FOXO transcription factors have been shown to be upstream of a number of genes, which are up-regulated during autophagy, a process that has been implicated in the survival of CML stem cells.[28] Targeting FOXO, potentially through inhibition of TGF-beta,[27] therefore, could be a means of inducing CML stem cells into cycle in order for them to be targeted by TKIs.

Promyelocytic leukemia protein

Promyelocytic leukemia protein (PML) is another protein that has been shown to be essential for maintaining quiescence of normal HSCs. It is a tumor suppressor protein, which is localized to PML nuclear bodies, and plays a role in cell cycle, senescence, and apoptosis.[29,30] The name is derived from acute promyelocytic leukemia (APL), where a t(15;17) chromosomal translocation results in an oncogenic fusion protein between PML and retinoic acid receptor alpha.[31] PML has been shown to be highly expressed in the HSC compartment, with primitive cells containing increased numbers of PML nuclear bodies.[32] Knockout of PML resulted in a loss of HSCs in control mice and of LSC in a mouse model of CML. Additionally, inhibition of PML by arsenic trioxide induced the LSCs into cycle and sensitized them to killing by cytarabine.[32] Thus, PML represents another key player in the maintenance of quiescence, which could be targeted to induce CML stem cells into cycle for killing by conventional therapies. Indeed a Phase II clinical trial has already been completed combining arsenic trioxide with imatinib in patients who failed to achieve complete cytogenetic response with imatinib.

Self-renewal

Wnt

In addition to strategies designed to promote entry of quiescent LSC into cell cycle to sensitize them to TKIs, an extension of this approach is to attempt to influence the outcome of subsequent cell division. Promotion of the generation of committed progenitor cells as progeny rather than identical daughter HSCs would have the effect both of depleting LSCs and of differentiating the cells to a stage amenable to TKI therapy. The Wnt signaling pathway is essential for normal stem cell maintenance, both in solid-organs and hemopoietic tissues.[33] Wnt proteins are secreted by HSCs and stromal cells within the niche. Binding of Wnt proteins to frizzled receptors on the HSC membrane activates the canonical pathway, in which β-catenin is stabilized, translocates to the nucleus, and effects transcriptional activation.[33] β-catenin has been shown to be essential for long-term maintenance of murine HSCs, and notably conditional β-catenin knockout *in vivo* blocks the development of BCR-ABL1 driven CML, whilst the generation of acute lymphoblastic leukemia (ALL) is unaffected.[34] Hu *et al.* confirmed β-catenin to be essential for serial transplantation of CML in mice.[12] Of interest, granulocyte-macrophage progenitors (GMP) from blast crisis CML patients have been demonstrated to have increased levels of nuclear β-catenin, and inhibition of β-catenin by axin reduced *in vitro* self-renewal.[35] Overexpression of β-catenin in blast crisis CML may be driven by missplicing of glycogen synthase kinase 3 beta (GSK3β).[36] Recently, the addition of indomethacin, a pharmacological inhibitor of β-catenin, to imatinib therapy has been demonstrated to deplete LSC *in vivo* and prolong survival in a BCR-ABL1+ve CML murine model.[37] Using an anti-sense RNA knockdown approach, Gregory *et al.* identified that non-canonical Wnt signaling, which involves calcium mobilization leading to activation of the transcription factor nuclear factor for activated T cells (NFAT), is active in BCR-ABL1 positive cells.[38] Inhibition of this pathway sensitized BCR-ABL1 positive cells to TKI therapy *in vitro* and *in vivo*; however, the involvement of non-canonical Wnt signalling in maintenance of chronic phase CML LSCs remains to be determined.

Hedgehog

Hedgehog signaling has also recently been established to be required for the maintenance of chronic phase CML LSCs. Secreted Hedgehog ligands bind to the transmembrane Patched (Ptch) receptors on the stem cell membrane, which de-represses Smoothened (Smo), a G-protein coupled receptor, which when active, positively regulates transcription of genes, including Ptc and Gli.[39] Both Gli1 and Ptch1 are overexpressed in chronic phase CML CD34+ cells compared with normal HSCs, and both Smo and Gli1 can be detected by immunohistochemistry in bone marrow of CML patients.[40] Notably, in a CML mouse model, Smo and Gli1 overexpression was found to be largely independent of BCR-ABL1.[40] Interest in pharmacological inhibition of Hedgehog signaling grew following the demonstration that the Smo inhibitor cyclopamine induced apoptosis and reduced colony-forming ability of BCR-ABL1+ve LSCs *in vitro.*[40] In addition, this group showed that Smo knockout inhibited the expansion and serial transplantation capacity of BCR-ABL1 transfected HSCs *in vivo*.[40] Zhao *et al.* reported similar results, with Smo knockout in HSCs decreasing the ability of BCR-ABL1 to induce CML-like disease in transplant recipients from 94 to 47%.[41] Conversely, constitutively active Smo promoted the development of CML in a BCR-ABL1 mouse model.[41] Both groups demonstrated the combination of a TKI and cyclopamine to inhibit colony formation of primary CML cells in LTC-IC assay by more than either drug alone.[40,41] Moreover, the addition of cyclopamine to nilotinib reduced BCR-ABL1 positive colony forming potential by 40% compared with nilotinib alone in a murine transplantation model.[40] We have reported similar results using the combination of nilotinib and another small molecule Smo inhibitor, LDE225,[42,43] suggesting the potential efficacy of this combination in eradicating CML LSCs. As a result, a Phase II clinical trial is now underway examining the safety and tolerability of dasatinib in combination with the Smo inhibitor BMS-833923 in CML patients resistant or with suboptimal responses to imatinib (ClinicalTrials.gov NCT01218477).

Stem cell niche

Normal HSC behavior is governed by cellular interactions with a specialized microenvironment within the bone marrow termed the 'stem cell niche'.[44] A complex network of paracrine signals control HSC homing to endosteal and perivascular locations within the bone marrow, in which self-renewal and quiescence are promoted. It is now appreciated that CML stem cells remain able to respond to pro-survival microenvironmental signals, which may contribute to chemoresistance. Inhibition of such physiological signaling has the

potential to prevent LSCs from accessing such sanctuary sites, or to mobilize them from these sites, and has received attention as a novel therapeutic strategy to re-sensitize LSCs to TKIs.

CXCR4

The most comprehensively investigated target to date is the CX chemokine CXCL12 signaling pathway. CXCL12 is expressed and secreted by stromal cells within the bone marrow, signals solely through the membrane receptor CXCR4, and has been reported to control HSC chemotaxis and quiescence.[45,46] CML CD34+ cells from chronic phase and blast crisis patients express significantly lower levels of CXCR4 than their normal counterparts, correlating with impaired migratory capacity towards CXCL12.[47] Jin *et al.* confirmed CXCR4 expression to be negatively regulated by BCR-ABL1, and restored to normal levels on imatinib treatment of blast crisis CML patients *in vivo*, leading to the hypothesis that TKI treatment may increase the ability of LSCs to access the stem cell niche. In support of this, imatinib treatment increased migration of CML cell lines towards CXCL 12/SDF-1 *in vitro* and elevated CXCR4 expression on CD34+ cells within the bone marrow of CML patients.[47] Preliminary work within our laboratory suggests that the CXCR4 antagonist plerixafor (AMD3100) sensitizes CML stem cells to TKI treatment *in vitro,*[48] and *in vivo* studies are ongoing.[49]

JAK

In addition to chemokines, cytokines are also implicated in BCR-ABL1-independent maintenance of CML stem cells. Hiwase *et al*. recently reported that a cocktail of Flt3-ligand, stem cell factor, interleukin (IL)-3. IL-6, and granulocyte colony-stimulating factor promotes survival of CML LSCs in the presence of TKI treatment.[50] This group, therefore, investigated a small molecule Janus tyrosine kinase (JAK), a cytokine signaling intermediary inhibitor, and demonstrated that it re-sensitized CML CD34+ cells to dasatinib in the presence of growth factors,[50] suggesting this class of agent to be worthy of further study.

Autophagy

Autophagy has also been shown to play a role in the survival of CML stem cells in response to TKIs. Autophagy is a cell survival process that mediates the breakdown of intracellular material to protect the cells from cell death during times of stress, such as starvation, which leads to loss of growth factor signaling.[51] Bellodi and colleagues noted that CML cell lines that survive TKI have an increased number of cytoplasmic vacuoles, an indicator of autophagy. Subsequent investigation confirmed the induction of autophagy in response to imatinib in CML cell lines and primary CML cells, an effect that appeared to be mediated by imatinib-induced ER stress. While autophagy can result in Type II cell death, in CML cells, it clearly induced a survival response as inhibition of autophagy by chemical or genetic means potentiated imatinib-induce cell death. Importantly, treatment of primary CML cells with TKIs in combination with the autophagy inhibitor chloroquine resulted in near ablation of the CML stem cells.[28] This suggested that inhibiting autophagy in combination with TKI treatment could be a means of eradicating CML[52] and has consequently led to the CHOICES Phase II clinical trial combining hydroxychloroquine with imatinib (ClinicalTrials.gov NCT01227135).

LSC versus HSC

Exploiting normal stem cell signaling pathways as therapeutic targets will inevitably impact on the normal HSC compartment. As within chronic phase CML patients, normal HSCs are reported to greatly outnumber LSCs.[53] A therapeutic window may exist for these novel therapeutic approaches to translate successfully to the clinic, so a degree of selectivity for LSCs is desirable. As BCR-ABL1 activity per se does not seem to be so important in the LSC compartment, targeting other proteins/pathways, which are deregulated in LSCs compared with normal HSCs, may be a means of eradicating this persistent population. A number of proteins have already been identified which could be targets for therapy.

Alox5

Alox5, the gene that transcribes for Arachidonate 5-lipooxygenase (5-LO), was shown to be upregulated in BCR-ABL1+ve LSCs but was not modulated by imatinib, suggesting its expression to be independent of BCR-ABL1 kinase activity.[54] This enzyme, which converts arachadonic acid into leukotrienes[55] and its products, has been shown to be upregulated in a number of cancers and is thought to be involved in proliferation and survival of tumor cells.[56] Inhibition of 5-LO in blast crisis CML cells *in vitro* resulted in an inhibition of proliferation and induction of apoptosis,[57] while knockout of 5-LO or inhibition with zileuton in a mouse model of CML affected LSC survival and inhibited CML development.[54] These results suggested that zileuton, which is already in clinical trials for the treatment of asthma, could be a novel therapy that would specifically target LSCs but not normal HSCs.[54] Indeed, there is an ongoing Phase I clinical trial looking at the safety of zileuton in combination with imatinib in CML patients.

PP2A

Another protein that is deregulated in CML is PP2A (protein phosphatase 2A). PP2A is a serine threonine phosphatise, which has been shown to have a role in cell survival, proliferation, and differentiation, and acts as a tumor suppressor protein.[58)] In CML chronic and blast crisis CD34+ cells, PP2A's activity was inhibited through upregulation of the phosphoprotein SET by BCR-ABL1. This inhibition was associated with disease progression and appeared to require increased phosphorylation of PP2A at tyrosine 307. Interestingly BCR-ABL1 activity itself was regulated by PP2A, and re-activation of PP2A interfered with the leukemogenic activity of BCR-ABL1.[59] Indeed an activator of PP2A, FTY720, which is already in Phase III clinical trials for multiple sclerosis, was shown to have efficacy in the treatment of blast crisis CML and BCR-ABL1 positive ALL *in vitro* and *in vivo.*[60] In addition, recent evidence suggests the

FTY720 and its derivatives are able to target BCR-ABL1+ve LSCs, while sparing normal HSCs,[61] making this a very interesting therapeutic agent for the treatment of CML.

IL-1RAP

IL-1RAP (IL-1 receptor accessory protein) is a component of the receptor complex for members of the IL-1 family of cytokines.[62] Using gene-expression profiling, Jaras and colleagues identified IL-1RAP as an *integral to plasma membrane* protein up-regulated in CML CD34+ cells in a BCR-ABL1-dependent manner. Within the primitive CML CD34+CD38- population, this protein was shown to be up-regulated only in the Ph+ve cells and not in the normal Ph-ve cells.[63] These results indicate that IL-1RAP could be a cell-surface biomarker for the identification and isolation of BCR-ABL1 positive LSCs. In addition, the authors generated an antibody against IL-1RAP, which exerted antibody-dependent cell-mediated cytotoxicity (ADCC) against CML CD34+38- cells but not normal cells.[63] These findings identify not only a cell surface biomarker for primitive CML stem cells, but also novel means of targeting this resistant population.

Epigenetic changes

Abnormalities in epigenetic control of gene expression are implicated in many human cancers. Within cell nuclei, DNA is associated with histone proteins, which undergo modification by acetylation and methylation, resulting in changes in chromatin structure, which ultimately determine gene transcription.[64] The p210 BCR-ABL1 fusion protein has been demonstrated to be associated with histone H4 hyperacetylation.[64] Histone deacetylase (HDAC) inhibitors have been shown to induce apoptosis of CML cell lines and blast crisis CML primary cells;[65–67] however, effects on the chronic phase LSC compartment have only recently been studied. Zhang *et al.* demonstrated that addition of the HDAC inhibitors LBH589 or LAQ824 to imatinib significantly enhanced apoptosis induction in quiescent primary CML CD34+CD38- progenitors, reduced engraftment potential of BCR-ABL1 progenitors in a murine transplantation model, and depleted BCR-ABL1 positive LSCs in a murine transgenic system.[68] HDAC inhibitors alone exerted minimal effects, demonstrating that whilst CML LSCs are not solely dependent on BCR-ABL1, inhibition of BCR-ABL1 along with a second target is likely to be required for LSC eradication. Further analysis of gene expression in CML progenitors treated with combination TKI and HDAC therapy revealed down-regulation of HOX-, MYC-, and WNT-related pathways.[68] Given the multitude of BCR-ABL1-independent survival mechanisms implicated in the maintenance of CML LSCs, therapies with the potential to simultaneously inhibit several signaling pathways are clearly desirable.

Rac GTPases

Very recently, evidence has emerged that the activity and function of Rac GTPases may differ between normal and CML stem cells. In normal HSCs, Rac GTPases regulate homing, survival, and proliferation.[69] In an inducible CML murine model, knockout of Rac2 GTPase reduced LSC generation and prolonged survival compared with Rac2 GTPase WT controls.[69] Notably, no significant effect on HSC number or proliferation was seen in BCR-ABL1 negative controls, suggesting a LSC-selective effect.

Conclusions

The demonstration that the persistence of LSCs in CML is not solely dependent on the oncoprotein BCR-

Target	Compound	Pre-Clinical Data	Clinical Status*
Arachidonate 5-lipoxygenase (Alox 5)	Zileuton	Zileuton inhibited CML development *in vivo* (53).	Phase I trial in combination with imatinib.
Autophagy	Hydroxychloroquine	Chloroquine + TKI caused near ablation of CML LSCs *in vitro* (28).	Phase II trial of imatinib ± hydroxychloroquine in patients with persistent MRD.
Hedgehog signalling	Smo inhibitor	Smo inhibition depletes CML LSCs *in vitro* and *in vivo* (40-43).	Phase II trial of Smo inhibitor (BMS-833923) in combination with dasatinib in patients refractory to or with suboptimal response to imatinib.
Histone Acetylation	Panabinostat Sodium valproate	HDAC inhibitor induce apoptosis of quiescent CML LSCs *in vitro* and reduce serial transplantation *in vivo* (65).	Phase I study in combination with imatinib in patients in CCR with evidence of residual disease. Phase II study of imatinib and sodium valproate in patients with persistent MRD.
PML	Arsenic trioxide	Sensitized LSCs to killing by AraC (32).	Phase II study of arsenic trioxide in combination with imatinib in patients failing to achieve CR on imatinib alone.

* Further details of clinical studies can be found at http://clinicaltrials.gov.

Table 1. Current status of investigational agents designed to eliminate CML stem cells in chronic phase CML patients.

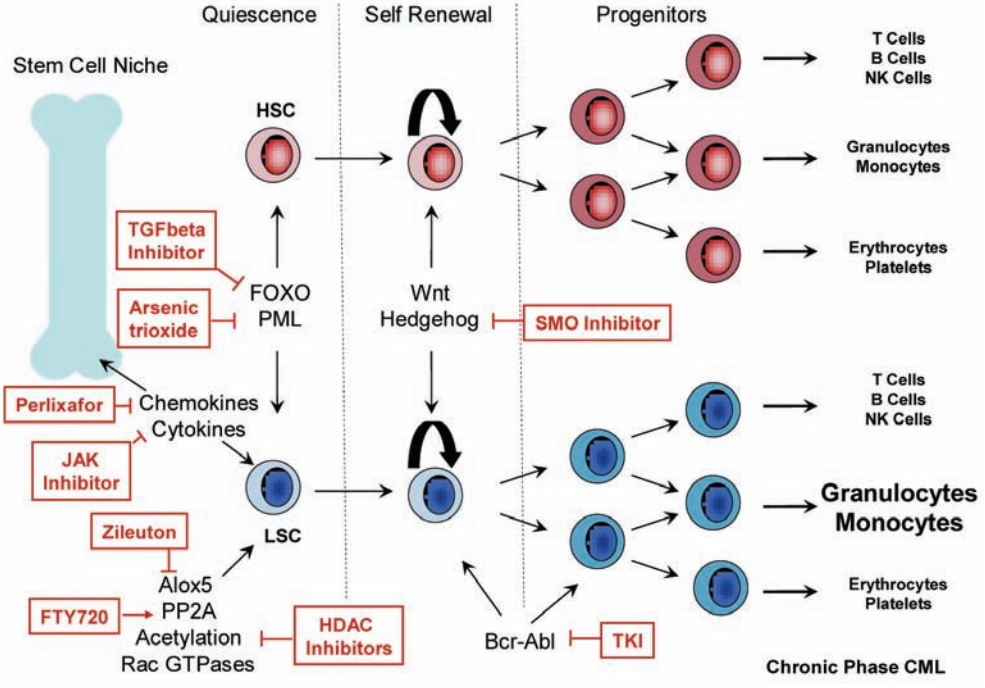

Figure 1. Summary of our current understanding of the molecular mechanisms that mediate leukemic stem cell resistance in chronic phase CML, and the therapeutic agents being used to target this resistant population.

ABL1 confirms that eradication of disease will require combination therapy. The research described herein, focusing on identifying and exploiting differences between normal and CML HSCs, and identifying ways to push the drug-resistant LSCs into cycle so that they can be targeted by TKIs, has led to the identification of several novel therapeutic targets (Figure 1). Consequently, a number of new and ongoing clinical trials have emerged from this basic research (Table 1). These findings and the resulting clinical trials combining LSC-directed therapy with TKIs represent an exciting step forward in the treatment of CML and lead us closer to the ultimate goal of curing the disease.

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Management of newly diagnosed chronic myeloid leukemia patients

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A B S T R A C T

The BCR-ABL1 tyrosine kinase inhibitor (TKI) imatinib has been the standard first-line therapy for chronic myeloid leukemia (CML) for almost 10 years. High-dose imatinib and imatinib in combination with interferon alpha are options to increase the efficacy of TKI therapy. Dasatinib and nilotinib, two newer drugs with higher potency than imatinib against BCR-ABL1 and activity against most imatinib-resistant BCR-ABL1 mutations, have each shown superior efficacy compared with imatinib for first-line treatment of chronic-phase CML in randomized phase 3 trials. With 18–24 months follow-up time, no differences in overall survival were observed between imatinib and the second generation drugs. Available data suggest no differences in efficacy between dasatinib and nilotinib. Compared with imatinib, dasatinib is associated with higher rates of pleural effusion and thrombocytopenia, but lower rates of edema, gastrointestinal, or musculoskeletal adverse effects, and rash. Nilotinib is associated with higher rates of dermatologic toxicity and biochemical abnormalities associated with hepatic and pancreatic toxicity compared with imatinib, but lower rates of edema, gastrointestinal adverse effects, muscle spasms, and neutropenia. New studies are in preparation or ongoing to test the option to cure CML with novel TKI alone or in combination.

Introduction

The evolution in the understanding of the biology of chronic myeloid leukemia (CML) that eventually translated into the development of specific, highly effective therapy is unparalleled in oncology. In 1960, a minute G-chromosome was identified in patients with CML.[1] In 1973, the abnormality was more specifically described as a balanced translocation between chromosomes 9 and 22,[2] with the formation of a chimeric gene, BCR-ABL1[3] that translates into a protein with increased and dysregulated tyrosine kinase activity.[4] The realization of the critical role this protein plays in the pathogenesis of CML[5] led to a search for specific inhibitors and resulted in the development of imatinib.[6] After a series of clinical trials, imatinib became standard therapy in CML. The IRIS trial in which patients with CML in chronic phase (CP) were randomized to receive imatinib or interferon alpha (IFNα) plus ara-C established imatinib as the standard therapy.[7] With 8 years of follow-up on this study, the results are outstanding. A complete cytogenetic response (CCyR) was achieved in 83% of patients, with a projected 8-year event-free survival (EFS) of 81% and overall survival of 85%.[8] In this trial, 17% of patients never achieved CCyR, approximately 15% achieved CCyR but eventually lost it, and nearly 5% were intolerant to imatinib. Thus, approximately one-third of all patients did not have an acceptable outcome.

Following the lead of imatinib development, second-generation tyrosine kinase inhibitors (TKI) were created shortly after imatinib failure began to be identified. An initial important step was the identification of mutations in the tyrosine kinase domain as the most common mechanism of resistance.[9] This was soon followed by the development of new agents with higher binding affinity to BCR-ABL1, even in the presence of most known mutants. Two of these agents, dasatinib and nilotinib, have been most extensively studied.

The excellent results obtained with imatinib when used as initial therapy and the availability of effective salvage therapy redefined the CML treatment algorithm. Nearly all patients are offered therapy with imatinib or a second generation TKI at diagnosis and for those with failure to therapy.[10] an alternative TKI is indicated. With this approach, the median survival for CML patients will probably exceed 20 years. Still, as we understand the disease better and improve the outcome of patients, we have uncovered new challenges and important questions that demand attention.

What should we aim for in chronic myeloid leukemia therapy?

For many years, IFNα was the standard therapy for CML (besides allogenic stem cell transplantation) as it induced cytogenetic remissions in a significant number of patients. Importantly, it was established that patients that achieved CCyR had a significant improvement in survival, with 78%

alive after 10 years.[11] Hence, CCyR became the goal of therapy. The value of achieving an improved molecular response had been suggested for patients treated with IFNα. Among patients that achieved CCyR, those with the least disease detectable by PCR had the best probability of a sustained response.[11,12] Major molecular response (MMR) has been defined as a decrease in tumor load equivalent to a 3-log reduction from a standardized baseline, which was established in the IRIS trial. Because of heterogeneity of PCR results, this is better expressed on the international scale (IS) being implemented throughout the world by standardizing PCR results so that MMR corresponds to a ratio of BCR-ABL1/control gene of ≤0.1%.[13] Definition of complete molecular response (CMR) depends on the quality of the samples analyzed. Thus, the sensitivity of the assay should be provided when CMR is provided. CMR may be considered when transcripts are undetectable in an assay with sensitivity of ≥4 log (≤0.01%; CMR^4) or ≥4.5 log (≤0.0032%; $CMR^{4.5}$).

It is important to consider whether achieving an MMR improves long-term outcome relative to achieving CCyR lacking MMR. Considering that more than 80% of patients achieve CCyR with imatinib, that we have highly effective salvage therapy for patients with imatinib failure, and that follow-up for imatinib-treated patients is relatively short, it is not surprising there is no evidence of improved survival for patients with MMR or CMR (also because 1 log could be close to the standard error of the technique at low levels). Initial reports from the IRIS trial suggested that among patients with CCyR, patients that achieved MMR by 12 months had a significantly better EFS probability than those without MMR.[14] With additional data, this difference was no longer detectable according to the 12-month response, but measuring response at 18 months, patients with MMR had an improved EFS probability at 72 months (95%) than those with CCyR but no MMR (86%).[15] The difference in probability of survival without transformation to accelerated or blast phase (AP/BP), although significant, was smaller.[15] Achieving CMR may further decrease the probability of relapse.[16,17] Perhaps more important, achieving a CMR offers the possibility of discontinuing imatinib. Preliminary results from the Stop Imatinib (STIM) trial in 69 patients who discontinued imatinib after having a sustained CMR for more than 2 years, 59% relapsed.[18] All relapses occurred within 7 months of discontinuation, were molecular relapses (no cytogenetic or hematologic relapses), and always responded again to imatinib.[19]

Current recommendations by the European LeukemiaNet (ELN) do not include failure to achieve MMR or loss of MMR as a criterion of failure to therapy.[10] An analysis of patients treated in the German CML Study IV did show an advantage for patients having achieved BCR-ABL1 levels according to the IS of more than 1% after 12 months with regard to event free survival, progression free survival, and overall survival. There was no additional benefit for patients with BCR-ABL1 levels between 0.1 and 1%.[19,20] In the IRIS trial, an advantage for EFS could be demonstrated for patients with BCR-ABL1 levels ≤ 0.1% (MMR) over patients with levels between 0.1 and 1% after 18 months of therapy.[15]

Strategies to improve response

Despite the excellent results achieved with standard-dose imatinib, approximately one-third of patients do not achieve the desired outcome. Thus, there is a need to improve these results.

High dose imatinib

Among the early strategies to improve the outcome was the use of high-dose imatinib. Several single-arm phase II trials suggested that patients treated with imatinib 600–800 mg rapidly achieved CCyR and MMR, at higher rates than expected with imatinib 400 mg/day.[21–24] In the phase III TOPS (Tyrosine kinase inhibitor OPtimization and Selectivity) study, adult patients (n=476) with newly diagnosed CML-CP were randomized to receive imatinib 800 mg/day (n=319) or 400 mg/day (n=157). The primary end point was the major molecular response (MMR) rate at 12 months. At 12 months, differences in MMR and complete cytogenetic response (CCyR) rates were not statistically significant (MMR, 46% vs. 40%; p=0.20; CCyR, 70% vs. 66%). However, MMR occurred faster among patients randomly assigned to imatinib 800 mg/d, who had higher rates of MMR at 3 and 6 months compared with those in the imatinib 400 mg/d arm (p=0.0035). CCyR also occurred faster in the 800 mg/d arm (CCyR at 6 months, 57% vs. 45%; p=0.0146). The most common adverse events were edema, gastrointestinal problems, and rash, and all were more common in patients in the 800 mg/d arm. Grades 3 to 4 hematologic toxicity also occurred more frequently in patients receiving imatinib 800 mg/d.[25]

In the German CML IV study, 1014 newly diagnosed CP-CML patients were randomly assigned to imatinib 800 mg/d (n=338), imatinib 400 mg/d (n=325), or imatinib 400 mg/d plus IFNα (n=351). Dose adaptation to avoid high grade toxicity was recommended. A higher rate of MMR at 12 months (primary end point) occurred more with tolerability-adapted imatinib 800 mg than with imatinib 400 mg (59% vs. 44%, p= 0.0003) or imatinib 400 mg plus IFNα (59% vs. 46%, p= 0.002). Median average daily imatinib dose in the 800 mg arm was 628 mg/d with a maximum of 737 mg/d during months 4–6 and a maintenance dose of 600 mg/d. All three treatment approaches were well tolerated, with similar grade 3 and 4 adverse events. Independent of treatment approach, MMR at 12 months was associated with better progression free survival (p=0.0143; 99% vs. 95% at 3 years) and better overall survival (p= 0.0156, 99% vs. 95% at 3 years). Thus, treatment of early phase CML with imatinib can be optimized. Early high-dose followed by rapid adaptation to good tolerability increases the rate of MMR at 12 months. Achievement of MMR by month 12 is directly associated with improved progression-free survival.[19] The effect of dose intensity might be modulated by the efficiency of the OCT-1 transporter. Patients with a less active transporter protein derived significant benefit from higher initial imatinib dose, while those with a more active transporter showed equivalent outcome with any dose.[26]

Imatinib in combination with other drugs

Another approach is to use imatinib-based combinations. Because of the established clinical benefit of IFNα, combining imatinib with IFNα became attractive. Early attempts with these combinations established their feasibility, albeit with the expected IFNα-related toxicity.[27] Results of at least three randomized trials using of imatinib with or without IFNα as initial therapy in CP CML have been reported. The French SPIRIT trial randomized patients to four treatment arms: standard-dose imatinib, high-dose imatinib (600 mg/d), imatinib plus cytarabine, and imatinib plus pegylated IFNα. Six hundred and thirty-six patients with untreated chronic-phase CML were randomized to receive imatinib alone at a dose of 400 mg daily, imatinib (400 mg/d) plus cytarabine (20 mg/m^2*d on days 15 through 28 of each 28-day cycle) or pegylated IFNα-2a (90 μg weekly), or imatinib 600 mg/d. Molecular and cytogenetic responses, time to treatment failure, overall and event-free survival, and adverse events were assessed. At 12 months, the rates of cytogenetic response were similar among the four groups. The rate of a superior molecular response (as defined by 4-log reduction in that study) was significantly higher among patients receiving imatinib and pegylated IFNα-2a (30%) than among patients receiving 400 mg of imatinib alone (14%) (P=0.001). Gastrointestinal events were more frequent among patients receiving cytarabine, whereas rash and depression were more frequent among patients receiving pegylated IFNα. As compared with other treatments, the addition of pegylated IFNα-2a to imatinib therapy resulted in significantly higher rates of molecular response in patients with chronic phase CML.[28] In the German CML Study IV, the 12-month rate of MMR was not different for standard-dose imatinib (42%) or imatinib with IFNα (45%).[19] In the third study, 94 patients received imatinib 800 mg/day for the first 6 months, and then were randomly assigned to continue high-dose imatinib alone or with pegylated IFNα-2b. After a median of 54 months, there were no differences in response rate, PFS, or survival between the two arms.[29]

Second generation tyrosine kinase inhibitors

Based on the higher *in vitro* potency of the second generation TKI, with lesser risk to trigger selection of mutations,[20] and their efficacy and adequate toxicity profile as second-line therapy, these agents became attractive candidates for frontline therapy. Three phase 2, single-arm studies have been reported using nilotinib or dasatinib as initial therapy. All three suggested that cytogenetic and molecular responses can be achieved rapidly, with CCyR reported in greater than 90% at 6 months.[31–33] For patients investigated, MMR occurred at 12 months in 71% of patients treated with dasatinib[32] and 81–85% with nilotinib.[31,33] Randomized trials were designed comparing imatinib to nilotinib, dasatinib, or bosutinib.

Nilotinib. In the Evaluating Nilotinib Efficacy and Safety in Clinical Trials-Newly Diagnosed Patients (ENESTnd) trial, patients received standard-dose imatinib or nilotinib at either 400 mg BID or 300 mg BID.[34] At 12 months of therapy, the rate of MMR (primary endpoint of this study) was 43% with nilotinib 400 mg BID, 44% with nilotinib 300 mg BID, and 22% with imatinib (p<0.0001). The key secondary end point was durable MMR at 24 months. After a median duration of therapy of 25 months, the median dose intensities of nilotinib delivered were close to planned dose at 594 mg/day on the 300 mg BID arm and 776 mg/day on the 400 mg BID arm. The median dose intensity of imatinib was 400 mg/day.[35]

The MMR rate by 24 months was significantly higher for nilotinib 300 mg BID (71%, p<0.0001) and nilotinib 400 mg BID (67%, p<0.0001) compared with imatinib 400 mg/d (44%). The achievement of MMR remained higher for both nilotinib arms across all Sokal risk groups with a minimum follow-up of 24 months. Achievement of CMR[4] was also significantly higher for nilotinib 300 mg BID compared with imatinib (44% vs. 20%, p<0.0001) and nilotinib 400 mg BID compared with imatinib (36% vs. 20%, p<0.0001). Additionally, the proportion of patients achieving CMR[4.5] was also significantly higher for nilotinib 300 mg BID compared with imatinib (26% vs. 10%, p<0.0001) and nilotinib 400 mg BID compared with imatinib (21% vs. 10%, p=0.0004).

Achievement of CCyR by 24 months was significantly higher for nilotinib 300 mg BID compared with imatinib (87% vs. 77%, p=0.0018) and nilotinib 400 mg BID compared with imatinib (85% vs. 77%, p=0.016). Based on a modified 2009 ELN definition of suboptimal response and treatment failure,[10] 4%, 4%, and 13% of patients had a suboptimal response and 3%, 3%, and 11% had failed treated by 12 months in the nilotinib 300 mg BID, nilotinib 400 mg BID, and imatinib arms, respectively.

Both doses of nilotinib significantly delayed time to progression to AP/BC compared with imatinib. When clonal evolution was considered as an event for progression, two additional patients in the nilotinib 400 mg BID arm and five additional patients in the imatinib arm had progression events. The estimated rates of patients free from progression to AP/BC (including clonal evolution) at 24 months were 99.3%, 97.3%, and 93.2%, in the nilotinib 300 mg BID, nilotinib 400 mg BID, and imatinib arms, respectively. When considering progression information on patients after discontinuation of study drug, an additional seven, two, and six progression events were observed in the nilotinib 300 mg BID, nilotinib 400 mg BID, and imatinib treatment arms, respectively. Progression-free survival on core treatment was higher for nilotinib 300 mg BID compared with imatinib (5 events (98.0%) vs. 12 events (95.2%), p=0.0736) and for nilotinib 400 mg BID compared with imatinib (4 events (97.7%) vs. 12 events (95.2%), p=0.0437). A trend for higher 24-month overall survival (OS) rates were observed for nilotinib 300 mg BID compared with imatinib (97.4% vs. 96.3%, n.s.) and nilotinib 400 mg BID compared with imatinib (97.8% vs. 96.3%, n.s.). The number of CML-related deaths was ten in the imatinib arm, five in the nilotinib 300 mg BID arm, and three in the nilotinib 400 mg BID arm. The rates of patients alive at 24 months were 96.7%, 98.9%, and 98.9% for the respective treatment arms. Grade 3/4 nonhematologic AEs were uncommon at either nilotinib dose or for imatinib. Pleural effusion was reported for six patients (two patients (<1%) in

each arm). A minimal change in myelosuppression rates was observed with additional 12 months follow-up. With a minimum follow-up of 24 months, only slight increases were observed in anemia (1% for both the nilotinib arms) and neutropenia (1% for each the nilotinib 400 mg BID and imatinib arms) compared with the 12-month minimum follow-up. Lipase and amylase elevation was rare and in most cases asymptomatic. Hyperglycemia occured significantly more often on nilotinib, whilst imatinib had the potential to reduce serum glucose levels. By 24-month, one patient in the imatinib arm had a QTcF greater than 500 msec but no patient in any of the nilotinib arms had a QTcF greater than 500 msec. No patient in the study had a decrease in left ventricular ejection fraction less than 45%. Discontinuations due to AEs occurred in 6% of patients on nilotinib 300 mg BID, 10% on nilotinib 400 mg BID, and 9% on imatinib. A total of 26 patients died (11 in the imatinib arm, 9 in the nilotinib 300 mg BID arm, and 6 in the nilotinib 400 mg QD arm).[34,35]

Dasatinib. In the international phase 3 DASISION trial, dasatinib showed higher and faster rates of CCyR and MMR versus imatinib in patients with newly diagnosed CML-CP. Patients were randomized to receive dasatinib 100 mg once daily (n=259) or imatinib 400 mg QD (n=260). Primary endpoint was confirmed CCyR (cCCyR; CCyR on 2 consecutive evaluations) by 12 months. After a median of 18 months of therapy, 81% and 80% of patients remained on dasatinib and imatinib, respectively. Response rates were higher for dasatinib versus imsatinib, including cCCyR by 18 months (78% vs. 70%; p=0.037) and MMR (57% vs. 41%; p=0.0002). Thirteen percent versus 7% achieved a BCR-ABL1 level less than or equal to 0.0032% ($CMR^{4.5}$). Six (2.3%) versus 9 (3.5%) patients transformed to accelerated/blast phase on study, and 18-month progression-free survival rates were 94.9% versus 93.7%. Rates of grade 3/4 nonhematologic AEs were less than or equal to 1%. Pleural effusion were seen only with dasatinib (2% grade 1, 9% grade 2, <1% grade 3) and did not impact efficacy. Most cytopenias (75%) occurred during first 4 months of treatment. Rates of grade 3/4 laboratory abnormalities were less than or equal to 3%, except hypophosphatemia (5% vs. 24%). For dasatinib versus imatinib, 6% versus 4% discontinued due to drug-related adverse events. Dasatinib shows superior efficacy over imatinib with an acceptable tolerability supporting the use of first-line dasatinib in newly diagnosed CML-CP.[36,37]

Bosutinib. Results of a trial of imatinib versus bosutinib were presented at the annual meeting of the American Society of Hematology in 2010.[38] Five hundred and two patients were randomly assigned to receive bosutinib (n=250) or imatinib 400 mg/d (n=252). The median follow up was 16 months. Bosutinib treatment did result in a superior rate of MMR at 12 months compared with imatinib based on the ITT and evaluable populations. However, bosutinib did not demonstrate a superior rate of CCyR at 12 months based on the ITT population, but was higher based on the evaluable population. Major side effects were frequent but transient diarrhea and liver enzyme induction. Patients on bosutinib appear to have lower rates of deaths due to CML progression, transformation to AP/BC, and discontinuations due to treatment failure compared with those on imatinib.

Evidently, longer follow-up is needed to assess fully the possible benefit of these agents above what is expected with imatinib. However, these results suggest that nilotinib and dasatinib may help reduce the percentage of patients who have an unacceptable outcome with imatinib and progression to advanced phases of CML, in particular.

What is the best strategy?

With the excellent results using second generation TKI as initial therapy, one important question is how these results may change the way we approach newly diagnosed patients. We have on one end, excellent results with imatinib with an 8-year follow-up that confirms the durability of responses and good tolerance for most patients, with no unanticipated adverse events with long-term use. On the other end, we have that one-third of patients treated with imatinib do not have the minimally accepted outcome, and the encouraging early results of studies using second generation TKI as initial therapy. Thus, we could envision two possible strategies to manage newly diagnosed patients with CML: one is to use imatinib for all patients and only change therapy for those with resistance (and, possibly, suboptimal response) or intolerance. The second option is to start all patients with second generation TKI.

Unfortunately, the available data only presents results for one intervention at a time (i.e., imatinib as frontline or second generation TKI after imatinib failure). The effect of sequential use of different treatment strategies is difficult to assess from the available literature. Based on IRIS data, 30–35% of patients would need to change therapy. Approximately 50% of patients who develop imatinib resistance will achieve CCyR with second generation TKI, and the 2-year PFS is 64–81%.[39,40] Thus, approximately 30–40% of patients who fail imatinib might be successfully rescued. When taken in isolation, the EFS after imatinib is 81%. However, accounting for patients successfully treated with subsequent TKI, nearly 90% of patients would be expected to be alive and in CCyR. Whether initial therapy with second generation TKI will provide a long-term outcome superior to what would be expected with sequential TKI therapy requires additional study and longer follow-up.

A central point of this debate is the significance of achieving earlier responses. The most obvious benefit yet from using new agents as initial therapy is that very early, most patients achieve CCyR. Although some analyses have suggested that for patients who achieve CCyR, the time to response has little impact on EFS,[39] it is clear that a patient that has not achieved an early response faces the competing probabilities of improvement to the desired response, or eventually progressing. It has been suggested that the longer it takes to achieve a CCyR, the lower the probability of achieving this response and the higher the probability of progression.[40] The early results from the randomized trial of imatinib versus nilotinib or dasatinib might support the benefits of an earlier response, as higher early response

rates have been associated with lower risk of transformation. In addition, population-based analysis have suggested that the rate of imatinib failure might be higher than reported in IRIS,[41,42] with a 5-year EFS of 63%.[41] With these results, the possible benefit of earlier responses could be magnified, provided these results can be reproduced in similar population-based analysis.

Treatment discontinuation and operational cure

Among the most intriguing clinical questions remaining in the management of CML is whether patients could eventually discontinue therapy and be cured. The current recommendation is to continue therapy indefinitely. Early attempts at treatment discontinuation among patients with CMR suggested that most patients relapsed.[43–45] However, some patients remained in remission and it was suggested that prior IFNα use could contribute to a sustained response.[43] As mentioned earlier, in the STIM trial, 59% of patients relapsed after treatment discontinuation.[18] Although longer follow-up is needed, the fact that approximately 40% of patients had not relapsed is a promising finding. An important task is to identify what characteristics make these patients remain in remission.

In aiming for treatment discontinuation and cure for all patients, two goals should be accomplished. One is to make treatment discontinuation available to all patients. Only patients with sustained CMR for greater than or equal to 2 years were eligible for the STIM trial. How often patients treated with imatinib reach this milestone is unclear. A recent analysis suggested that, after a median follow-up of 79 months, only 32% of all imatinib-treated patients achieved sustained CMR,[17] but other studies have suggested that approximately two-thirds of patients may reach this hallmark.[15] One approach to increase the number of patient achieving CMR is the use of vaccines to trigger an anti-CML immune response. Several approaches have been reported, including a junction BCR-ABL1 peptide,[46] a proteinase-3-derived peptide (PR1),[47] heat-shock protein,[48] or GM-CSF-transfected K562 cells.[49] Although immune responses have been reported with all of them, the clinical results have been mixed. A study using the BCR-ABL1 junction peptide reported 41% of patients achieving CMR after vaccination.[50]

The second important element is to develop approaches that decrease the probability of relapse after treatment discontinuation. In one study, patients received IFNα as they discontinued imatinib. Three patients achieved CMR after imatinib discontinuation and, after more than 2 years of follow-up, 75% of patients remained in remission.[51] Intriguingly, some patients did not relapse despite the persistence of low levels of BCR-ABL1 transcripts. Although preliminary, these results suggest that IFNα might aid in maintaining responses. Alternative strategies are looking at targets directed to the leukemic stem cell, such as inhibition of the Smo/Hedgehog pathway.[52] Trials with these agents have been initiated. However, for the moment, all patients should continue therapy indefinitely unless they are enrolled in clinical trials exploring treatment discontinuation.

Toward the path to a cure: achievement and maintenance of CMR

Several groups are assessing the discontinuation of TKIs following the achievement of sustained CMR. The STIM study, the most mature of its kind, prospectively assessed imatinib discontinuation in 100 CML patients in CMR[5] more than 2 years in duration.[18] n the interim analysis (median 17 months follow-up after imatinib discontinuation), 42 of 69 (61%) patients relapsed, with 98% of relapses occurring in the first 7 months following discontinuation of imatinib. At 12 months, the probability of persistent CMR was 41%. All patients who relapsed responded to reintroduction of imatinib, with 26 achieving sustained CMR. Interestingly, the kinetics of response in these patients was heterogeneous. Along with factors identified in a univariate analysis for prediction of relapse (Sokal score, gender, duration of imatinib therapy), perhaps the time to and duration of prior CMR might also influence the rate and kinetics of relapse in patients on discontinuation studies. Whatever the path to CMR, the ultimate goal should be to use the achievement and maintenance of CMR as a marker for allowing patients to stop TKI therapy, in the context of a clinical trial. Such a pan-European clinical study is currently in preparation by the ELN.

The need for CMR definition and standardization

With more patients likely to achieve CMR moving forward and with more treatment cessation studies to come, it is essential that standardized definitions of CMR are used. CMR is defined by the ELN as undetectable BCR-ABL1 mRNA transcripts by reverse transcriptase quantitative PCR (RQ-PCR) and/or nested PCR in two consecutive high-quality samples (sensitivity $>10^4$).[10] In laboratories with the most sensitive RQ-PCR techniques available, CMR typically constitutes confirmed and consistent undetectable BCR-ABL1 transcript levels with an assay sensitivity of 4-, 4.5- or 5-logs (CMR^4, $CMR^{4.5}$, or CMR^5, respectively). DNA-based PCR of BCR-ABL1 has been evaluated previously to monitor patients for any detectable presence of the Philadelphia chromosome in cells that may not be actively expressing BCR-ABL1 mRNA.[53] This study found that a proportion of patients with undetectable transcript levels and RQ-PCR negativity demonstrated positivity for BCR-ABL1 via DNA-based PCR. This was presumably due to the presence of dormant CML stem cells expressing very low levels of BCR-ABL1 RNA below the limit of detection. The alternative was also observed in two patients, where patients were positive for BCR-ABL1 by RQ-PCR but demonstrated DNA negativity. These examples raise questions of assay sensitivity and sample quality, as well whether DNA and RNA samples should be required from the peripheral blood and bone marrow. Still, results from these studies suggest that DNA-PCR, coupled with traditional RQ-PCR, may provide additional information to help the clinician make an informed decision on therapeutic approaches for individual patients.

Any discussion about the definition of CMR also raises the question "what is loss of CMR?" Loss of CMR has been defined as detection of any transcripts at least twice (to rule out false positives) in a patient who was confirmed CMR at one time. One issue with the definition of loss of CMR is that the reliability of the results obtained when operating at the lower limits of assay sensitivity may be dubious. Indeed, there have been reports of patients fluctuating into and out of CMR, yet remaining in complete disease remission and even discontinuing TKI therapy without relapse. As clinical trials in CML move toward the use of CMR as a primary endpoint, it will be important to understand the relevance of loss of CMR and its potential impact on duration or MMR, CCyR, and long-term survival outcomes. To this end, the European Treatment and Outcome Study (EUTOS) group is currently working in collaboration with regional and local investigators, as well as industry sponsors to understand and hopefully define CMR, and loss thereof better, to help to create studies of curative potential.

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Imatinib-resistant chronic myeloid leukemia. Definitions and management

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Imatinib mesylate, a selective BCR-ABL1 tyrosine kinase inhibitor (TKI), has revolutionized the treatment of patients with Philadelphia (Ph)-positive chronic myeloid leukemia (CML). In newly diagnosed patients in chronic phase, imatinib therapy is associated with a cumulative best complete cytogenetic response (CCyR) rate of 85%, an estimated 5-year CCyR rate of 67%, and an estimated 7–10 year survival rate of 80–85%; 90–93% if only CML-related deaths were accounted for.[1–5] Despite this success, about 2–5% of patients develop imatinib resistance requiring alternative therapies. This failure rate figure varies depending on how failure is defined; for example, whether it includes only patients who develop hematologic resistance or transformation to accelerated or blastic phase (AP-BP), or whether it includes additional other events, such as loss of complete or major cytogenetic response, TKI related toxicities preventing further continuation of imatinib, or other events. In the original IRIS trial, the reported failure rate, defined as progression to AP-BP or death on imatinib, or loss of complete hematologic or major cytogenetic response on imatinib, was 2–4% annually.[3] In recent trials, randomizing patients to nilotinib versus imatinib, or dasatinib versus imatinib, the early rate of being off TKI therapy was surprisingly high, in the range of 8–20%.[6,7] This may be due to the particulars of the study protocols, the definitions of events resulting in patients not continuing on the TKI, or the availability of multiple TKIs, which lowered the threshold for changing therapy by both the treating physicians and the patients.

Several treatment options are highly effective in patients with CML after failure of imatinib therapy. However, the strict definition of imatinib failure, either resistant disease clearly associated with worse long-term outcome, or severe intolerance to imatinib precluding further therapy even after dose adjustments and maximum supportive care, is important. This is in order not to discard an effective therapy, imatinib, which soon will become available in generic formulations at a lower cost. In this review, the definition of imatinib failure versus resistance, and the treatment options, including second and third generation TKIs, non-TKI therapies, and allogeneic stem cell transplantation (SCT) will be discussed.

Definitions of imatinib failure

Aside from imatinib severe or moderate-chronic toxicity interfering with patient quality of life, imatinib failure should be defined as an event that predetermines a worse long-term outcome. The latter could be measured by overall survival, progression-free survival (PFS), event-free survival (EFS), transformation-free survival, survival without AP-BP, or other clinically relevant definitions. These definitions have become extremely confusing, accounting for progressions, events, or even deaths in different ways. For example, deaths could be reported as all-cause, or CML-related. Also, progression/event may not be accounted for in patients who discontinue TKI therapy for toxicities, if progression/event, or even death, occur beyond 1–2 months after discontinuation of TKI therapy.[8,9] In the classic definition of EFS by the IRIS (event = death from any cause on imatinib, development of AP-BP, loss of complete hematologic response (CHR), loss of major cytogenetic response, increasing white blood cell count),[2] the 8-year EFS rate was 81%. With more inclusive definitions of EFS (event = any occurrence resulting in discontinuation of TKI; intention to treat analysis), the estimated 5-year EFS rates ranged from 63–80%.[8,10]

Achievement of CCyR has been clearly associated with improved long-term outcome, measured by survival, PFS, and EFS with interferon alpha therapy and with TKIs.[1,11] Therefore, achievement of CCyR, in our opinion, is the only reproducible surrogate endpoint for long-term prognosis in patients who have received imatinib therapy for more than 1 year. The European Leukemia Network (ELN) guidelines clearly define imatinib resistance (Table 1). Patients who do not achieve CHR (or perhaps a minor cytogenetic response) at 6 months, a major cytogenetic response (Ph-positive less than 35%) at 12 months, or CCyR beyond 1 year of therapy, or who lose CCyR or CHR at any point beyond this, are clearly patients with imatinib resistance requiring a change of therapy[12] (Table 1).

The question of when to change imatinib therapy has been muddied by the proposal of definitions of sub-optimal versus optimal response. A sub-optimal response is a status,

Table 1. Recommendations of the European Leukemia Network for definitions of failure and sub-optimal response to frontline imatinib therapy in chronic myeloid leukemia.

Time (mo)	Response		
	Failure	Suboptimal	Optimal
3	No CHR	≥5% Ph+	<65% Ph+
6	No cytogenetic response (>95% Ph+)	>35% Ph+	≤35% Ph+
12	>35% Ph+	1-35% Ph+	CCyR
18	No CCyR	No MMR	MMR
Any	Loss of CHR Loss of CCyR insensitive mutations clonal evolution in Ph+cells	Loss of MMR sensitive mutations	Stable or improving MMR

CHR, complete hematologic response; Ph+, Philadelphia chromosome positive metaphases; MMR, major molecular response; CCyR, complete cytogenetic response.

which we may not be happy with, but which may or may not predict for imatinib resistance or for worse long-term prognosis. The issue has become important because, in community practice (based on referrals to leukemia centers), a common reason for changing imatinib therapy is a sub-optimal response to imatinib (or misinterpretation of the significance of the molecular studies in patients still in CCyR). This is most often the case in patients on long-term imatinib therapy who are in CCyR but who have a "sub-optimal response", defined by significant rises in molecular levels, failure to achieve a major molecular response (MMR=BCR-ABL1 ratio by International Scale[IS] less or equal 0.1%), or by loss of MMR. The ELN recommendations suggest that for sub-optimal response, patients may continue on imatinib, increase the dose of imatinib, or change to second generation TKIs. This is problematic because it may discard an effective (and in the future less expensive) therapy in favor of equally effective but perhaps more expensive therapies (second generation TKIs). Four large-scale studies have so far analyzed the significance of achievement of MMR in patients in CCyR.[10,13–15]

While some have reported better PFS or EFS rates among patients achieving CCyR plus MMR versus those achieving CCyR without MMR, there was no difference in survival among the two groups in any of the four studies. This could be attributed to effective second generation TKI salvage implemented at the time of imatinib resistance (cytogenetic or hematologic relapse) and suggest that this approach is as effective (and perhaps less expensive) than instituting second generation TKI therapies in situations of sub-optimal response. While anecdotal reports and one single arm study[16] suggest that second generation TKIs improve and optimize a sub-optimal response, no studies have shown a benefit in terms of long-term survival. Of note, no randomized studies of patients with sub-optimal response randomized to continuation of imatinib therapy versus changing to second generation TKIs showed a difference in outcome between these two strategies.

Based on the above discussion, changing therapy for imatinib failure should today be based on a clear-cut evidence of imatinib resistance, as defined by the ELN recommendations. It should not be based on a sub-optimal response or increasing molecular levels, as is often practiced in the oncology community. The latter practice is further compounded by the variability of the molecular studies and the different recommendations for monitoring and changing treatments based on different levels of increased BCR-ABL1 ratios.[14,17–19] There is evidence that changing therapy at the time of cytogenetic rather than hematologic relapse would be associated with improved outcome,[20] which emphasizes the need for close monitoring of patients on imatinib therapy for the possibility of cytogenetic relapse.

Second generation tyrosine kinase inhibitors as first salvage therapy

At present, three second generation TKIs have demonstrated high efficacy among patients with CML after failure of imatinib therapy. These include nilotinib (a more potent selective BCR-ABL1 kinase inhibitor), and dasatinib and bosutinib (both dual SRC and BCR-ABL1 kinase inhibitors). Both nilotinib and dasatinib are approved by the EMA and FDA for the treatment of different phases of CML post imatinib failure. Bosutinib is still an investigational agent. These TKIs usually produce major cytogenetic response rates of 55–65% in patients post imatinib failure, CCyR rates of 40–50%, MMR rates of 28–43%, and are associated with 2-year PFS rates of 64–80% and 2-year survival rates of 87–91% (Table 2).[21–24] In a phase 3 study randomizing 670 patients with chronic phase CML post imatinib failure to dasatinib 100–140 mg orally daily, in single or twice daily dose schedules, dasatinib 100 mg once daily showed equivalent efficacy results to dasatinib 70 mg twice daily with less toxicity. The major cytogenetic response rates were 61–63%; the CCyR rates were 50–54%. Among patients achieving CCyR, 78–93% remained in CCyR at 2 years. The MMR rates were 38%; among patients in CCyR, the MMR rates were 66–72%. The estimated 2-year PFS rates were 75–80%. The estimated 2-year survival rates were 88–94%. Nilotinib 400 mg orally twice daily was given to 321 patients with CML in chronic phase post imatinib failure. The overall major cytogenetic response rate was

Table 2. Results of second generation tyrosine kinase inhibitors salvage therapy after imatinib failure.[21–24]

Outcome	Dasatinib		Nilotinib		Bosutinib	
	Imatinib resistance	Imatinib intolerance	Imatinib resistance	Imatinib intolerance	Imatinib resistance	Imatinib intolerance
MCyR, %	55–59	74–82	56	66	60	73
CCyR, %	44–48	67–78	41	51	46	59
MMR, %	35	43	28	–	–	
24-month PFS, %	76–80		64		77	86
24-month survival, %	88–91		87		92	99

MCyR = major cytogenetic response; CCyR = complete cytogenetic response; PFS = progression free survival.

59%; the CCyR rate was 44%. Among patients achieving CCyR, 56% also achieved an MMR. Cytogenetic responses were durable with 84% of patients achieving CCyR maintaining it at 24 months. The estimated 24-month survival rate was 87%, PFS rate 64%, and EFS rate 55%. At present, 124 of 321 patients (39%) continue on nilotinib therapy.[21] Bosutinib 400–600 mg was given orally daily to 294 patients with CML in various phases post imatinib failure. Their median age was 52 years. The median CML duration was 4 years. The median duration of prior imatinib therapy was 2.3 years. Imatinib resistance was documented in 69%; 45% had mutations. Overall, the CHR rate in patients in chronic phase was 78%; the major cytogenetic response rate 58%; the CCyR rate 46%; and the estimated 12-month survival rate 95%.[24] The estimated survival rates with second generation TKIs salvage at 2–3 years are encouraging, considering that the mortality rate post imatinib failure ranged from 10–20% before the availability of second generation TKI salvage therapy.[25]

Role of allogeneic stem cell transplant SCT in frontline and in salvage therapy

Allogeneic SCT was frontline therapy among eligible patients prior to the introduction of imatinib therapy in 1999. Since then, allogeneic SCT has been considered generally as a second or third line salvage therapy. However, several important considerations emphasize the continued importance of allogeneic SCT as a major component of CML therapy. With an incidence of CML in the United States (US) of 4,000–5,000 cases annually, and the reduced annual mortality to 1–2%, the prevalence of CML will continue to increase until it reaches a plateau of about 250,000 cases by 2040 (a time when the annual incidence will be equal to the annual mortality). Considering a failure rate of 2–5% annually, and a current prevalence of about 80,000 cases in the US, the potential annual number of allogeneic SCT post imatinib failure in CML now ranges from 2–4,000 cases and could become as high as 10,000 cases a year. This highlights the importance of continued research to improve the efficacy and tolerability of allogeneic SCT in CML, as it will remain a highly curative element in CML therapy, albeit post imatinb failure rather than as frontline therapy. In this context of transplanting patients with imatinib resistance, and the expectation of possibly higher rates of CML relapse post-allogeneic SCT in patients transplanted in later CML phases and after imatinib failure, it becomes important to design post-allogeneic SCT maintenance strategies, for example with TKIs, which may in the future become mainstay components of post-SCT maintenance therapy to improve the long-term cure rates in such patients. This is even more important considering that many patients post imatinib failure may have components of CML transformation at the time of SCT.

The role of allogeneic SCT in frontline CML therapy is changing. In our small experience in patients with p190 chronic phase CML treated with imatinib, the failure rate was high. Therefore, such patients may still be considered for frontline allogeneic SCT.[26] The current costs of imatinib therapy may far exceed the cost of allogeneic SCT. For example, imatinib annual therapy costs about $40–50,000 in the US; in contrast the cost of allogeneic SCT in many nations may be as low as $30–100,000. Therefore, in some situations or countries, it may be more economically beneficial, on average, to offer allogeneic SCT as a frontline one time, fixed-cost curative option, rather than to continue imatinib therapy for decades. This may change with the availability of generic imatinib.

Another important question is when to consider second generation TKIs as long-term second salvage therapy versus only as an interim bridge therapy to reduce minimal residual disease status prior to allogeneic SCT. This generally depends on several factors: 1) CML status at imatinib failure (chronic versus AP-BP); 2) the presence or absence of mutations or clonal evolution; 3) the initial cytogenetic response to second generation TKIs; and 4) the patient age and availability of matched siblings versus unrelated versus unmatched donors. Among patients who progress post imatinib failure to AP-BP, second generation TKIs should be considered only as an interim salvage approach to reduce CML burden and optimize patients for allogeneic SCT. Among patients with T315I mutation, second generation TKIs are ineffective and such patients should proceed to allogeneic SCT as soon as possible. Patients with mutations or clonal evolution post imatinib failure have generally shorter PFS and EFS and should be considered for allo-

geneic SCT if they are young and if an acceptable donor is available.[27–30] In contrast, patients without mutations or clonal evolution, particularly if they achieve a major cytogenetic response within 12 months of second generation TKI therapy, may consider continuing on therapy until there is evidence of failure on TKI therapy before considering allogeneic SCT (as third salvage option). Finally, patients who are over the age of 65–70 years and those with poorly matched donors may decide to forego a curative but toxic allogeneic SCT option in favor of several years of CML control with combinations of TKIs and standard agents (hydroxyurea, cytarabine, decitabine) or novel investigational therapies.

Third generation TKIs and other investigational therapies

Ponatinib (AP24534; ARIAD) is a multikinase inhibitor with *in vitro* efficacy against wild-type and mutated CML, including T315I. Early efficacy results in a phase 1 study of ponatinib were encouraging. Ponatinib was given at dose ranges of 2–60 mg orally daily. Among 64 patients with CML or Ph-positive acute lymphocytic leukemia treated, 95% had failed at least two TKIs and 65% had failed at least three TKIs. Among 38 patients treated in chronic phase CML, 25 (66%) achieved major cytogenetic response and 20 (53%) achieved CCyR. Among 9 patients with T315I mutation treated in chronic phase, a major cytogenetic response rate was obtained in all 9 (100%) and CCyR in 8 (89%).[31] The dose-limiting toxicities were pancreatitis and thrombocytopenia at the dose level of 60 mg orally daily. Ongoing FDA pivotal trials (PACE studies) are using ponatinib 45 mg orally daily in patients post multiple TKI failures and those with T315I mutation. DCC-2036 is another unique multikinase inhibitor with binding properties to the switchpocket of the BCR-ABL1 kinase, thus making it unaffected by the bulky isoleucine residue of the T315I. It has demonstrated preclinical efficacy in mutant CML lines, and is undergoing a phase 1 study evaluation.[32]

Omacetaxine (previously known as homoharringtonine) is a Chinese herbal medicine derivative drug with *in vitro* efficacy against CML including T315I, and with documented anti-CML efficacy, as well as activity in acute myeloid leukemia and myelodysplastic syndrome. Following a long-term developmental process seeking an indication in an orphan niche, studies have been recently completed with omcetaxine 1.25 mg/m^2 subcutaneously twice daily for 7 days (induction) and shorter maintenance schedules every 1–2 months in patients with CML post multiple TKI failures and with T315I mutations. A major cytogenetic response rate was obtained in 25% of patients in chronic phase post 2 TKI failure and in 14% of patients post 3 TKI failure.[33]

Finally, many agents have been partly neglected because of the exciting data of TKIs, including classic standard agents like hydroxyurea, cytarabine, interferon alpha, and more novel ones like decitabine. These agents have definite anti-CML activity and may be used alone or in combinations in patients with CML post imatinib failure to provide disease control and maintenance of chronic phase for potentially many years.

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The power of diversity: hematopoietic stem cell heterogeneity and its clinical relevance

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A B S T R A C T

Multiple subsets of functionally distinct hematopoietic stem cells (HSCs) have recently been identified in murine studies. Individual HSCs can differ in their potential to generate certain mature blood cell lineages, repopulating capacity, and turnover rate. Changes in the clonal representation of distinct HSC subsets in the bone marrow are associated with aging and certain types of hematopoietic disorders. Therefore, understanding and addressing HSC heterogeneity is of major clinical importance and may lead to the development of superior treatment protocols. For instance, HSC transplantations should ensure re-establishment of a heterogeneous HSC pool in recipient patients. Although heterogeneity in the stem cell pool has been studied less extensively in humans than in mice, clonal tracking in recent gene therapy trials serves as an important source of information on stem cell behavior. Development of novel barcoding and deep sequencing technologies can provide more detailed and quantitative data on stem cell clonality.

Introduction

More than 10^{11} blood cells are produced daily in the process of hematopoiesis.[1] The turnover of these cells is assured by the enormous proliferative capacity of hematopoietic stem cells (HSCs) residing in the bone marrow. HSCs are capable of generating and regenerating at least nine distinct types of mature blood cells in the complex process of differentiation (Figure 1). The current prevailing view implies that during hematopoiesis, HSCs undergo several unidirectional division steps in which they generate distinct intermediate cell populations[2] and gradually lose their ability to self-renew. The ability for self-renewal and multilineage differentiation is the unique feature of HSCs.

Traditionally, it has been assumed that all stem cells possess similar developmental potential and are equally capable of producing cells of all lineages (Figure 1). Multiple strategies were developed that were aimed to purify a homogeneous stem cell population, where each and every single stem cell possessed equal potential. However, the notion of stem cell homogeneity was challenged by data indicating a high level of heterogeneity of murine HSCs in terms of differentiation program,[3,4] repopulating capacity,[5,6] and turnover rate.[7,8] Although much effort is put in uncovering improved ways to characterize and (prospectively) isolate the putative hematopoietic stem cell, a growing body of data points out that THE hematopoietic stem cell does not exist. In contrast, intrinsic heterogeneity may be a key characteristic of HSCs.

Existence of stem cells with different behavior provides a new perspective on current views of normal and pathological hematopoiesis. Recent data on HSC heterogeneity suggests that the equilibrium of different stem cell subsets can be crucial in maintaining blood homeostasis, while the clonal disbalance of HSC underlies abated hematopoietic function in aging and disease. Although evidence for intrinsic heterogeneity of the HSC compartment originates from studies in mice, there is no reason to assume that a similar extent of heterogeneity is absent in human HSCs. Understanding the facets of HSC heterogeneity in humans is important to ensure both the current and future success of stem cell therapies and for the apprehension of blood disorders, such as various types of leukemias and other proliferative and dysplastic bone marrow diseases. The unique opportunity to track individually marked human HSCs provided by gene therapy trials, combined with rapid development of high-throughput sequencing analysis, holds great promise to obtain insight in such cellular heterogeneity. In the current review, we will address recent advances in our understanding of HSC heterogeneity and will highlight putative clinical consequences.

HSC heterogeneity

Stem cells comprise less than 0.01% of all bone marrow cells and are morphologically very difficult to distinguish from progenitor cells. HSCs can be partly characterized by immunophenotype, but unlike, for instance, the situation in the intestine, where Lgr5 can be used as a single stem cell marker,[9] such mono-markers have neither been found in

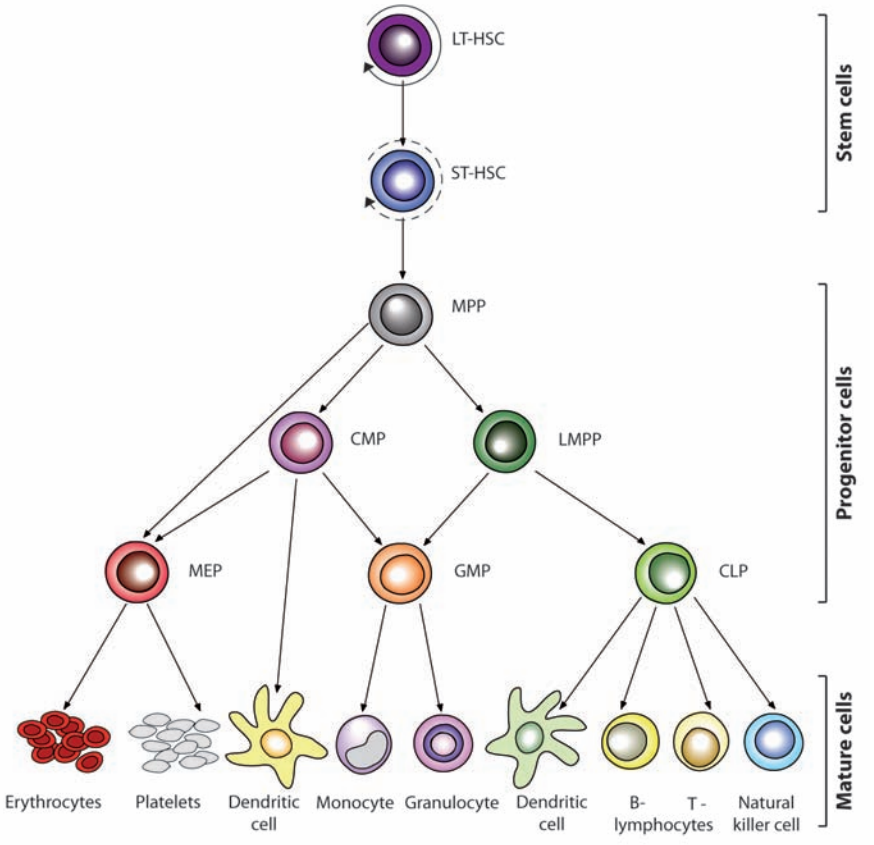

Figure 1. Traditional model of differentiation and commitment of hematopoietic stem cells. Long-term (LT-HSC) and short-term (ST-HSC) HSCs reside at the top of hematopoietic hierarchy. Both these populations have multilineage developmental potential, but ST-HSCs have lower self-renewal capacity.[75] Upon differentiation, HSCs are equally capable of producing all mature blood lineages via a chain of progenitor populations with restricted lineage commitment. MPP - multipotent progenitor, CMP - common myeloid progenitor, LMPP - lymphoid-primed multipotent progenitor, MEP - megakaryocyte-erythroid progenitor, GMP - granulocyte-macrophage progenitor, CLP - common lymphoid progenitor. Self-renewal is indicated in the round arrows.

the hematopoietic system,[10] nor in most other tissues. In a young mouse, differential expression of several surface molecules allows isolation of populations containing up to 50% of long-term repopulating HSCs.[11–13] In the human system, the knowledge of HSC surface proteins is much more limited, and typically only a few markers are used in purification protocols. These include lineage negative, CD34, CD90/Thy1,[14] CD38[15] and CD45RA.[16]

The most stringent way to demonstrate the "stemness" of a cell is to test its function by transplantation into a recipient in which endogenous blood cell formation is destroyed or otherwise impaired. Several criteria, based on either long-term repopulating ability, self-renewal capacity in serial transplantation, or multilineage differentiation potential, were proposed to define a stem cell. In the mouse system, a very common definition of a HSC requires a cell to be able to contribute at least 1% of chimerism in both myeloid and lymphoid lineages for 16 weeks post transplantation into a recipient animal.[6,17] Yet, several substantially different populations of stem cells fit this definition. In a series of *in vivo* transplantation studies, a spectrum of different subcategories in the HSC pool was uncovered by scrutinizing three key properties of HSCs – multilineage developmental capacity, proliferative potential, and self-renewal ability.

The first insights into differences of clonal behavior of distinct HSCs were provided by experiments on retrovirally transduced stem cells that demonstrated a high degree of diversity in the HSC pool. Contributions of individual HSC clones to myeloid and lymphoid lineages and their engraftment kinetics were strikingly different.[5,18] Since then, the development of murine HSC enrichment methods, allowing isolation of stem cells with sufficient purity,[19] has propelled single-cell HSC studies.

It was demonstrated that individual HSCs possess markedly diverse self-renewal capacity and repopulating activity upon transplantation into irradiated host mice.[6,20] For instance, in competitive repopulation assays, single purified cells could contribute from as low as 2.5% to as high as 63% of white blood cell chimerism in recipient mice.[6] Interestingly, the size of the HSC clone was correlated with the ability of an HSC for self-renewal. The cells with the highest repopulating activity were also more likely to produce transplantable progeny in secondary transplantations.[6] However, the ability to produce cells of myeloid, T- or B-lymphoid lineages varied significantly, even between cells with similar repopulating capacity.

Notably, this fundamental feature allowed classification of each HSC: data on both limiting dilution[3] and single-cell transplantations[4] have shown existence of at least three stem cell developmental subtypes (Figure 2A). These cells differ in their ability to produce mature cells of the myeloid or lymphoid lineage. The HSCs are either myeloid-biased (or alpha), balanced (or beta), or lymphoid-biased (or gamma) stem cells.[3,4] This "differentiation program" is inherited by the progeny of the original cells and exhibited upon serial transplantation[3,4] and therefore, strongly suggests epigenetic differences to be the cause of observed variation.

Another level of HSC heterogeneity originates from differences in stem cell turnover rate, which underlies the

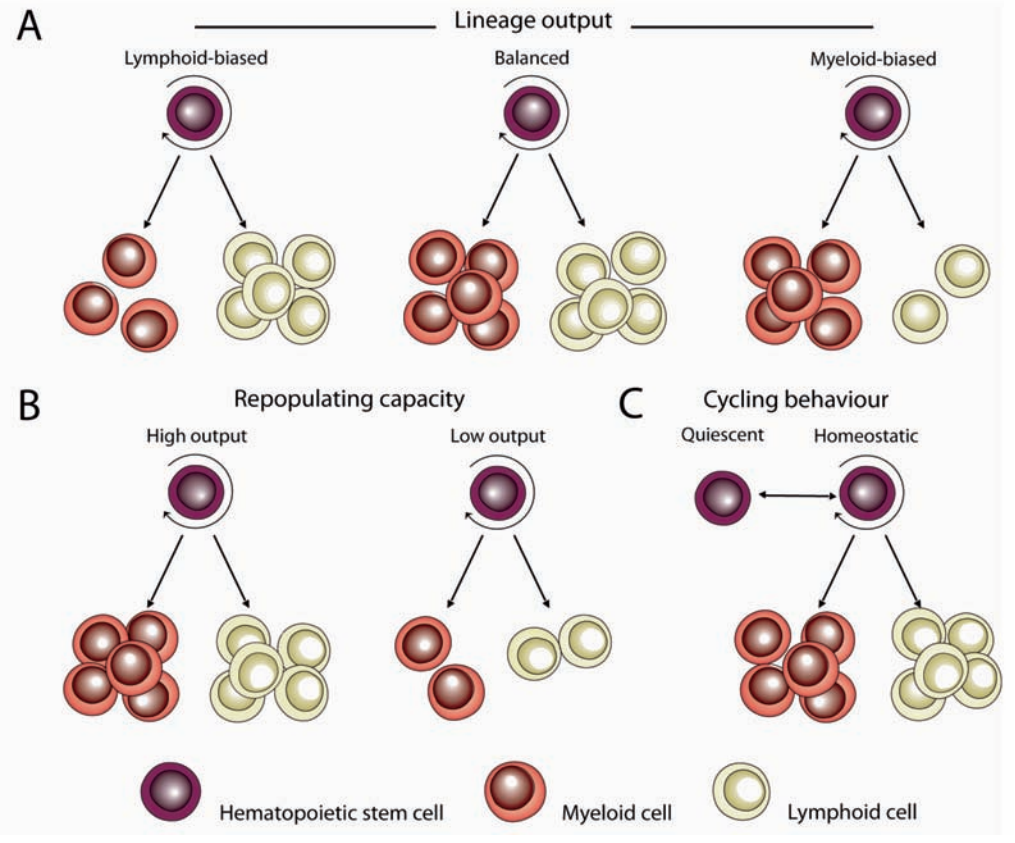

Figure 2. Different levels of heterogeneity in the HSC compartment. Although the behavior of individual cells taken from any of these categories can be very different, certain stem cell qualities are related. For instance, quiescent HSCs are more likely to provide high output in repopulating assay than their counterparts. Myeloid bias, high repopulating capacity, and quiescence have all been associated with high self-renewal.[6–8,31]

proliferative capacity of the HSCs.[7,8] It has been proposed that a high repopulation potential of HSCs is associated with slow turnover, and that proliferative quiescence protects these cells against mutagenic stress and prevents their exhaustion.[20–22] Slow cycling was confirmed by analysis of phenotypically characterized primitive cells.[7,23,24] Seventy percent of most primitive HSCs were found to be in the G_0 stage of the cell cycle, in contrast to less than 10% in more differentiated progenitor cells.[7]

The concept of dormancy has been discussed for several decades. For example, it has been shown that the most primitive HSCs survive a very high dose of the S-phase specific cytotoxic drug 5-fluorouracil (5-FU)[25] and that cytokines can be used to increase sensitivity of HSCs towards 5-FU.[26] However, recently this concept was proven more directly, when two laboratories have combined label-retaining assays, flow cytometry, and mathematical modeling to demonstrate that less than 30% of HSCs are contained in a dormant state.[7,8] In these studies, cells were labeled *in vivo* by traceable markers that upon cellular division were diluted and became undetectable. Cells that retained the label after a prolonged period of time represented slowly cycling cells. Measuring percentages of these label-retaining cells (LRCs) within the stem cell population at different time points allowed the authors to deduce the cell cycling history. Mathematical modeling of these data demonstrated that the stem cell pool was constituted by two cell populations with different cycling times: "dormant" HSCs that divided just once in approximately 170 days and "homeostatic" HSCs, which divided every approximate 30 days[7,27] (Figure 2C).

To test whether "dormant" label-retaining HSCs had a higher proliferative potential than their actively cycling "homeostatic" counterparts, the authors have isolated these HSCs populations by flow cytometry and transplanted them into irradiated recipients.[7,8] While all LRCs exhibited ability to repopulate both primary and secondary transplant recipients, only a minor part of non-LRCs were able to do so.[7,8] Remarkable differences in multilineage reconstitution capacity confirmed the long-standing hypotheses linking dormancy and proliferative potency.

Although HSC heterogeneity is multidimensional, different aspects of it are correlated (Figure 2). What drives distinct behaviors in individual HSC clones is a topic of frequent discussion.

One of the fundamental questions related to HSC heterogeneity is the role of deterministic, "programmed" decisions and stochastic factors, or biological noise, in stem cell decisions. It is tempting to divide HSC subtypes into binary categories: quiescent/cycling, myeloid-/lymphoid-biased, and so on. However, these states can be temporal and interchangeable for individual cells. Moreover, additional levels of heterogeneity within each of the stem cell subtypes indicate that the HSC pool could be a continuum of cells with a gradient of features. In concordance with this idea, a recent paper from Takizawa *et al.*[28] provides experimental evidence on the inter-changeability of quiescent and cycling states of HSCs. When fast-cycling cells (more than five divisions over 14-week period in the primary recipients) were re-transplanted into non-irradiated secondary recipients, some of them remained quiescent

over the period of 6 weeks, demonstrating that stem cells can regulate their division rate.[28] This notion is further supported by mathematical models and simulations. For instance, Glauche *et al*. have re-analyzed the original data on stem cell quiescence/activation by Wilson *et al*.,[7] taking into consideration individual decisions that single stem cells make.[29] The authors have demonstrated that rather than being separated into categories with distinct cell cycle, individual HSCs could adapt their turnover rate in response to demands in blood production.[29] Although general changes occurring during aging or in disease are likely to shift HSC pool in a certain direction, some authors propose that it is impossible to predict the behavior of an individual stem cell.[30]

HSC heterogeneity may be an important attribute of balanced hematopoiesis. Both data of clonal skewing of lymphomyeloid potential during aging in mice,[31–33] and models of clonal expansions preceding leukemia development[34] support this notion. Consequently, HSC transplantations should ensure re-establishment of a heterogeneous HSC pool in the recipient patient.

Clinical relevance of HSC heterogeneity

Understanding the biology of HSCs is important for the potential development of more efficient treatments to fight human blood diseases and to compensate for aging-related loss of hematopoietic function. Several hematopoietic disorders have been already directly linked to defects in HSCs, and more evidence arises implicating their role in other diseases. Since different subtypes of HSCs exist, expansion or loss of clones of stem cell with distinct properties during the lifespan of the organism can predispose to the development of hematopoietic malignancies[34] and contribute to impaired hematopoietic function in the elderly. Data demonstrating changes of HSC compartment in aging will be summarized below.

Aging of the hematopoietic system

In humans, aging is associated with an elevated occurrence of anemias, a decline in immune response, and an increased frequency of hematopoietic malignancies and certain autoimmune diseases.[35] Molecular studies in mice provide cues about how changes in the HSC pool can contribute to impaired hematopoiesis in aging. The hallmarks of this process include skewing of hematopoiesis to produce more myeloid cells at the expense of lymphoid cell production,[36,37] elevated HSC numbers,[36–39] impaired function per stem cell,[36,37,40] and decreased homing efficiencies after transplant[41] (reviewed in[42]).

Hematopoietic aging is orchestrated by the interplay between intrinsic and extrinsic factors, which are likely to be related both to the aging environment (hematopoietic niche) and functional changes in the HSCs. Strikingly, the number of phenotypically characterized HSCs increases through the lifespan of a mouse.[36–39] Several studies report a 7- to 16-fold expansion of the HSC pool.[42] However, the repopulation potential of individual aged stem cells declines, possibly due to accumulation of DNA damage.[43,44] The ability of HSCs to generate cells of different lineages also changes and becomes "skewed" towards myeloid cells, while lymphoid lineage output declines.[37,38,41] Recently, it has been demonstrated that this effect is accompanied by clonal expansion of myeloid-biased HSCs in the aging bone marrow.[31–33,45] It was proposed that both higher self-renewal potential[31] and unequal cytokine response[32] allow myeloid-primed HSCs to outperform their lymphoid-biased counterparts. Further, a significantly higher proportion of the aged HSCs are cycling,[28,37,38,40] in contrast to predominantly dormant young HSCs.

Changes of the HSC pool during aging in humans have been much less studied. In general, human hematopoiesis is associated with decreased bone marrow cellularity during aging.[46] As in mice, the frequency of primitive cells identified by immunophenotype ($CD34^+CD38^-$ or $lineage^-CD34^+CD38^-CD90^+$) increases over the lifespan.[47–49] Although it is not certain whether the repopulating ability of the individual human HSCs decreases over time, the quality of the whole bone marrow decays with age. A study of 6978 bone marrow transplantation patients demonstrated that older donor age is significantly associated with lower disease-free survival in transplant recipients.[50]

Interestingly, lineage changes in the murine HSC compartment mirror the human aging phenotype, as reflected in immunological decline and increased frequency of myeloid cancers. It is likely that age-dependent changes in the human HSC pool are caused by these clinically important features. Moreover, if the composition of the HSC pool predisposes to hematological disease, one may expect that the same oncogenic mutation will cause a distinct type of disease in old and young patients. This argument is fueled by data indicating that transformation of cells with BCR-ABL, the oncogene often associated with chronic myeloid leukemia (CML), causes myeloproliferative disease (MPD) in old mice, while young mice develop both B-cell leukemia and MPD.[51]

Complementary, heterogeneity of leukemias in the elderly population could be a reflection of heterogeneity in underlying HSC pool. The current models of leukemic development in aging presume that over the lifespan, HSCs accumulate mutations and combination of several events by chance could lead to malignant transformation.[52] Clonal selection of myeloid-biased HSCs could explain the elevated proportion of AML during aging.[52]

Besides a general aging-related clonal shift, hematopoietic injuries by infections or cytotoxic agents could affect the balance of dormant and active cells in the organism.

Loss of HSC quiescence – the evil and the good

Since dormant HSCs are a reservoir preserving most of hematopoietic repopulating activity, clinical implications could arise if this compartment is disrupted. Stem cell proliferative potential is not unlimited, and self-renewal is lost upon long-term divisions. For instance, serial transplantations of HSCs are only possible for four to six rounds.[53] It seems plausible that repeated hematopoietic stress will lead to recurrent HSC proliferation, which has been shown to decrease HSC quality.[23] As a consequence, certain treatments, such as repeated

chemotherapy, are likely to result in activation of dormant HSCs. It remains to be investigated what the long-term effects of this therapy will be. Several recent studies indicated that activation of quiescent stem cells by cytokines associated with viral and bacterial infection rendered them vulnerable to competition with HSCs resistant to stimulation.[54,55]

Conversely, in some cases, it could be clinically beneficial to activate quiescent stem cells. Certain types of stress, such as radiation-induced damage, are more detrimental to dormant than to cycling cells, making them prone to apoptosis.[56] Pre-stimulation of quiescent HSCs before radiotherapeutic treatment could possibly help to decrease the damage.[57] Moreover, use of agents activating HSCs have been proposed to break the resistance of quiescent leukemic stem cells to chemotherapeutic drugs, and consequently to increase response to chemotherapeutics (reviewed in[10]). The first clinical study employing a combination of G-CSF and imatinib mesolate is underway.[58]

Factors mediating the exit of HSCs from the quiescent state are mostly unidentified but are likely to be cytokines and growth factors. Current known signaling molecules include granulocyte colony-stimulating factor (G-CSF),[59] interferon-α,[55] and interferon-γ.[54] It is also likely that molecules that were used many years ago to explore their potential as radioprotective agent, such as stem cell factor (SCF),[26] interleukin-1 (IL-1),[60] and FLT3-ligand[61] will activate dormant HSCs.

HSC transplantation

Transplantation of HSCs is used for treatment of a range of congenital and acquired diseases, including hematopoietic cancers (leukemias, lymphomas, and myelomas), myelodysplastic and myeloproliferative disorders, anemias, and immunodeficiencies. The studies referred to above that document heterogeneity of bone marrow-derived HSCs allow speculation about the quality of cells derived from different sources (bone marrow, peripheral blood, or cord blood).

It can be expected that cord blood will bear the cells with both the higher proliferative capacity[62] and lymphoid developmental potential than HSCs from mobilized blood or adult bone marrow, and with increased donor age, the HSC pool will lose the ability to generate lymphoid cells and will have an overall reduced repopulating ability. Longer neutrophil and platelet recovery times in recipients of UCB graft compared with peripheral blood HSC or bone marrow HSCs could also reflect more quiescent, slower cycling behavior of UCB stem cells.[63] Interestingly, one of HSC activating molecules, G-CSF[59] is routinely used to mobilize HSCs for bone marrow transplantations. It still remains to be investigated whether heterogeneity within the mobilized HSC pool in the blood reflects the bone marrow situation and how this could affect recipients of the HSC transplants.

Clonal behavior of human hematopoietic cells – lessons from gene therapy

Correction of genetic defects by introducing a therapeutic gene in a patient's own stem cells – gene therapy – is one of the most promising applications of stem cells. HSC gene therapy has already been proven successful for correction of both hematopoietic and non-hematopoietic diseases, including X-linked severe-combined immunodeficiency (SCID-X),[64] β-thalassemia[65] chronic granulomatous disease (CGD),[66] adenosine deaminase deficiency,[67] and adrenoleukodystrophy.[68] The essence of the treatment is the isolation of patients' own HSCs, which are corrected by (retroviral) gene transfer *ex vivo* and infused in the blood stream. However, the wide-spread usage of this technique has been hampered by the observation of a high incidence of leukemia in a severe combined immunodeficiency gene therapy trial – 4 of 10 patients in a French SCID-X study developed T-cell acute lymphoid leukemia (T-ALL).[64] One additional case of leukemia was registered in a similar trial of 10 patients in the United Kingdom.[69,70] Leukemia development was associated with activation of pro-oncogenes (including LMO2 and CCND2) after integration of the virus that was used for gene delivery in close proximity to these genes, resulting in expansion of clones marked by these integration sites. Similarly, clonal dominance preceded the development of myelodysplasia and leukemia in two patients with X-linked CDG treated with gene therapy.[66] Clonal restriction was triggered by retroviral integration in proximity to EVI1 and MDS-EVI1 loci.[66]

The development of safer therapeutic vectors and the establishment of methods to control dynamics of retrovirally marked clones was a prerequisite for the restart of gene therapy trials. Several methods for tracking individual HSC clones by analysis of integration sites have been reported. The most advanced methods combine detection of insertion sites with high-throughput sequencing. Integration site analysis is based on the cutting of genomic DNA isolated from transduced cells by restriction enzymes. Subsequently, the fragments containing parts of viral sequences are amplified by polymerase chain reaction (PCR) and analyzed by sequencing, allowing identification of viral insertion site. If the relative contribution of an integration site over time increases, such clonal expansion can mark development of a myeloproliferative disease. Coupling of integration site analysis with high-throughput sequencing allows simultaneous detection of multiple clones in a single sample. For instance, a longitudinal study of clonal fluctuations in the French SCID-X study reported variation in 9767 integration sites in the blood of eight patients.[71] However, these data need careful interpretation. Aspects, such as efficiency of integration site recovery, sensitivity of the method, robustness against sequencing noise, and statistical analysis can influence the scope of reported integration sites.

Importantly, clonal tracking in gene therapy studies would provide a unique opportunity to assess the heterogeneity in the human HSC pool. By analyzing the presence of different HSC clones in subtypes of mature blood cells (granulocytes, B and T cells) over time, it would be possible to distinguish different human HSC types. Remarkably, a study of a patient who underwent HSC gene therapy for treatment of β-thalassemia, demonstrated long-term (33 months) labeling of erythroid and myeloid, but not lymphoid cells with vector insertion in the HMGA2 gene, indicating possible gene transfer into myeloid-biased stem cell.[65]

Data on clonal changes in the blood of gene therapy patients can be an important source of information on HSC heterogeneity in humans. Multiclonality observed in two patients in X-linked adrenoleukodystrophy trial[68] provides an exciting starting point for observations of clonal changes. Development of unbiased quantitative methods for clonal tracking is an important factor to ensure reliability of such information. Unfortunately, currently used methods of integration site analysis are non- or semi-quantitative, and integration site discovery is biased to the shorter size of DNA fragments (less than 500 DNA base pairs[72]). Furthermore, even extensive analysis fails to detect 30–40% of the clones.[73]

Recently, our laboratory developed and validated a novel cellular barcoding technique, which can be used for tracking clonality in transduced cell populations.[74] We have shown that in contrast to previously available techniques, the barcoding method provides unbiased, quantitative results. The barcoding technique and its possible application in gene therapy trials will be discussed in the next paragraph.

Cellular barcoding as a powerful tool to study stem cell clonality

Our cellular barcoding method is based on introduction of a short 27 nucleotide-long random sequence tag (barcode) in the retroviral vector (Figure 3A). Upon viral integration, a barcode provides a heritable traceable label into the genome of transduced cells. Optimal design of the barcode tag allows construction of barcoded vector libraries with complexity up to several million combinations. Although the barcode tag itself is highly variable, the structure of the vector backbone is constant, allowing amplification of equal size barcode fragments with primers against internal virus sequences. After PCR amplification of genomic DNA, barcode tags can be detected by sequencing. In contrast with integration site analysis methods, our barcoding technique permits unbiased analysis of clonal composition in the sample. Simplicity of barcode tag design allows introducing such a tag in virtually all vector systems.

Previously, we have described barcode analysis based on Sanger sequencing. Recently, we coupled cellular barcoding to the Illumina Solexa high-throughput sequencing method, which allowed us to observe fluctuations of more than 100 different clones in transduced bone marrow cultures *in vitro* (Bystrykh, *et al.*, unpublished data, 2010). Tracking clonality of hematopoietic cells by barcoding provides a setup, which can be easily applied in clinical gene therapy studies and would allow extracting data on human HSC behavior with more precision. Both crucial targets of clonal tracking in gene therapy could be addressed: first, resolution and sensitivity of the method allow detailed analysis of the polyclonal hematopoiesis (Figure 3B); second, quantitativeness of barcode detection can be utilized to monitor expansion of (potentially malignant) clones (Figure 3C).

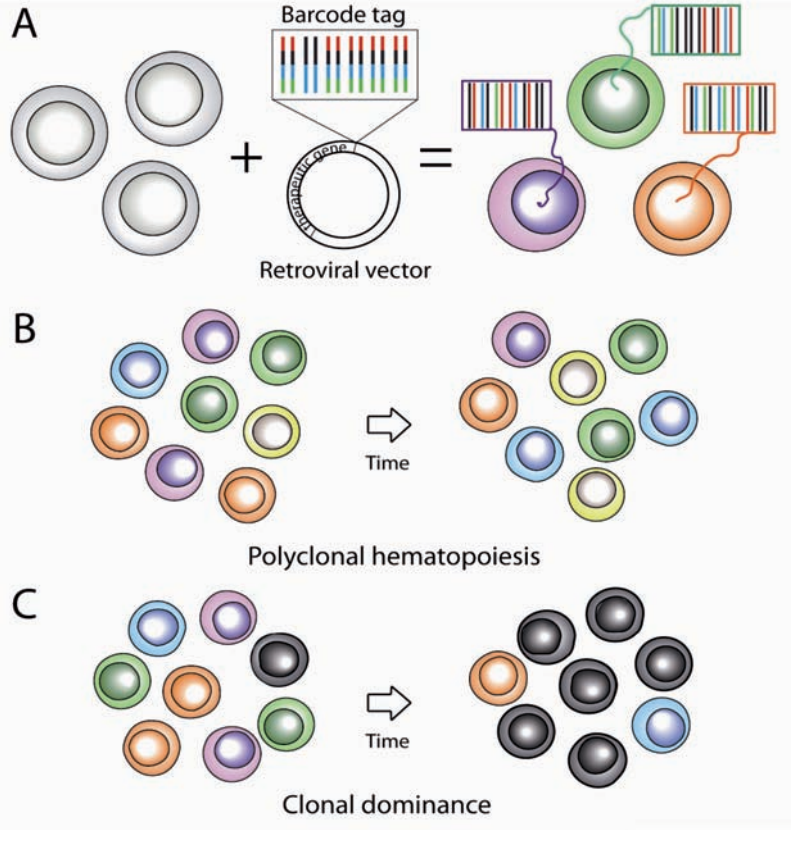

Figure 3. Use of barcoded vectors for tracking clonal changes in hematopoietic system. Under normal circumstances, HSCs are genetically identical (A, left), therefore, analysis of the output of HSC clones is impossible. Cellular barcoding uniquely labels individual HSCs enabling clonal tracking of their progeni (A). Barcode analysis can be used in gene therapy trials both to study the clonal fluctuations in the polyclonal system (B) and to detect clonal expansion events (C).

Conclusions

1. Heterogeneity in the HSC compartment involves all the crucial features of stem cells - their repopulating ability, self-renewal capacity, cycling time, and multilineage differentiation potential. Maintenance of heterogeneity might be required to ensure tissue homeostasis.
2. Clonal selection is associated with development of hematopoietic disorders and function loss during aging
   – Hematopoietic aging is accompanied by clonal expansion of myeloid-biased cells.
   – Viral and bacterial infections activate dormant cells.
3. Gene therapy provides a context in which HSC clonal changes in humans can be investigated.

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Neural and immune regulation of the hematopoietic stem cell niche

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A B S T R A C T

Hematopoietic stem cells (HSC) reside in specific bone marrow areas often referred to as niches. These niches regulate the proliferation, differentiation and migration of HSC. The cellular constituents comprising this niche are the subject of active investigations. Here we briefly review current knowledge on the major candidate cellular components, and discuss its regulation by the sympathetic nervous system and bone marrow macrophages, which exert antagonistic functions in HSC retention within the niche.

Introduction

In the adult, blood-forming hematopoietic stem cells (HSC) reside in the bone marrow (BM), but retain migratory properties. HSC continuously circulate from the BM to blood and tissues,[1–3] where they may participate in immunity[1] and tissue regeneration.[2,4] In the BM, HSC reside in defined areas that maintain, support, and regulate them.[5–10] The specific microenvironment is now commonly referred to as the HSC "niche", as initially proposed in 1978 by Schofield.[11] Although the HSC niche has been extensively studied in murine models, there are notable anatomical differences between human and murine hematopoiesis. For example, murine hematopoiesis is sustained throughout life in the marrow of long bones, whereas in humans, after puberty, hematopoiesis is progressively lost from the epiphyseal portion of long bones and persists in parts of long bone metaphyses and the axial skeleton.[12]

In the past decade, our knowledge of the major cellular components that form the niche and of how these cells regulate HSC function has increased dramatically. However, the understanding of how the niche is regulated and integrates signals from the periphery is much more limited. Parallels between the healthy and dysregulated niche may shed insights on the development and propagation of malignancies.[13–15] Here, we review the putative cellular constituents of the HSC niche and discuss recent data showing that the sympathetic nervous and the innate immune systems regulate the niche in an antagonistic manner to direct HSC trafficking.

Cellular components of the hematopoietic stem cell niche

Studies of the HSC niche have been hampered by its enclosure in bone, making direct observation difficult. Advances have been made through imaging of microenvironments and genetic models, in which specific cells and molecules have been deleted *in vivo*. Several recent excellent reviews discuss the different components of the niche and of how it regulates HSC.[5–10] In this section, we will provide an overview of the major candidate niche cells.

The osteoblast

The fact that HSC localize within bone structures suggests a role for specific bone constituents in their maintenance. Studies decades ago suggested that progenitor activity was enriched in proximity to the endosteal surface[16,17] and, after transplantation, preferentially home to BM endosteal areas.[18] Further, osteoblasts can support HSC expansion *in vitro*.[19,20] These observations led to the hypothesis that cells belonging to the osteoblastic lineage were niche cells. This hypothesis was supported through *in vivo* experiments.[21,22] Mice in which the Collagen α1 (*Cola1*) gene promoter directs the expression of an activated form of the parathyroid hormone receptor in osteoblastic cells showed an increase in the number of trabeculae and this was associated with an increase in total HSC numbers.[21] Further, treatment of wild-type mice with PTH also led to increased trabecular bone and HSC, presumably by activating Jagged-1, a Notch ligand, in osteoblastic cells.[21] Similarly, conditional *in vivo* inactivation of bone morphogenetic protein receptor IA (*Bmpr1a*) in total bone marrow cells led to increased trabeculae and HSC numbers.[22] Moreover, *in vivo* ablation of osteoblastic cells with gancyclovir treatment of Col2.3Δtk transgenic mice caused a progressive reduction in HSC numbers.[23,24] Cells of the osteoblastic lineage can also synthesize factors that promote HSC maintenance, such as CXCL12 (SDF-1α),[25] Angiopoietin-1 (Angpt1),[26] Thrombopoietin[27,28] and Osteopontin, which affect HSC quiescence.[29] These data

demonstrated that changes in osteoblastic lineage cells could affect the niche size and HSC numbers.

Presuming that the concept of niche was valid, only a very small subset of stromal cells should be endowed with niche activity. A population of bone-lining, spindle-shaped, N-cadherin$^+$ osteoblastic cells has been suggested to be physically associated with BrdU-retaining cells by immunofluorescence staining,[22] but these cells have not been functionally evaluated. The fact that calcium sensing receptor expression is required for homing to the endosteal region suggests that bone turnover areas may play a role[30] and that perhaps the most active osteoblasts may provide a niche. Areas of bone remodeling also include other cell types, such as osteoclasts, which have also been suggested to promote HSC release from the BM.[31] Although the previously mentioned genetic approaches are thought to limit expression to osteoblasts, the number of other stromal cells potentially targeted by these promoters remains relatively large. Changes in HSC numbers may potentially be explained by changes in expression in other stromal cell types rather than osteoblasts. Furthermore, other studies have found that *in vivo* depletion of active osteoblasts did not necessarily lead to acute reductions in HSC numbers.[24,32,33]

The endothelial cell

While many studies had focused on the presence of HSC near the endosteum, the development of new stem cell markers allowed fresh histological analyses of the spatial localization of HSC in marrow.[34] These studies surprisingly revealed that most CD48$^-$CD41$^-$CD150$^+$ HSC were in fact located near BM vessels.[34] Like osteoblasts, cultured endothelial cells (EC) can support *in vitro* HSC expansion,[35–37] probably by inducing Notch activation in HSC.[38] Co-transplantation of HSC and EC[39] or endothelial progenitors[40] enhanced hematopoietic engraftment. Regeneration of BM sinusoids preceded and was necessary for BM regeneration.[41] However, a definitive proof of EC as niche cells is a difficult challenge because they are essential for vascular integrity and nutrient transport. Interestingly, mice lacking the gp130 signaling subunit of the IL-6 family of cytokines specifically in endothelial cells (inasmuch as the *Tie2* reporter is specific within non-transplantable stromal cells) showed progressive BM failure and died 12 months after birth.[42] Additionally, conditional expression of a constitutively active form of Akt in endothelial cells resulted in increased HSC numbers in mice, and constitutive activation of Akt in cultured endothelial cells increased their ability to support HSC *ex vivo*.[43] Vascular microdomains expressing E-selectin have been described in the bone marrow,[44] a finding that suggests the existence of distinct functional units among ECs. Although these results suggest that EC participate in the HSC niche, the question of which subset of EC is endowed with niche properties remains.

Perivascular mesenchymal stem/progenitor cells

The chemokine *Cxcl12* is a major regulator of HSC trafficking. Thus, stromal cells expressing it would likely play important roles in the niche. In mice genetically engineered to express GFP under the control of *Cxcl12* regulatory elements, highly fluorescent cells (so-called Cxcl12 abundant reticular or CAR cells) were located near blood vessels.[45] In addition, most HSC (94–97%) were in direct cell contact with at least one CAR cell.[45] Further characterization of these cells has revealed that CAR cells were are perivascular adipo-osteoprogenitor cells.[46]

A role for osteoprogenitor cells in organizing hematopoiesis had been shown in the human bone marrow, where self-renewing CD146$^+$ CFU-F clonally expanded mesenchymal progenitors could reconstitute hematopoiesis in heterotopic ossicles.[47] These cells are also found in multiple other organs.[48] In fetal bone marrow, other studies have found that CD45$^-$CD105$^+$Thy1$^-$ cells could generate bone and a hematopoietic microenvironment after ectopic transplantation under the kidney capsule.[49] Niche formation in these studies was dependent on endochondral ossification (generation of bone through a cartilage intermediate) since *in vivo* inhibition of this process by osterix or VEGF blockade abolished the ability of CD45$^-$CD105$^+$Thy1$^-$ cells to form the hematopoietic microenvironment.[49] These reports demonstrated that osteoblastic precursor cells had the ability to form hematopoietic stem cell niches in the bone marrow.

As the HSC niche is regulated by neural signals (see below), tracking the stromal target of neural fibers was predicted to provide useful insight on the nature of niche cells. Most sympathetic innervation in the bone marrow is associated with blood vessels.[50] In the course of investigations to identify putative niche cells, cells expressing GFP under the control of elements from the *Nestin* promoter, a rare subpopulation of BM stromal cells accounting for approximately 0.08% of total BM cells, were recently found to fulfill several characteristics of a *bona fide* niche, namely a low frequency in the bone marrow with a striking physical association with HSCs, high expression of key factors that regulate HSC maintenance and function (*Cxcl12*, *Kitl*, *Vcam1*, and *Angiopoietin1*), the downregulation of these factors upon HSC mobilization with G-CSF, and alterations in HSC number and homing to BM after deletion of the Nestin$^+$ cells.[51] *In vitro* and *in vivo* assays demonstrated that Nestin$^+$ cells are self-renewing mesenchymal stem cells (MSC) capable of forming both bone and cartilage and reconstitute a hematopoietic microenvironment in heterotypic ossicles.[51] Further evidence for a function of osteoprogenitors in the niche was demonstrated in follow up analyses of CAR cells.[46] These cells express osteoprogenitor markers and differentiate into mesenchymal lineages.[46] Specific depletion of CAR cells by diphtheria toxin (DT) injection in mice expressing the DT receptor under the *Cxcl12* promoter caused a reduction in HSC numbers.[46] Importantly, CAR cells are more prevalent in the BM than Nestin$^+$ cells,[46,51] suggesting that CAR cells may contain both the Nestin$^+$ MSC population and more committed osteoblastic progenitors. Whether uncommitted MSC capable of self-renewal or a more committed osteoprogenitor, or both, contribute to the HSC niche is a matter of ongoing investigations.

Neural and immune regulation of the hematopoietic stem cell niche

The mechanisms that control HSC migration have provided important knowledge on regulatory mecha-

nisms of the niche. HSC continuously traffic from the BM to the blood, and this process can be enhanced by stimulation with various agents, including granulocyte colony-stimulating factor (G-CSF).[3] Modulation of Cxcl12 levels in the BM microenvironment has emerged as a common mechanism for several mobilization stimuli.[45,52–54] In the remaining section, we will review emerging data implicating the nervous and immune systems.

Niche regulation by the sympathetic nervous system

The bone marrow is extensively innervated by both myelinated and non-myelinated nerve fibers.[50,55–57] The vast majority of these fibers are intimately associated with the BM vasculature,[50] although they also reach into the bone marrow stroma[56] and the endosteal surface of the bone.[58,59] In addition, electron microscopy studies have shown that BM nerves are separated from perivascular stromal cells by a layer of endothelial cells.[57] These cells are connected to the nerves through abundant gap junctions, thus forming a "neuroreticular complex" that was proposed to act as a single functional unit that may transduce neural signals to hematopoietic cells.[57] Further, multiple receptors for neurotransmitters are expressed by hematopoietic cells, and *in vitro* and *in vivo* experiments indicate that these molecules can influence hematopoiesis by acting directly on hematopoietic cells (reviewed in[60] and[61]).

Following the finding that fucoidan, a sulfated glycan, could elicit rapid progenitor release from the BM in a manner that did not depend on its ability to bind to selectins,[62,63] we hypothesized that endogenous sulfated glycans in the bone marrow might regulate HSC/progenitor trafficking. Sulfatide, a sulfated galactolipid with biological properties similar to those fucoidan,[64] was tested as a candidate because it was reportedly expressed in hematopoietic cells.[65] Indeed, both G-CSF- and fucoidan-induced mobilization were impaired in $Cgt^{-/-}$ mice lacking the enzyme UDP-galactose:ceramide galactosyltransferase, which is required for the synthesis of sulfatide and galactocerebrosides.[66] A pivotal observation revealed that G-CSF induced remarkable acute suppression of osteoblast function in young wild-type mice whereas osteoblasts were constitutively suppressed in young $Cgt^{-/-}$ mice,[66] which suggested common mechanisms. Because osteoblasts neither express the Cgt enzyme nor the G-CSF receptor (encoded by *Csf3r*), it strongly suggested an indirect effect. The Cgt enzyme is highly expressed in the nervous system by oligodendrocytes and Schwann cells, contributing for the rapid transmission of the action potential; $Cgt^{-/-}$ mice show defects in nerve transmission and die before sexual maturity.[67,68] The neurological phenotype of $Cgt^{-/-}$ mice, and the fact that the SNS had previously been shown to regulate bone mass through effects on osteoblasts[69–71] and osteoclasts,[71] led to further investigation of the SNS. These studies using pharmacological and genetic gain- or loss-of-function experiments indicated that an intact SNS was required for optimal HSC/progenitor mobilization.[66] Further investigations have revealed that the SNS was also important in steady-state HSC egress, where release is controlled by circadian oscillations. These oscillations are driven by adrenergic signals from the SNS transmitted by the β3 adrenergic receptor into Cxcl12-producing stromal cells leading to circadian fluctuations of the chemokine expression in nearly antiphase with the number of progenitor/HSC in blood.[72] A direct function of bone marrow nerves in regulating the niche is consistent with the neuroreticular complex structure coined by Yamazaki and Allen.[57] β3 adrenergic receptor expression in the BM is relatively restricted, being highly enriched in $Nestin^{+}$ niche MSCs,[51] which respond to G-CSF or β3-adrenergic signaling by downregulating the expression of *Cxcl12*, *Kitl*, *Angiopoietin-1*, and *Vcam*-1[51] genes that regulate HSC retention in the BM.[73] Thus, the sympathetic nervous system acts on $Nestin^{+}$ MSC niche cells to induce downregulation of HSC retention factors, and HSC egress to the periphery (see scheme in Figure 1).

Niche regulation by bone marrow macrophages

Although G-CSF-induced mobilization requires a functional SNS,[66] other data have indicated that G-CSF receptor expression was required on a transplantable hematopoietic cell for efficient mobilization.[74] However, in wild-type recipient mice co-transplanted with a mixture of wild-type and $Csf3r^{-/-}$ bone marrow donor cells, G-CSF injections induced HSC mobilization equally in both strains, indicating the requirement of *Csf3r* on hematopoietic cells distinct from HSCs.[74] Investigations trying to uncover this hematopoietic population have led to three independent reports, suggesting that bone marrow macrophages act on niche cells to regulate HSC trafficking.[75–77]

Winkler and colleagues observed that G-CSF-induced mobilization caused a reduction in the number of bone-associated macrophages, a population previously referred to as "osteomacs",[78] and hypothesized that this reduction was playing a role in mobilization.[75] To test this hypothesis, they depleted BM myeloid cells using

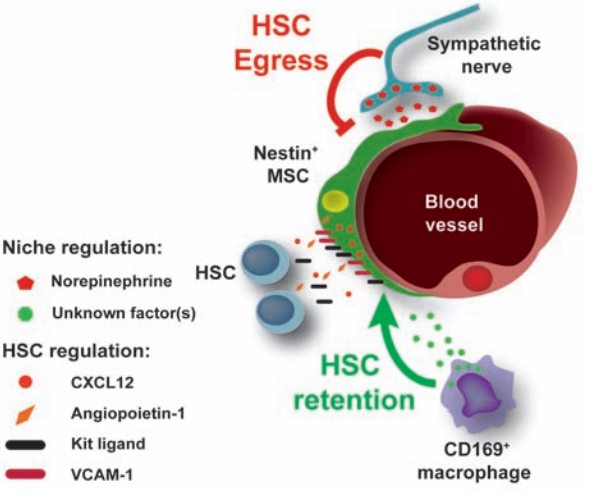

Figure 1. Regulation of the hematopoietic stem cell niche by the sympathetic nervous and innate immune systems. $Nestin^{+}$ niche cells express regulatory molecules (CXCL12, Angiopoietin-1, Kit ligand, and VCAM-1) that retain HSC in the niche. Expression of these molecules is negatively regulated by the sympathetic nervous system (SNS) through the release of norepinephrine. By contrast, BM $CD169^{+}$ macrophages secrete an unidentified factor that increases the expression of retention molecules by $Nestin^{+}$ niche cells. These antagonistic arms regulate the trafficking of HSCs to and from the niche.

"Mafia" transgenic mice in which a suicide protein under the control of the c-fms (CD115) promoter can be activated by systemic injection of a dimerizer agent,[79] and by injection of Clodronate-loaded liposomes, which specifically depletes phagocytes.[80] Both treatments significantly increased HSC/progenitor numbers in the blood and reduced the number of macrophages and osteoblasts (as determined by Osteocalcin immunohistochemistry) in bone-lining surfaces.[75] Clodronate-liposomes injection also reduced BM mRNA levels of CXCL12, Kit ligand, and Angiopoietin-1, indicating reduced function of hematopoietic stem cell niches.[75]

In a separate study, transgenic mice in which the *Csf3r* gene was under the control of the CD68 promoter were generated to evaluate the role of the G-CSF receptor on BM phagocytes.[77] These mice were bred into a *Csfr3r*$^{-/-}$ background, thus generating mice (CD68:G-CSFR) in which the *Csfr3r* gene was only expressed by monocyte/macrophages. In these mice, G-CSF injection elicited HSC mobilization, demonstrating that Csf3r expression within the monocyte/macrophage lineage was sufficient to confer mobilization signals.[77] Since G-CSF-induced mobilization alters osteoblast function[66,81] and reduces osteoblast numbers,[82] the role of macrophages in osteoblast function was investigated *in vitro*. In agreement with a previous report,[78] co-culture of BM macrophages with stromal cells increased osteoblast growth.[77] Moreover, culture of macrophages with osteoblasts enhanced the production of Cxcl12 and Osteocalcin through the release of an unidentified soluble factor from BM macrophages.[77]

Other studies analyzed whether mononuclear phagocyte function collaborated with the SNS during HSC/progenitor mobilization.[66] Monocyte/macrophage populations in the BM were separated by Gr-1, CD115, and F4/80 expression. BM macrophages were identified as CD115^{+}Gr1^{-} F4/80^{+}CD169^{+} cells, whereas two monocyte populations, CD115^{+}Gr1^{+}CD169^{-} and a CD115^{+}Gr1^{-}CD169^{-}, could be distinguished. Ablation of monocyte/macrophage cells was accomplished through three different *in vivo* models: Clodronate-liposome injection, "Mafia" mice, and diphtheria toxin (DT) injection in mice expressing the diphtheria toxin receptor (DTR) under the control of the CD11b promoter (CD11b-DTR mice[83]). In all models, monocyte/macrophage depletion was associated with Cxcl12 downregulation and HSC/progenitor mobilization.[76] Downregulation of Cxcl12, which is not produced by BM monocytes/macrophages, suggested that HSC niche function was indirectly affected by their depletion. Indeed, the depletion of mononuclear phagocytes led to significant reductions in the expression of the retention factors *Cxcl12*, *Angpt1*, *Kitl*, and *Vcam1* by Nestin^{+} niche cells, but not by osteoblasts. *In vitro* experiments suggested that BM macrophages secreted an unidentified soluble protein factor that upregulated Cxcl12 production in BM stromal cells.[76] The specific role of BM macrophages in regulating the niche was shown using mice expressing DTR under the CD169 promoter[84] where the specific depletion of macrophages, but not monocytes, was sufficient to increase the numbers of circulating HSC/progenitors. Taken together, these findings highlight two opposing regulatory arms of the HSC niche (Figure 1) in which BM CD169^{+} macrophages produce a yet unidentified soluble protein factor(s) that upregulate(s) HSC retention genes in Nestin^{+} cells, promoting HSC retention in the BM, whereas adrenergic stimulation activates β-adrenergic receptors on Nestin^{+} cells, which downregulates the expression of the same retention genes and promotes HSC/progenitor egress.[51,66] Among the several possible targets of G-CSF, it likely modulates these two arms by increasing the sympathetic tone and inhibiting the macrophage influence on the niche.

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Genetic defects in hematopoietic stem cells. Focus on myeloid cell differentiation and function

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Neutrophil granulocytes were first identified when Paul Ehrlich, aged 25 years, developed staining techniques. He described three different types of granulocytes: basophilic, neutrophilic, and eosinophilic polymorphonuclear cells.[1] Independently, Elie Metchnikoff's studies in starfish larvae revealed that certain cells are capable of phagocytosis.[2] Both investigators shared the Nobel Prize in 1908 for their discoveries, laying the foundation of cellular immunity.

Diseases related to dysfunction of neutrophil granulocytes were discovered around the turn of the 19th and 20th centuries, when Philip King Brown first described a patient with lethal pharyngitis due to agranulocytosis.[3] Similar cases were reported thereafter,[4] many associated with the medical use of aminopyrines becoming available as a drug at that time. In 1950, the Swedish pediatrician Rolf Kostmann published the first report of inherited neutrophil deficiency.[5]

Kostmann recognized severe congenital neutropenia ("*agranulocytosis infantilis hereditaria*") as an autosomal recessive trait characterized by severe neutropenia and "maturation arrest" in the bone marrow.[5,6] Children with severe congenital neutropenia (SCN), usually defined as an absolute neutrophil count of less than 500 /mL, present with invasive bacterial infections, such as omphalitis, skin abscesses, pneumonia, or septicemia. Characteristically, sites of infection lack formation of pus. Today, we recognize a great variety of genetic defects that can lead to congenital neutropenia.[7] The gene encoding neutrophil elastase (*ELANE*) was the first found to be mutated in patients with cyclic neutropenia[8] and severe congenital neutropenia.[9] *ELANE*-mutated patients represent the largest group among Caucasian patients with SCN.[10] Neutrophil elastase belongs to the class of serine proteases and is expressed exclusively in mature myelomonocytic cells and their committed immature precursor cells. Cells expressing a mutated *ELANE* allele induce the so-called unfolded protein response, a physiological rescue mechanism to prevent toxic effects by improperly folded proteins. The unfolded protein response signal cascade is initiated by three ER-localized protein sensors: IRE1alpha (inositol-requiring 1alpha), PERK (double-stranded RNA-dependent protein kinase (PKR)-like ER kinase, and activating transcription factor 6 (ATF6).[11] In case these rescue mechanisms cannot prevent undue ER stress, cells undergo apoptosis. Neutrophils from patients with mutations in *ELANE* show increased signs of ER stress and apoptosis[8,12,13] suggesting that ER stress is critically involved in the pathophysiology of severe congenital neutropenia associated with mutations in *ELANE*.

Using a genome-wide linkage study and candidate gene sequencing in consanguineous pedigrees with severe congenital neutropenia, loss-of-function mutations in *HAX1* (HCLS1 associated protein X-1) have been identified.[14] *HAX1* is also the gene mutated in the patients originally identified by Rolf Kostmann. Originally, HAX1 was cloned as an interacting partner of HCLS1, a kinase involved in B-cell receptor signal transduction. HAX1 is predominantly localized at the mitochondria membrane and stabilizes the mitochondrial membrane potential. In the absence of HAX1, cells are prone to undergo apoptosis. Interestingly, two isoforms of HAX1 (A and B) with tissue-specific expression patterns have been described. Biallelic mutations affecting both isoforms (A+B) lead to severe congenital neutropenia and associated neurological problems, whereas mutations affecting only isoform A lead to a phenotype of isolated congenital neutropenia.[15]

Patients with mutations in the zinc finger molecule GFI1 (growth factor independent-1) also present with congenital neutropenia.[16] GFI1 is a master-regulator of hematopoiesis and coordinates transcription[17] and splicing[18] in hematopoietic, as well as in non-hematopoietic cells. Similar to mutations in *ELANE, GFI1*-mutations are inherited in an autosomal dominant pattern. Mutations affect the DNA-binding domain, generate a dominant-negative variant of GFI1, and thus interfere with transcriptional networks on multiple target genes and regulatory RNAs. As a consequence, patients show not only a severe maturation arrest of myeloid cells but also aberrations in monocytoid and lymphoid cells.

Mutations in the X-chromosome encoded *Wiskott–Aldrich Syndrome* (*WAS*) gene typically cause loss-of-function of the Wiskott–Aldrich Syndrome protein (WASP). As a consequence, affected boys suffer from a combined immunodeficiency syndrome associating thrombocytopenia, autoimmunity, and immunodeficiency. WASP is a cytosolic adaptor protein expressed in all nucleated blood cells and is required for actin polymer-

ization. In WASP deficiency, lymphoid and myeloid cells are functionally perturbed in multiple aspects. Rare human patients were found expressing a constitutively active variant of WASP, leading to increased actin polymerization and congenital neutropenia.[19] Increased WASP activity also causes defective cytokinesis, increasing the risk of myelodysplasia.[20]

Neutrophil granulocytes critically depend on glucose metabolism. This is highlighted by mutations in *SLC37A4*[21] and *G6PC3,*[22] respectively. SLC37A4 encodes the glucose-6-phosphate transporter (G6PT) mediating transition of G6P from cytosol to the endoplasmic reticulum, whereas G6PC3 encodes a ubiquitously expressed homologue of glucose-6-phosphatase. Whereas in G6PT, deficiency congenital neutropenia is associated with metabolic complications (glycogen storage, hypoglycemia), G6PC3 deficiency causes congenital neutropenia in conjunction with variable structural defects or the cardiovascular and urogenital system. This disease is phenotypically quite variable and may also present with inner ear deafness or growth failure. Interestingly, in contrast to glycogen storage disease type Ia and type Ib, G3PC3 deficiency does not result in metabolic complications, presumably because glucose homeostasis is regulated mainly be G6PC1 and G6PT.

A number of other rare genetic defects have been reported to control differentiation, migration, and viability of neutrophil granulocytes. For example, Hermansky–Pudlak syndrome type II, caused by defects in *AP3B1,* is associated with congenital neutropenia and hypopigmentation.[23] The gene encodes for a subunit of the adaptor protein 2 complex controlling vesicular trafficking. Mutations in *P14/MAPBPIP*, encoding an endosomal protein, have been discovered in a single pedigree with congenital neutropenia, combined immunodeficiency, and hypopigmentation. In contrast to patients with severe congenital neutropenia, patients with defects in AP3 and MAPBPIP do not have a myeloid maturation arrest. The mechanism of neutropenia remains to be resolved.

In American Indians, a rare variant of congenital neutropenia has been identified that is associated with poikiloderma, hyperkeratosis, and nail dystrophy.[24] Using a targeted next generation sequencing approach, Volpi *et al.* identified mutations in C16Orf57 as the cause for Clericuzio-type neutropenia.[25] Recently, Clericuzio *et al.* confirmed that C16Orf57 is also the mutated gene in Athabascan patients with poikiloderma and neutropenia.[26] The function of the mutated protein is still unknown.

Some other monogenetic diseases, such as warts-hypogammaglobulinemia, immunodeficiency-myelokathexis (WHIM) syndrome, Cohen syndrome, Shwachman Diamond syndrome, cartilage-hair-hypoplasia, or Barth syndrome may also be associated with congenital neutropenia (Table 1).

Table 1. Synopsis of disease with congenital neutropenia and related animal models.

Severe Congenital Neutropenia			
Disease	**Mutated Gene (Reference)**	**Comments**	**Animal models (Reference)**
SCN and Cyclic neutropenia	ELANE/ELA2[9,10]	Increased ER stress and premature apoptosis of myeloid progenitor cells	Mouse: Ela2 knockout[28] – normal neutrophil counts but decreased function
			Mouse: Ela2V72N knockin[29] – normal neutrophil counts and function
SCN	HAX1[14]	Instability of mitochondrial membrane potential and premature apoptosis of myeloid cells Neurodevelopmental and cognitive disorder related to genotype	Mouse: Hax1 knockout[30] neuronal degeneration and lymphopenia
SCN	GFI1[31]	Impaired neutrophil differentiation	Mouse: Gfi1 knockout,[16,32] neutropenia, increased immature monocytes, defect in hematopoietic stem cells, T-cells, B-cells, DC, neuronal cells, endocrine cells
SCN	WAS[19]	Constitutive activation of WASP and actin polymerization cause genomic instability and premature apoptosis of myeloid cells	Mouse: Was knockout,[33] defects in lymphocytes, myeloid cells, thrombocytes
			Mouse:[34] Expression of constitutive active mutant causing genomic instability
SCN	unknown	Myeloid maturation arrest	unknown
SCN with lymphoid deficiency (Reticular Dysgenesis)	AK2[35,36]	Increased apoptosis of myeloid progenitor cells associated with lymphopenia and sensorineural hearing loss	Drosophila: knockout[37] growth delay and death at larval stage
G6PC3-deficiency	G6PC3[22]	Cardiac, vascular and urogenital malformations, increased apoptosis of myeloid cells involving GSK3beta	Mouse: G6PC3 knockout,[38] neutropenia, increased apoptosis of myeloid progenitor cells
Glycogen storage disease type Ib	SLC37A4[21]	Hypoglycemia, liver adenoma, neutrophil dysfunction	Mouse: SLC37A4 knockout, glycogen storage and neutropenia

Table 1. Synopsis of disease with congenital neutropenia and related animal models.

Congenital neutropenia and hypopigmentation			
Disease	**Mutated Gene (Reference)**	**Comments**	**Animal models (Reference)**
Hermansky-Pudlak syndrome, type 2	AP3B1[23]	Hypopigmentation, defect in cytotoxicity Defective function of endosomes due to absence of adaptor-protein-3 complex	Mouse: Ap3b1 knockout, no neutropenia reported Dogs: AP3B1 mutations[39] Cyclic canine neutropenia
Griscelli syndrome, type 2	RAB27A[40]	Hypopigmentation Defective cytotoxicity due to deficient exocytosis Variable neutropenia	Mouse: spontaneous mutation,[41] no neutropenia reported
Chediak–Higashi syndrome	LYST[42–44]	Defective function of lysosomes	Mouse: spontaneous mutation ("beige") defective function of lysosomes
p14(ROBLD3) deficiency	p14/ROBLD3[45]	Hypopigmentation, lymphoid immunodeficiency, growth failure, congenital neutropenia Aberrant function of late endosomes associated with mislocalization in cytoplasm	Mouse: p14 constitutive knockout is embryonic lethal
Complex phenotypes associated with congenital neutropenia			
Disease	**Mutated Gene (Reference)**	**Comments**	**Animal models (Reference)**
Autosomal-dominant WHIM syndrome	CXCR4[46]	Warts, hypogammaglobulinemia, immunodeficiency, myelokathexis, Constitutive activation of chemokine receptor CXCR4	Truncated Cxcr4 xenotransplantion of human cells into NOD/SCID mice, myelokathexis
Autosomal-recessive WHIM syndrome	unknown[47]	unknown	Unknown
Cohen syndrome	VPS13B[48]	Skeletal and mental anomalies, Congenital neutropenia vesicle-mediated sorting	none
Shwachman–Diamond syndrome	SBDS[49]	Ribosomal protein, exocrine pancreatic insufficiency, congenital neutropenia, predisposition to leukemia	Mouse: SBDS knockout[50] embryonic lethal Saccharomyces cerevisiae: SDO1/YLR022C,[51] deficiency in pre-60S ribosome maturation Danio rerio:[52] Abnormal pancreas and myeloid development
Cartilage hair hypoplasia	RMRP[53]	Ribosomal protein, skeletal dysplasia, hypoplastic hair, gastrointestinal dysfunction, neutropenia and lymphoid immunodeficiency	none
Barth syndrome	TAZ[54]	Cardiomyopathy, skeletal myopathy, congenital neutropenia Control of cardiolipin metabolism	Mouse: Taz knockout[55] polycystic renal disease, emphysema Saccharomyces cerevisiae: growth defects on nonfermentable carbon sources Drosophila: TAZ ortholog null,[56] Movement disorder, male sterility, phagocytic cells not investigated
Poikiloderma with neutropenia	C16Orf57[25]	Papular erythematous rash and neutropenia, discovered in Athabascan indians	none
Charcot Marie Tooth	DNM2[57]	Peripheral neuropathy, Charcot–Marie–Tooth Disease Endocytosis and membrane trafficking, actin assembly and centrosome cohesion	none
Pearson's syndrome	Mitochondrial deletion[58]	Bone marrow failure, myopathy	none

Currently the epistatic relationships between these variant proteins and their role in governing differentiation and homeostasis of neutrophil granulocytes remain obscure. Cross-species perspectives may open novel horizons in our understanding of life and death of neutrophil granulocytes.[7,27]

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Biology of Hodgkin's lymphoma

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A B S T R A C T

The Hodgkin and Reed/Sternberg (HRS) tumor cells in classical Hodgkin lymphoma (HL) are derived from mature B cells. However, they have largely lost the B cell-specific gene expression program and express a mixture of genes typical for various hematopoietic cell types. A grossly deregulated network of transcription factors contributes to this mixed lineage phenotype. The transforming events involved in the generation of the malignant HRS cells are only partly understood, but multiple genetic lesions in members of the NF-κB signaling pathway have been identified, including frequent mutations in the TNFAIP3 tumor suppressor gene. Recent studies revealed that the frequent genomic amplification of the chromosomal region 9p24 in HRS cells involves at least four pathogenetically relevant genes: JAK2, PD-1 ligands 1 and 2, and JMJD2C. There is currently a controversial discussion whether HRS cell clone members with cancer stem cell features exist, and if so, whether such cells are present among the typical HRS cell population or have a distinct phenotype.

Introduction

In the current WHO lymphoma classification, Hodgkin lymphoma (HL) is subdivided into a classical form and a nodular lymphocyte predominant form.[1] Classical HL, which accounts for about 95% of cases, is further subdivided into nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte depleted HL. This subclassification is largely based on differences in the morphology of the tumor cells and the histological picture.

HL is a very peculiar and hence fascinating malignancy, due to several specific features. First, the tumor cells, named Hodgkin and Reed/Sternberg (HRS) cells in classical HL and lymphocyte predominant (LP) cells in nodular lymphocyte predominant HL (NLPHL), are rare in the lymphoma tissue and usually account for only about 1% of the cells. The vast majority of other cells in the lymphoma tissue resembles an inflammatory infiltrate and is composed of T cells, B cells, plasma cells, neutrophils, eosinophils, histiocytes, mast cells, and others. Although the tumor cells are rare, HL is still a fatal disease if left untreated (with some exceptions for NLPHL).[2] Second, whereas in all other lymphomas, the tumor cells retain key immuno-phenotypic and gene expression similarities with their cells of origin, HRS cells in classical HL show a very "mixed" phenotype, which does not resemble any normal cell of the hematopoietic system.[3] Third, although deregulation of numerous signaling pathways is a hallmark of all leukemias and lymphomas, it appears that HRS cells are rather unique in the extent to which multiple signaling pathways show a deregulated and partly aberrant activation in these cells.

Deregulated transcription factor networks in HRS cells

The detection of rearranged and somatically mutated immunoglobulin (Ig) variable (V) region genes in isolated HRS and LP cells unequivocally established the mature B cell origin of these cells, as Ig gene rearrangements and somatic hypermutation are B cell specific-processes.[4–7] LP cells of NLPHL also show a mature B cell phenotype, with expression of key B cell transcription factors (e.g., Bcl-6, Pax-5, Oct-2) and differentiation markers (e.g., CD20).[8] However, HRS cells of classical HL express only few B cell markers and express multiple markers of other hematopoietic cell types, which was one reason why the origin of HRS cells has been enigmatic for a long time.[9–11] As such a dramatic "reprogramming" to a mixed-lineage phenotype is unique among lymphoid malignancies, this is likely a key factor for HL pathogenesis. Thus, there is much interest in understanding the mechanisms that cause this loss of the B cell phenotype and the upregulation of genes not normally expressed by B cells. Several factors contributing to the "reprogramming" have been identified in recent years. The B cell transcription factors Oct-2, BOB1, and Pu.1 are strongly downregulated in HRS cells, which explains why many of their target B cell genes are also not expressed.[10,12,13] The silencing of these and other factors is also influenced by epigenetic mechanisms, including DNA methylation in the promoter regions of these genes.[14] Another main B cell transcription factor, E2A, is still expressed, but its activity is blocked in HRS cells by high levels of the two E2A inhibitors ABF-1 and ID2.[15,16] Notably, ID2 is normally expressed by natural killer and dendritic cells, supporting their

differentiation and inhibiting a B cell development of lymphoid precursors. HRS cells also express the important myeloid colony stimulating factor 1 receptor (CSF1R).[17] The mechanism for its aberrant expression in the HRS cells involves the reactivation of an endogenous retrovirus located upstream of the CSF1R gene.[17] The lost suppression of this retroviral element is at least partly caused by downregulation of the corepressor CBFA2T3.[17] This downregulation appears to affect the expression of many other endogenous retroviral sequences, so that potentially many genes may be aberrantly expressed in HRS cells through the activity of reactivated retroviral elements. HRS cells also express the T cell transcription factors, Notch1 and GATA3.[18–20] Notch1 is a main T cell lineage differentiation factor, and in early lymphocyte development, suppresses B cell genes and thus a B cell development, and promotes T cell differentiation. GATA3 is typically expressed by T helper type 2 cells. We recently showed that the high activities of Notch1 and NF-κB in HRS cells contribute to aberrant GATA3 expression in these cells and that GATA3 activity plays a role in the cytokine expression and signaling pattern of these cells.[21] Further factors that have been implicated in the downregulation of B cell genes in HRS cells include the polycomb group gene BMI-1, active STAT5, and in the Epstein–Barr virus (EBV)-positive cases, the EBV-encoded genes latent membrane protein 1 (LMP1) and latent membrane protein 2a (LMP2a) (reviewed in ref.[3]).

Thus, multiple factors contribute to the grossly deregulated gene expression program in HRS cells. However, it is still not clear how this "reprogramming" of the HRS cells is initiated, and whether so far undetected oncogenic events are involved in this process.

Mechanisms causing constitutive NF-κB activity

The transcription factor family NF-κB consists of five members – Rel, RelA (p65), RelB, p50, and p52 – which function as homo- or heterodimers.[22] A canonical and a non-canonical NF-κB signaling pathway is distinguished. In the canonical pathway, NF-κB is kept inactive by binding to IκBα or other members of the IκB family in the unstimulated stage, which retains the NF-κB dimers in the cytoplasm. Upon activation of the NF-κB signaling pathway, IKK kinases induce the degradation of the IκB factors, so that the NF-κB dimers can translocate to the nucleus and activate the transcription of their target genes.[22] In the non-canonical pathway, inactive precursor proteins are expressed in the absence of stimulatory signals. Upon stimulation of this pathway, the NIK kinase processes the p100 precursor of p100/RelB heterodimers into the active p52 form, which then translocate as p52/RelB dimers into the nucleus. NF-κB activates multiple genes involved in inflammation, survival, and proliferation, including IL6, IL13, CCL5, BclXL, and FLICE inhibitory protein (FLIP). In normal B cells, NF-κB is only transiently activated. However, several types of B cell lymphomas, including HL, show a constitutive activation of NF-κB. The pathogenetic role of this activation is evident from the observation that inhibition of NF-κB in HL cell lines causes the apoptotic death of the cells.[23]

Multiple mechanisms likely contribute to the constitutive NF-κB activation in HRS cells. First, HRS cells express several surface receptors known to activate NF-κB, including CD30, CD40, and RANK.[8] HRS cells are often in direct contact with CD40L expressing T cells, and CD30L-positive eosinophils, and mast cells are regularly seen in the HL microenvironment, suggesting ligand-mediated activation of the CD30 and CD40 receptors.[24–26] Signaling through Notch1, TACI, and BCMA presumably also contributes to NF-κB activation.[8] Second, in about 30–40% of cases of classical HL, the HRS cells are infected by EBV, and in these cases, LMP1 is expressed, which is known to mimic an activated CD40 receptor and activate NF-κB.[27] Third, genetic lesions in HRS cells play an important role in the deregulated NF-κB activity. These lesions include genomic gains of the NF-κB factor Rel and of the NF-κB activating kinase NIK, and inactivating mutations in the genes NFKBIA (encoding IκBα) and NFKBIE (encoding IκBε). Gains of Rel and NIK are found in about 40% and 20% of cases, respectively,[28–30] whereas NFKBIA and NFKBIE mutations have been detected in approximately 10% of cases.[31–35] We and others recently identified mutations in the TNFAIP3 gene, encoding the NF-κB inhibitor A20, as a frequent genetic lesion in HRS cells: 40% of HL cases showed such mutations.[36,37] TNFAIP3 mutations are also frequent in classical HL cell lines, as four out of six lines in the initial analysis showed inactivating TNFAIP3 mutations.[37] In the SUP-HD1 classical HL cell line, we also recently detected an inactivating mutation in exon 2 (Figure 1). Interestingly, most mutated HL cases were EBV-negative, and TNFAIP3 inactivation was seen in 70% of EBV- cases of classical HL.[37] These findings also prompted the analysis of further regulators of the NF-κB pathway for mutations, but mutations in the NF-κB inhibitors CYLD and TRAF3 were rare. CYLD inactivation was found in one of eight HL cell lines analyzed, but in none of ten primary cases of classical HL.[38] Similarly, destructive TRAF3 mutations were identified in one of six classical HL cell lines studied, but not in isolated HRS cells from seven cases of classical HL (own unpublished data).

Considering the various types of genetic lesions in components of the NF-κB pathway in HRS cells, the question arises whether these are cooperating or mutually exclusive events. Indeed, for many cancers, the concept has been proposed that there is usually only one genetic lesion per oncogenic pathway,[39] and the observation that TNFAIP3 mutations are largely restricted to EBV-negative cases of classical HL (see above) shows that these NF-κB activating events are largely mutually exclusive. For the other lesions, this issue cannot be answered with primary cases, as for all the genes analyzed by us and others, independent collections of cases were studied. However, we have a clearer picture for the HL cell lines (Table 1). Notably, several line show genetic lesions in more than one of the oncogenes and tumor suppressor genes of the NF-κB pathway.[32–34,37,38] Therefore, it appears that we have the unusual situation that multiple genetic lesions cause the deregulation of one transcription factor. As some mutations affect the canonical and others the non-canonical NF-κB pathway, it appears to be a selective advantage for the HRS cells to deregulate both arms of this pathway, which likely

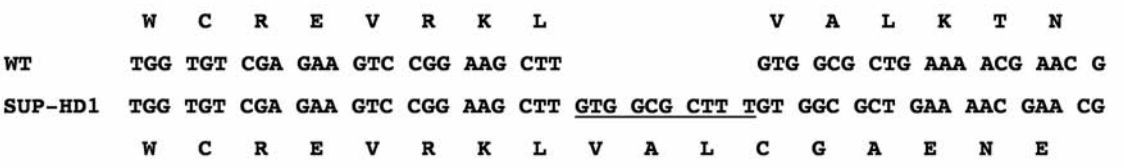

Figure 1. TNFAIP3 mutation in HL cell line SUP-HD1. PCR amplification and sequence analysis of the coding exons of TNFAIP3 in the classical HL cell line SUP-HD1 according to the protocol published in ref.[37] revealed that SUP-HD1 harbors a frameshift-causing insertion (which is largely a duplication of the sequence further 3') in exon 2. The 10 bp insertion is underlined. As only the mutated sequence was obtained, this is either a homozygous mutation (perhaps caused by an uniparental disomy event), or the other allele of TNFAIP3 is deleted. In any case, no functional A20 can be generated. The corresponding aminoacid sequences are given above the wild type and below the SUP-HD1 DNA sequences. The 3' part of exon 2 is shown. The SUP-HD1 exon 2 sequence has been submitted to the EMBL database under accession number FR775799.

Table 1. Multiple genetic lesions in regulators of NF-κB activity in HL cell lines.

HL cell line	NFKBIA	NFKBIE	TNFAIP3	CYLD	TRAF3	REL
L428	+	+	-	-	-	+
L591	-	n.a	-	-	n.a.	n.a.
L1236	-	n.a.	+	-	-	+
KMH2	+	n.a.	+	+	-	+
HDLM2	-	n.a.	+	-	-	n.a.
UHO-1	n.a.	n.a.	+	-	+	n.a.
SUP-HD1	n.a.	n.a.	+	-	-	n.a.

+ denotes presence of a genetic lesion, - a wildtype sequence. For NFKBIA, NFKBIE, TNFAIP3, CYLD, and TRAF3, inactivating point mutations and deletions are considered; for REL chromosomal gains. L591 is the only EBV-positive HL cell line. n.a.: not analyzed

leads to deregulation of more NF-κB target genes than through activation of only one of the pathways. Moreover, one may speculate that because of the multilevel regulation of NF-κB activity, a mutation in only one component would not be sufficient to cause the very strong constitutive NF-κB activity in HRS cells.

A recent gene expression profiling study of isolated LP cells of NLPHL revealed that also these lymphoma cells show constitutive NF-κB activity.[40] However, the mechanisms for this activity appear to be quite distinct from those identified in HRS cells of classical HL. LP cells do not express CD30, they are not surrounded by CD40L expressing T cells, they are virtually never EBV-infected, there is no indication for Rel gains, and inactivating mutations in NFKBIA or TNFAIP3 were not found in a molecular analysis of ten cases of NLPHL and the LP cell-derived cell line DEV.[41]

The pathogenetic role of the 9p24.1 amplification in HRS cells

In 2000, Joos and colleagues reported that gains or amplifications of the chromosomal region 9p23-p24 are frequent in HRS cells and can be found in about a third of cases.[42] Later, they showed that such gains were also present in three of four HL cell lines analyzed.[43] 9p24 gains are also frequent in primary mediastinal B cell lymphoma (PMBL), a B cell lymphoma with numerous similarities to classical HL.[8,44] The amplified region harbors the JAK2 gene, an important factor of the JAK/STAT cytokine signaling pathway. This pointed to a potential role of the JAK/STAT pathway in HL pathogenesis. Constitutive activation of this pathway was indeed validated, as active forms of STAT3, STAT5, and STAT6 were found in HRS cells.[45–48] Moreover, inhibition of STAT activity in HL cell lines impaired cell proliferation.[46,49,50] A role of JAK/STAT deregulation in HL pathogenesis was further supported by the finding that HRS cells in about 40% of HL carry somatic mutations in the SOCS1 gene, a main negative regulator of STAT activity.[51] Activation of the JAK/STAT pathway in HRS cells also involves signaling through cytokines, in particular IL13 and IL21.[47,52,53] Thus, there is strong evidence that activation of the JAK/STAT signaling pathway through cytokine signaling and genetic lesions is a major factor in HL pathogenesis.

Two recent publications now indicate that the 9p24 amplifications have additional pathogenetic consequences. Green *et al*. revealed that the programmed death-1 (PD-1) ligand genes 1 and 2 (PD-L1 and PD-L2, respectively), which are also located in the amplicon, show an increased expression in the HRS cells of those HL cases with gains of the 9p24 region.[54] PD-1 is an inhibitory receptor expressed on T cells, and there is evidence that HL-infiltrating T cells are functionally impaired through PD-1 ligand/PD-1 signaling.[55,56] Thus, increased PD-L1 and PD-L2 expression by HRS cells likely contributes to the immunosuppressive microenvironment in classical HL. Enforced PD-1 ligand expression in HRS cells with 9p24 gains is not only due to increased gene dosage of the PD-L1 and PD-L2 genes, but also through further transcriptional upregulation of these genes by JAK2.[54]

Based on an RNA interference screen of genes located in the 9p24 amplicon region, Rui and colleagues identified a further gene in this region with pathogenetic relevance.[57] Downregulation of expression of the histone demethylase JMJD2C was toxic for a HL cell line and cell lines of PMBL, which harbored the 9p24 amplification. Importantly, not only JMJD2C modulates histones, but also JAK2 has been reported to modify histones, by phosphorylating histone H3. Indeed, further experiments showed that both proteins modulate the epigenetic state in HL and PMBL, and that they cooperate in this regard to promote proliferation and survival of HRS and PMBL cells.[57]

Taking these findings together, the pathogenetic role of the amplification of 9p24 in HRS cells (and PMBL) involves at least four genes, and the tumor-promoting

mechanisms include activation of the JAK/STAT cytokine signaling pathway (JAK2 gains), suppression of tumor-infiltrating T cells (PD-L1 and PD-L2 gains), and epigenetic remodeling (JAK2 and JMJD2C gains).

The search for HRS stem cells

In several types of cancers, there is convincing evidence that not all tumor cells have the same proliferative capability and that a small subset of cancer stem cells is mainly responsible for sustaining the tumor clone.[58] As cancer stem cells show differences in their gene expression to the bulk of the tumor clone and often appear to be more chemotherapy resistant than their descendents, the identification and characterization of cancer stem cells is also of high clinical relevance.

In HL, years before the issues of cancer stem cells became an important topic, there was a discussion whether the rare morphologically visible HRS cells indeed account for the whole tumor clone, or whether other tumor clone members might exist among the many other cells in the lymphoma microenvironment. It was also debated what the relationship between the mononuclear Hodgkin and the multinucleated Reed/Sternberg cells is.

The detection of rearranged immunoglobulin heavy and light chain V region genes firmly established the derivation of HRS cells from mature B cells.[5,6] Moreover, as most rearrangements carry a high load of somatic mutations, and as the process of somatic hypermutation, which generates such mutations, is linked to antigen-activated B cells proliferating in germinal centers,[59] HRS cells derive from germinal center B cells or their descendents, with the pattern of mutations suggesting a derivation from germinal center B cells that normally would have undergone apoptosis.[5,60] Importantly, as all HRS cells of a clone carry the same Ig V gene rearrangements and (with very few exceptions) the identical somatic mutation pattern, putative HRS stem cells – if they exist – must carry the same Ig V gene rearrangements and mutations and hence must also derive from mature B cells.

Regarding the relationship between the mononucleated Hodgkin and the multinucleated Reed/Sternberg cells, there is now firm evidence from studies with HL cell lines that Hodgkin cells give rise to Reed/Sternberg cells through endomitosis.[61–63] Cell fusion is not involved in the generation of Reed/Sternberg cells from Hodgkin cells, or the generation of the HRS cell clone as such.[64,65] Reed/Sternberg cells had little proliferative capacity in *in vitro* studies,[61–63] and it has been suggested that nuclear disorganization and telomere loss in Reed/Sternberg cells causes their failure to undergo further cell division.[66] Thus, the mononucleated Hodgkin cells represent or harbor the proliferative pool of tumor cells that give rise to more Hodgkin cells and to Reed/Sternberg cells.

The question whether the HRS tumor clone consists of more cells than the typical CD30+ HRS cells was addressed in several studies. First, in two HL cases in which the HRS cells showed numerical chromosomal abnormalities, it was analyzed whether cells with such abnormalities were also present among CD30- cells in the HL microenvironment. A few CD30- cells with trisomies as seen in the respective HRS cells were reported, arguing for the existence of clone members among the CD30-negative cells.[67] However, numerical abnormalities are not a stringent clonal marker, and increased frequencies of normal B cells with chromosomal abnormalities have actually been reported for HL.[68] Second, it was argued that in EBV+ cases of HL, in which the HRS clone shows a monoclonal viral infection pattern, putative HRS clone members not visible as CD30+ HRS cells must also be EBV-infected. However, in a detailed study of microdissected HRS cells and CD30-EBV+ cells, few, if any of the small EBV-infected cells belonged to the HRS clone, arguing against the existence of HRS clone members among CD30- cells in the HL tissue.[69] In a third study, it was reported that clonotypic B cells can be found in the peripheral blood of HL patients. These cells had a B cell phenotype (CD19+ and surface Ig+) and expressed the putative stem cell marker ALDH (aldehyde dehydrogenase).[70] However, this study was criticized as none of the data presented unequivocally demonstrated a clonal relationship between the ALDH+CD19+ B cells in the peripheral blood and HRS cells in the tissue.[71] In another study that searched for HRS clone members in the peripheral blood of HL patients, using a highly sensitive PCR with HRS clone specific Ig V gene primers, no HRS cell-specific amplificates were obtained, arguing that HRS clone members are very infrequent or absent in the peripheral blood.[72] It is also important to consider that B cell clones generated in a germinal center can be very large.[73] Thus, one may potentially find with highly sensitive assays other memory B cell descendents from a germinal center B cell clone that gave also rise to an HRS cell clone. These cells, although clonally related to the HRS cells, may be non-malignant B cells, or pre-malignant cells that share some transforming events with the HRS cells. As these clone members will likely differ in the Ig V gene somatic mutation pattern from the HRS cells, a detailed study of the Ig gene rearrangements is needed to distinguish between putative HRS stem cells and pre-malignant clone members (Figure 2).

Another approach to search for a subpopulation of cells among the HRS cells with specific features in terms of proliferation and chemotherapy resistance involves a flow-cytometric strategy. The increased chemoresistance of some cancer stem cells appears to be closely related to their expression of drug transporters of the ABC family, which expel chemotherapeutical drugs from the cells. As ABC transporters also extrude the Hoechst dye 33342, negativity for Hoechst dye staining has been used to identify ABC+ cells, which are called 'side population cells', in flow cytometry studies.[74,75] Although cancer stem cells and side population cells are defined through different features of tumor cell subpopulations, these populations appear to overlap in several instances.[74,75] Two recent studies addressed the issue whether side population cells exist in HL cell lines. Side population cells were indeed found, accounting for less than 1% of cell line cells. These cells were small (i.e., Hodgkin cells), chemoresistant, and could repopulate a mixed population of Hodgkin cells and Reed/Sternberg cells.[76,77] Thus, these cells fulfill key criteria of tumor stem cells.[78] However, not all HL cell lines harbored side population cells,[77] arguing against a general role of these

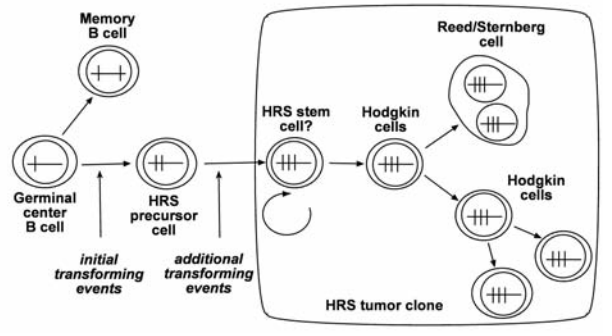

Figure 2. HRS cells and their precursors. The HRS tumor clone is most likely derived from germinal center B cells. As lymphoma development is a multi-step process, one can postulate that HRS precursor cells exist that carry some, but not all of the transforming events of the HRS clone. These cells may also persist in the patient. Among the tumor cells, the mononucleated Hodgkin cells are the proliferative compartment that give rise to more Hodgkin cells and through endomitosis to multinucleated Reed/Sternberg cells. These latter cells have little if any proliferative capacity. It is currently debated whether HRS stem cells exist that feed the HRS tumor clone. As HRS cells of a given clone carry identical Ig V gene rearrangements and an identical somatic mutation pattern of these rearrangements, also putative HRS stem cells must carry the same V region gene sequences. The pre-malignant HRS precursor cells carry the same Ig V genes and may have identical or partly different mutation patterns. As germinal center B cells clones can give rise to very large populations of long-lived memory B cells, cells with the same Ig V gene rearrangement as the HRS cells (but presumably different mutation patterns) may also be found among normal memory B cells. The horizonal lines in the cells indicate an Ig V gene rearrangement and the vertical lines exemplify somatic point mutations.

cells for the maintenance of the HRS tumor clone, and it remains to be shown whether side population cells clonally related to the HRS cells also exist *in vivo*.

Taken together, although the identification of side population cells in some HL cell lines and some other observations might indicate that HRS stem cells exist, it still remains unclear whether such cells exist *in vivo* in the patient, and if so, what their role is in the establishment and perpetuation of the HRS tumor clone. Moreover, it will be important to clarify whether side population cells are responsible for treatment failure in some patients.

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The treatment of Hodgkin's lymphoma in adults

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The treatment of Hodgkin lymphoma (HL) is one of the success stories of hematologic oncology.[1] Cure rates have risen steadily over the last 6 decades, following the introduction first of extended field irradiation, then of combination chemotherapy. Overall survival figures are now around 80% at 10 years in most Western countries, and the dominant question for many patients is less about cure, which is often assumed in all but the worst cases, and more about the avoidance of short-term side effects and long-term morbidity caused by the treatment. There nonetheless remains an unfortunate minority for whom cure is not achieved, and the pursuit of novel therapies remains highly relevant both for these and to devise less morbid treatment regimens for the future.

The toxicity of treatment

The short-term toxic effects of treatment for HL are very similar to those of any other malignancy: they are in direct proportion to the intensity of treatment, with the dose-limiting effects in general being myelosuppression, or organ tolerance in the radiotherapy field. Most patients experience fatigue, which is the most under-reported side effect, and almost all experience major disruption to their lives for the duration of therapy.

The late effects have assumed increasing importance as the likelihood of cure has risen. Myocardial damage and accelerated coronary artery disease are recognized dose-related effects of anthracycline chemotherapy and mediastinal radiation.[2] Pulmonary fibrosis is most often related to bleomycin exposure but is not simply dose-related, with some idiosyncratic sensitivity and a progressive increase in risk with age.[3] Bone marrow toxicity and the risk of myelodysplasia or acute myeloid leukemia appear to be most closely related to exposure to alkylating agents and extended field irradiation.[4] Radiotherapy has also been shown to raise the risk of epithelial malignancy within the treatment field, in particular, breast cancer for women undergoing extended field radiotherapy to the thorax below the age of 30,[5,6] lung cancer for smokers who undergo thoracic irradiation, and bowel cancer for those receiving extended field treatment of the abdomen.[7–9]

Finally, for a young population with a high chance of cure, the question of fertility is a critical consideration, particularly for young women, for whom the option of gamete cryopreservation is still in its infancy. There is a direct inverse relationship between the intensity of chemotherapy, particularly exposure to alkylating agents, and the maintenance of reproductive health.

All these types of toxicity may be averted or mitigated to some extent by the choice of therapy, and this has become a dominant theme for the management of patients. If increasing the cure fraction with initial therapy were the only goal of treatment, the choice would be relatively simple: treatment at the maximal intensity allowed by the immediate side effects. The reality, however, is more complex, and some patients will opt to exchange a slightly lower chance of initial cure for a reduced likelihood of long-term morbidity.

Features at presentation

In thinking about the balance between intensity of treatment and likelihood of cure, this field has relied heavily upon baseline prognostic features, in particular the anatomical extent of disease and its systemic effects. A formal lymph node or other tissue biopsy remains the investigation of choice for making the diagnosis of HL. Needle biopsies may be acceptable where no other approach is feasible, but the heterogeneity of cellular composition and the constraints upon further analysis imposed by such a limited sample make it far from ideal. Aspiration cytology is wholly inadequate and cannot be relied upon for this diagnosis.

The histologic classification of HL has become less of a dominant theme as the effectiveness of treatment has risen, so that the two sub-types of nodular sclerosis, mixed cellularity, lymphocyte-rich classical and lymphocyte deplete types, are managed similarly under the overall description of classical HL. The exception is Nodular Lymphocyte-Predominant HL, which is increasingly treated in ways similar to low-grade B-cell non-Hodgkin lymphoma.[10]

The broad anatomical and functional prognostic categories remain important in strati-

fying patients for whom different approaches are ideal, making initial staging an important step in management. Apart from a full history and physical examination, the minimum investigations include full blood count and differential, biochemistry screen including albumin, liver enzymes, and calcium; Erythrocyte Sedimentation Rate (or plasma viscosity, if this is not available); and contrast-enhanced CT scans of the chest, abdomen, and pelvis. FDG-PET scans are increasingly used as a matter of routine, mainly for comparison after treatment. Bone marrow biopsy is reserved for those with systemic symptoms, advanced disease, or abnormalities of the blood count. Other tests, such as bone scans, are only used when there are clinical grounds to suspect involvement at relevant extranodal sites.

Based upon these investigations, patients may be stratified into early or advanced stage disease, and within early disease, many clinicians will further sub-divide into favorable and unfavorable stages. Different analyses have yielded various means of making these divisions (Table 1). Within advanced stage disease, the generally-accepted prognostic scoring system is that developed by the German Hodgkin lymphoma Study Group (GHSG),[11] but the choice of therapy based upon prognostic features at presentation is increasingly superseded by a response-adapted approach, using FDG-PET imaging to determine the prognosis after an initial trial of therapy.

It is important to emphasize that in many cases, even if the initial therapy is not curative, second line treatment may nonetheless succeed. This was often so when the initial treatment was with radiation alone, but it is also true for many patients treated with chemotherapy. For this reason, inferior progression-free survival may not necessarily result in lower overall survival, a finding particularly seen in early stage disease. There may be more than one chance for cure: although in general patients will prefer initial therapy, which carries the highest chance of success and may choose to trade less toxicity and late side effects for a slightly reduced chance of outright cure, in the hope of effective salvage if required. There is no substitute for careful discussion of the options on the basis of evidence from clinical research and evaluation of the patient's priorities, for example, the preservation of fertility, particularly in young women.

Classical Hodgkin's lymphoma: favorable early stage disease

The results of therapy for this group are extremely good, whether through the use of radiotherapy, chemotherapy, or the combination of both. Extended field irradiation was originally shown to achieve high cure rates by encompassing all the areas of known disease with a margin to take account of sub-clinical involvement. However, this approach results in the exposure of significant areas of healthy tissue to radiation, and randomized trials have shown that combined modality treatment gives a higher progression free survival rate even when the radiotherapy field is reduced. Standard chemotherapy regimens, such as ABVD, MOPP-ABV or COPP-ABVD, were used in trials to reduce the amount of disease present, allowing the restriction of the radiation field to the involved nodal field without loss of efficacy.[12–15]

A further step is to eliminate the use of radiation altogether and give only chemotherapy. A single-centre series suggested that six cycles of ABVD was effective and safe, but in the absence of a combined modality comparator arm, it is hard to interpret this data.[16] In one randomized trial at least, the progression-free survival following ABVD alone was inferior to that seen after subtotal nodal irradiation.[17] For those patients prepared to tolerate a more prolonged exposure to cytotoxics to avoid radiation this may be a feasible idea, but it appears difficult to give less than a full course of standard ABVD chemotherapy without worse results.[18] The Memorial Sloan Kettering Cancer Center conducted a randomized trial of six cycles of ABVD ± IFRT in favorable localized and stage IIIA disease. No significant difference emerged, although there was a trend in favor of the combined modality arm.[19]

Several trials have made comparisons of these approaches, and the most recent evidence suggests that a cure rate of around 95% may be expected using two cycles of ABVD combined with 20 Gy of radiotherapy to involved nodes, with no apparent advantage to longer chemotherapy or higher doses of radiation.[20] Combined chemo-radiotherapy has the merit of minimizing exposure to either modality, although there remain strong advocates of chemotherapy-alone treatment. Recent studies have focused upon the possibility of using FDG-

Table 1. Prognostic factors distinguishing favorable and unfavorable early Hodgkin lymphoma.

	EORTC	GHSG
Risk factors	a) Mediastinal mass (>1/3)	a) Large mediastinal mass
	b) Age ≥ 50	b) Extranodal disease
	c) ESR ≥ 50 without B symptoms, or ESR ≥ 30 with B symptoms	c) ESR ≥ 50 without B symptoms, or ESR ≥ 30 with B symptoms
	d) ≥4 nodal areas involved	d) ≥3 nodal areas involved
Favorable	CS I-II supradiaphragmatic with no risk factors	CS I-II without risk factors
Unfavorable	CS I-II supradiaphragmatic with ≥1 risk factor	CS IA or IIA with ≥1 risk factor CSIIB with c) or d) but not a) or b)

PET scanning to try and identify a group in which radiation may safely be omitted following chemotherapy based upon complete metabolic response, but the results of these studies are not yet mature.[21]

Classical Hodgkin's lymphoma: early unfavorable disease

The presence of adverse features, such as bulky nodal disease, raised sedimentation rate, or multiple sites of involvement, makes it less likely that limited therapy of the type described for favorable disease above will be curative. In some centers, such cases are treated with the same protocols as advanced disease, but most groups have designed specific regimens for this category, recognizing their distinct features. Randomized trials have investigated whether the duration of chemotherapy, the dose of radiation, or the extent of the radiotherapy field can influence cure rates,[12,13,18,22] and in summary, the findings suggest that four cycles of ABVD with 30–36 Gy of involved field radiotherapy offer the best 5-year progression-free survival at 85–90%, with no apparent advantage to the use of more intensive chemotherapy, such as the MOPP-ABV or baseline BEACOPP regimens, more prolonged chemotherapy, or more extensive radiotherapy fields. Interestingly the GHSG study H11 has shown that there is some trade-off between intensity of chemotherapy and dose of radiation in this setting, in that the results after ABVD and BEACOPP were equivalent when 30Gy IFRT was given, but if only 20 Gy was used, then ABVD resulted in inferior progression-free survival.[23] Other studies have shown that there is a minimum threshold of intensity below which the results are less good,[13,24] but it is not clear that there is a continuing dose-response above this. Some preliminary evidence from the GHSG HD 14 study suggests that two cycles of escalated BEACOPP and two ABVD prior to radiotherapy may improve the progression free survival when compared with four ABVD, but this is at the expense of significant toxicity, with no overall survival advantage.[25] Once again, we await the results of the response-adjusted therapy trials to see whether FDG-PET may be useful to guide de-escalation of treatment, and at present, the consensus is for the use of combined modality therapy.

Classical Hodgkin's lymphoma: advanced disease

Combination chemotherapy completely changed the outlook for patients with advanced HL when MOPP chemotherapy produced remissions in more than half of cases.[26] The ABVD combination[27] was shown to be superior to MOPP, both in the remission rate and in its side-effect profile,[28,9] and is now widely used as a standard treatment, albeit not universally. A series of attempts have been made to improve further, using multiple chemotherapy agents, dose-intense schedules, and even myeloablative doses.

To summarize the results of many randomized trials, the addition of multiple agents in alternating or hybrid regimens, such as MOPP/ABV or ChlVPP/EVA increases toxicity without improving remission rates;[30,31] the use of weekly regimens, such as Stanford V requires the use of significant amounts of radiotherapy but does not improve remission rates;[32,33] and the use of high dose therapy in first remission has also not increased the proportion of cures, even when patients with poor prognostic features are selected.[34,35]

The exception to these observations is the escalated BEACOPP regimen developed by the GHSG. Initial studies showed this to produce results superior to alternating COPP-ABVD or a baseline regimen with the same drugs,[36] and two subsequent trials by Italian groups comparing it with ABVD have shown superior progression-free survival, albeit without differences in overall survival.[37,38] A large trial by the EORTC comparing ABVD to eBEACOPP in patients with adverse prognostic features at presentation has recently been completed, and the results are awaited with interest. Overall, the trials reported to date suggest that if ABVD is capable of producing a 5-year progression free survival of around 70% in patients with advanced HL, eBEACOPP can be expected to improve this figure by around 10–15%, but whether this translates into differences in overall survival is still not certain. What is certain, however, is that eBEACOPP carries the risk of increased toxicity, with rates of grade 3/4 myelosuppression and neutropenic fever approximately doubled in the short term, and relatively high rates of infertility in the long term. This makes it an unattractive option particularly for younger patients, who in general will retain their reproductive capacity after ABVD. The reports of myelodysplasia and acute leukemia associated with eBEACOPP have also caused concern, although the number of patients affected has not been large.[39]

Another area of uncertainty is over the role of consolidation radiotherapy in advanced HL. It should be noted that many of the trials conducted to compare chemotherapy regimens have also included radiation of residual masses in the protocol, and in most studies, this has been used in at least half of the patients. The utility of radiation appears to relate to the effectiveness of the antecedent chemotherapy, so that in studies using the Stanford V regimen, optimal results were only seen after irradiation of more than 75% of patients.[32,33,40] A retrospective analysis of the UK LY09 study suggested that patients who did not receive radiotherapy after ABVD or multidrug regimens were at a significantly higher risk of recurrence, even from complete remission (CR), and that this was reflected in reduced overall survival.[41] On the other hand, a randomized trial by the EORTC showed that omission of radiotherapy after eight cycles of alternating MOPP-ABV had no impact on recurrence from CR, although patients in partial remission (PR) had a clear benefit.[42] A study comparing radiotherapy with two further cycles of chemotherapy by the GELA group also concluded that irradiation added nothing for patients in CR.[43]

As in early HL, the use of FDG-PET scanning is being actively investigated as a means to address some of these important areas of uncertainty. Retrospective analyses suggest that patients who reach a metabolic CR after two cycles of ABVD have a very favorable prognosis even if they presented with apparently poor

prognosis HL, whilst those with residual FDG-avid disease have a high chance of recurrence regardless of their baseline risk.[44,45] In this respect, FDG-PET provides significantly better prognostic information than CT scanning, in which residual fibrotic masses frequently confound interpretation and make the distinction of CR and PR somewhat subjective. The GHSG has recently reported the first results of their HD15 study, in which patients with residual masses of more than 2.5 cm on CT scans underwent FDG-PET scanning, and only those 21% with increased uptake received consolidation radiotherapy. The outcomes in the un-irradiated group were no worse than patients in conventional CR, albeit with relatively short follow-up. In the PET-positive group who underwent irradiation, the success rate was also relatively high, with 85% disease free; suggesting that for residual active disease, irradiation can play a valuable role.[46]

Based upon these findings, several studies currently underway are using FDG-PET after two cycles of treatment to guide escalation or de-escalation of therapy. An international collaborative study between the UK, Italian, Nordic, and Australasian groups is testing whether patients PET-negative after two ABVD can have de-escalation by omission of bleomycin, while those with positive scans will have treatment escalated using eBEACOPP or BEACOPP-14 regimens (*http://www.clinicaltrials.gov/ct2/show/NCT00678327*). Conversely the GHSG is starting with maximally intensive eBEACOPP and testing de-escalation for those with negative scans. The results of these approaches, which are complementary, will give an indication as to whether maximal intensity of treatment needs to be used at the beginning, or can be reserved for those with the most difficult-to-treat disease.

Management of recurrent disease

Fortunately, the progressive improvement in primary treatment has made the management of recurrent HL a diminishing problem, although this remains an important area, particularly since primary treatment failures in many cases can be salvaged with appropriate therapy. A variety of chemotherapy regimens, often incorporating Etoposide, Platinum, Ifosfamide, and Gemcitabine have been used in this setting, although few comparative trials have been performed and the results are apparently more or less equivalent, with generally high overall response rates of 60–90%, depending upon patient selection (Table 2). A recent randomized trial tested the conventional approach of two cycles of DHAP followed by BEAM high dose chemotherapy against a more intensive high-dose sequential program prior to BEAM, which showed no difference in the progression free or overall survival.[47] The results of treatment for patients with primary refractory HL, in general are much less promising, and it is in this group particularly that new approaches are most needed.

In the last few years, several new agents with potential activity have entered clinical trials. The CD30 antigen, which is expressed in Reed-Sternberg cells, has long been considered an attractive target for antibody-based therapy, but studies using antibody alone were disappointing. More recently, an immunotoxin has been developed, which links an anti-CD30 to the tubulin toxin monomethyl auristatin E, giving an immunoconjugate, brentuximab vedotin (SGN-35). This has shown promising activity in a large phase II study for patients with recurrent and refractory disease with responses in 95% of patients, all of whom had previously undergone high dose therapy.[48] Several trials are planned or in progress to determine where this agent may best be incorporated into management of HL to best effect.

Other new agents, such as small molecules targeting histone deacetylase[49] and the mammalian target of rapamycin,[50] have also shown some activity in early phase trials, but more evidence is needed to determine their potential role.

The principal aim of second-line chemotherapy in the great majority of cases is to reach a point at which myeloablative therapy with autologous progenitor cell rescue can be given, since this is the strategy that appears able to produce long-term remissions in at least 50% of cases.[51,52] There is increasing evidence of the value of FDG-PET evaluation prior to high dose therapy in this group, since those with residual FDG-avid disease appear to have much less favorable outcomes,[53–55] and should be considered for alternative approaches such as allotransplantation.

Table 2. Results of selected salvage chemotherapy regimens.

Regimen	No of Patients	Responses (%)			Grade ¾ toxicity (%)			Toxic deaths (%)
		CR	PR	ORR	Neutropenia	Thrombocytopenia	Vomiting	
Dexa-BEAM[52]	144	27	54	81	NS	NS	NS	5
Mini-BEAM[51]	55	49	33	82	86	60	NS	2
ESHAP[66]	22	41	32	73	59	NS	NS	4
ICE[67]	65	26	59	85	NS	NS	NS	0
DHAP[68]	102	21	68	89	88	69	26	0
GDP[69]	23	17	52	69	9	13	13	0

Nodular lymphocyte predominant Hodgkin's lymphoma

This sub-type, which accounts for only 5% of HL, has important phenotypic differences from classical HL, and is recognized as requiring a different approach to its management.[10] The histology is distinct, with Reed–Sternberg cells rarely present, but lymphocytic proliferation with histiocytes (L&H cells) often seen. The immunophenotype includes expression of B-cell antigens, such as CD20 and CD79a, but not CD15 and CD30, and clonal rearrangements of immunoglobulin genes are present. This type is more frequently seen in males than classical HL, is more often localized at presentation, and is recognized with increasing frequency, although the underlying incidence is probably not rising.

Retrospective series have identified the relatively indolent behavior of this type of lymphoma, with overall 10-year survival of around 90%.[56,57] Several studies have suggested that the risk of death from radiation or chemotherapy-induced toxicity, such as second malignancies or ischemic heart disease, exceeds the risk from the lymphoma.[10,58] In this context, a conservative approach is justified, with watchful waiting for those patients who present with localized disease that is fully excised by the diagnostic biopsy. Radiotherapy has excellent results in localized disease, with 10-year progression free survival of 85% for stage I and 65% for stage II disease reported in a recent series.[59]

Those patients requiring systemic therapy have been treated with a variety of regimens, often the same as those used for classical HL, although in general, the results have not been especially encouraging, with high rates of recurrence. The expression of CD20 has led to investigation of rituximab as a single agent, and the results of treatment for a small series of patients with relapsed disease showed a 94% response rate, although the median time to recurrence was only 33 months.[60] The combination of rituximab with low toxicity regimens, such as CVP, has some attraction and is being tested prospectively.

An important risk for patients with NLPHL is that of transformation to diffuse large B-cell lymphoma, often of the T-cell rich type the cumulative incidence of which in an extended French series was 12% at 10 years.[56] These patients require treatment with combination therapy, such as R-CHOP, and in general the results are good, with around 75% disease-free survival, although the occurrence of transformation is a poor prognostic event by comparison with other patients who have only NLPHL.

Management of Hodgkin's lymphoma in older patients

Although HL is a highly curable disease in younger patients, the changing demographics of the population mean that increasing numbers of cases present in much older patients, where they pose a considerable challenge. The presence of co-morbidities, such as cardiovascular, respiratory, or metabolic disease, and the poor tolerance of older patients for cytotoxic drugs, all present difficulties in the administration of curative treatment. Analysis of trials which included older patients has shown that they do not benefit from treatment intensification, and that toxicity is a major impediment to cure.[61] The VEPEM-B regimen was designed to deliver relatively dose-intense treatment at short intervals in the hope of improving tolerability,[62] but first results from a randomized trial suggested inferior results to ABVD.[63] A small series of patients treated with CHOP has been reported, with a 76% progression-free survival at 3 years.[64] The GHSG has recently reported results with the BACOPP regimen, which produced 60% progression-free survival, but at the expense of significant toxicity: more patients died of causes related to treatment than lymphoma.[65]

It is clear that conventional cytotoxic treatment is unsatisfactory for this group of patients, and it is to be hoped that newer agents, such as the immuntoxins, might offer an alternative approach in the future.

Conclusions

While great progress has been made in the treatment of HL, it is evident that significant challenges remain. This is a field in which clinical trials have previously made a notable contribution to outcomes and to the definition of therapy, which strikes the correct balance between toxicity and efficacy. The results of the current portfolio of studies will be equally important.

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Autologous and allogeneic stem cell transplantation in the management of Hodgkin's lymphoma

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A B S T R A C T

The prognosis of patients with Hodgkin's lymphoma (HL) who relapse after first-line therapy remains poor in many cases. The use of autologous stem cell transplantation (ASCT) is considered the standard of care for the vast majority of chemosensitive patients in first relapse. Two randomized trials showed significant benefit in freedom from treatment failure for ASCT over conventional chemotherapy for relapsed disease. The results of these trials have resulted in the recommendation of ASCT at time of first relapse for even the most favorable patients, although salvage radiotherapy can offer an effective treatment for selected subsets. Results of ASCT in refractory patients are significantly poorer. Alternative strategies should be offered in this setting. Allogeneic stem cell transplantation (allo-SCT) is considered a therapeutic alternative for those patients deemed to do poorly with an ASCT or who relapse after it. The recent use of reduced intensity protocols has been able to reduce significantly the exceedingly high non-relapse mortality classically associated to conventional conditionings. There seems to be a clinically beneficial graft-versus-HL effect associated with the development of chronic graft versus host disease. Nevertheless, disease relapse still remains the major cause of failure, and innovative strategies have to be developed to improve the results.

Introduction

Newly diagnosed patients with advanced stage Hodgkin's lymphoma (HL) have an excellent prognosis, as the vast majority of them can be cured with initial treatment.[1] In contrast, the prognosis of patients relapsing after first-line therapy with either combination chemotherapy (CT) or CT followed by radiotherapy (RT) remains poor in many cases. In most of these cases, high-dose chemotherapy and autologous stem cell transplantation (ASCT) is nowadays considered to be the treatment of choice.

Autologous stem cell transplantation in refractory/relapsed Hodgkin's lymphoma

Autologous stem cell transplantation for relapsed HL

The use of ASCT is now considered the standard of care for relapsed HL patients.[2] In several phase II studies, ASCT has been shown to produce between 30 to 65% long-term disease-free survival (DFS) in selected patients with refractory and relapsed HL.[3–9] Two randomized trials showed significant benefit in freedom from treatment failure (FFTF) for ASCT over conventional CT for relapsed disease.[10,11] The results of these trials have resulted in the recommendation of ASCT at time of first relapse for even the most favorable patients, although salvage RT can offer an effective treatment for selected subsets of patients with relapsed or refractory HL. The lack of a survival benefit in these randomized trials has been attributed to patients in the non-transplant arm undergoing transplant at the time of second relapse.

Randomized trials

The first randomized trial of transplant for relapsed disease was from the British National Lymphoma Investigation comparing ASCT with BEAM as a preparative regimen to mini-BEAM without autologous transplantation[10] in patients with active HL, for whom conventional therapy had failed. Twenty patients were assigned treatment with BEAM plus ASCT and 20 mini-BEAM. All had been followed up for at least 12 months (median 34 months). Five BEAM recipients died (two from causes related to ASCT and three from disease progression) compared with nine mini-BEAM recipients (all disease progression). That difference was not significant ($p = 0.318$). However, both 3-year EFS and PFS showed significant differences in favor of BEAM plus ASCT ($p = 0.025$ and $p = 0.005$, respectively). There were no differences in OS. This trial was prematurely closed as recruitment became increasingly difficult because patients refused randomization and requested an ASCT.

In the second randomized trial performed by investigators of the German Hodgkin's disease Study Group, 161 patients between 16 and 60 years of age with relapsed HL were randomly assigned two cycles of Dexa-BEAM and either two further courses of Dexa-BEAM or high-dose BEAM.[11] Only patients with chemosensitive disease (CR or

partial remission (PR)) after two courses of Dexa-BEAM proceeded to further treatment. Of the 117 patients with chemosensitive relapse, there was a significant improvement in 3-year FFTF for patients undergoing ASCT compared with four cycles of Dexa-BEAM (55% vs. 34%, p = 0.019). With a median follow up of 39 months (range, 3–78), the 3-year FFTF was significantly better for patients treated with BEAM, regardless of whether first relapse had occurred early (< 12 months) (41% vs. 12% p = 0.007) or late (> 12 months) (75% vs. 44%, p = 0.02). There was no statistically significant difference in OS for any subgroup of patients. After a median follow up of 7 years, results continue to show an advantage for high-dose therapy (49% vs. 32%, p = 0.02) but still no difference in 7-year OS rates. The absence of differences in OS might be partly due to the fact that about one-third of the patients receiving conventional salvage CT received an ASCT after further relapse. As in the first reported analysis, there were no differences in 7-year FFTF for patients with multiple relapses prior to trial entry (32% for Dexa-BEAM vs. 27% for BEAM-HSCT). Of note, no excess rate of myelodisplastic syndromes (MDS)/secondary acute myelogenous leukemia (sAML) was reported in the high-dose arm.[12]

To improve the results of ASCT in relapsed or refractory HL patients, the GHSG has employed a sequential high dose CT prior to the intensive procedure.[13] Treatment started with two cycles of DHAP to reduce tumor burden. Patients achieving a CR or PR subsequently received a high-dose CT program with cyclophosphamide (4 g/m^2 iv), methotrexate (8 g/m^2 iv), vincristine (1.4 mg/m^2 iv), and etoposide (2 g/m^2 iv). Patients were then autografted using BEAM. Response rate after the final evaluation was 80% (72% CR, 8% PR). With a median follow up of 40 months (range, 3–84), FFTF, and OS for patients with early relapse were 62% and 78%, respectively and for patients with late relapse, 63% and 79%, respectively. The promising results coming from this phase II trial prompted the GHSG to develop a prospective phase III clinical trial looking at relapsed patients with HL being treated either with the conventional salvage approach (DHAP x two cycles plus ASCT with BEAM) versus DHAP plus high-dose sequential protocol plus ASCT. Somewhat unexpectedly, there were no significant differences in terms of PFS, FFTF, and OS between both arms.[14] Length of treatment was significantly longer, protocol violations higher, and mean administered total dose of chemotherapy lower in the experimental group.

Autologous stem cell transplantation in primary refractory disease

Prognosis of patients with primary refractory disease (PRD) is extremely poor. Nevertheless, and as opposed to non-Hodgkin lymphoma (NHL), where chemorefractory patients are not salvaged by transplant, there seems to be a general consensus that even patients who fail first- and second-line CT may still enjoy a 20–30% chance of cure with ASCT.

In the EBMT analysis published by Sweetenham *et al*.,[15] 175 PR-HL patients were presented; actuarial 5-year PFS and OS were 32% and 36%, respectively. In the ABMTR analysis on 122 patients undergoing ASCT after an induction failure (IF),[16] actuarial probabilities at 3 years were 38% and 50% for PFS and OS, respectively. The reasons for these discrepancies are not clear but one must be aware of the fact that under the definition of IF, different subsets of patients with different long-term outcome can be included. In the ABMTR analysis, almost 50% of the patients whose response to salvage CT prior to ASCT was known had a chemosensitive disease before transplantation. Lazarus *et al*.[16] found that the presence of B symptoms at diagnosis, as well as Karnofsky status at ASCT correlated with survival, and that the absence of these two factors was associated with an excellent 2-year survival of 87%. In the EBMT analysis,[15] patients receiving more than one line of CT before transplantation did worse, both in terms of OS and PFS.

The GELTAMO Cooperative Group presented the results of 62 patients treated with an ASCT for an IF.[17] One-year transplant related mortality (TRM) was 14%. Response rate at 3 months after ASCT was 52% (CR in 21 patients (34%), PR in 11 patients (18%)). Actuarial 5-year TTF and OS were 15% and 26%, respectively. The presence of B symptoms at ASCT was the only adverse prognostic factor significantly influencing TTF. The presence of B symptoms at diagnosis, MOPP-like regimens as first line therapy, bulky disease at ASCT, and two or more lines of therapy before ASCT adversely influenced OS.

Fermé *et al*. reported on 157 patients with either IF, PR of less than 75%, or relapse after doxorubicin-based chemotherapy plus/minus RT.[18] All patients received MINE as second-line therapy followed by ASCT with BEAM as the preparative regimen. The 5-year OS rates were 30% for patients with IF versus 72% for patients with less than 75% PR and 76% for patients with relapsed disease following first-line therapy. Of the 101 patients who went on to transplant, the 5-year FF2F rate for patients with a response to MINE was 64% versus 25% for those not responding to MINE. Of the 64 patients with IF, 40 responded to second- or third-line salvage therapy and 32 of these patients went on to transplant. Of the 24 patients not responding to salvage, 9 went on to transplant, only 1 of whom achieved a CR with ASCT.

The long-term outcome of 75 consecutive patients with biopsy-confirmed HL at the completion of primary therapy has been summarized by the Memorial Sloan Kettering Cancer Center group.[19] All patients underwent standard-dose salvage therapy followed by involved field RT (IFRT). Patients without progression went on to receive high-dose etoposide, cyclophosphamide, and either total lymphoid irradiation (if no prior RT) or carmustine (if prior RT) followed by bone marrow or peripheral stem cell rescue. Seven patients were excluded from transplant because of progression on standard salvage therapy and had a 4 month median survival. Patients with less than a 25% decrease with standard salvage therapy (n = 27) had a 10-year EFS of 17% versus 60% for those with at least a 25% decrease to standard second-line therapy (n = 48).

However, as indicated by all the previously shown analyses and as highlighted by Josting, reports of ASCT for PRD are subject to significant selection bias.[20] Patients with rapidly progressive disease, poor perform-

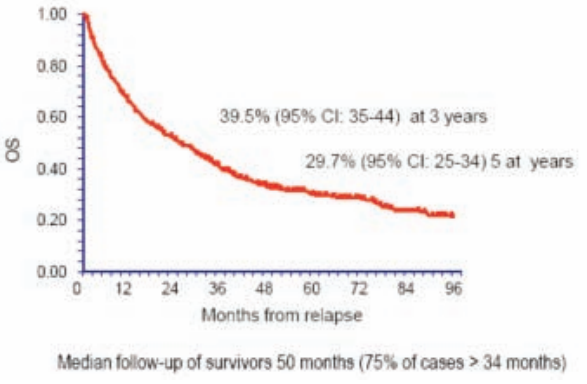

Figure 1. Long-term outcome of patients with Hodgkin's lymphoma who relapse after autologous stem cell transplantation. Results from the Lymphoma Working Party of the EBMT (Martínez *et al*., unpublished results).

ance status, older age, and poor stem cell harvest are not included in the reports. The GHSG, in a landmark analysis comparing patients with PRD who did or did not receive transplant within 6 months of progression, and excluding all patients who survived less than 6 months, showed no advantage to ASCT overt those treated with conventional salvage therapy.[20]

Prognosis after failed ASCT

The median survival for patients with relapse post-transplant is approximately 2 years, with the most important predictor of outcome being response to salvage therapy.[21,22] The GELTAMO recently reported the long-term outcome of a group of 175 patients who relapsed at a median time of 10 (4–125) months after ASCT.[23] OS and PFS were of 35±4% and 23±4% at 3 years, respectively. Advanced clinical stage at relapse and a short time interval between ASCT and relapse less than or equal to 12 months were independent adverse prognostic factors for PFS. Patients with both features had 3-year PFS of 14% compared with 48% for patients without either factor. Advanced clinical stage at relapse, extranodal disease and hemoglobin level less than or equal to 100 g/l at relapse were significant adverse prognostic factors for OS. More recently, the Lymphoma Working Party (LWP) of the EBMT reviewed 462 adult patients registered in the EBMT database who experienced a relapse after an ASCT in order to identify prognostic factors at relapse with an impact on long-term outcome after the procedure. Median time from ASCT to relapse or progression was 7 months (range, 1–78). After a median follow-up of 49 months, OS was 32% at 5 years (Figure 1). In multivariate analysis, independent risk factors for OS were early relapse, stage IV, bulky disease, poor performance status, and age 50 years or older at relapse. Patients with no risk factors OS at 5 years were 62% compared with 37% and 12% for those having 1 and 2 or more factors, respectively. This score was also predictive for outcome in each group of rescue treatment after ASCT failure.[24]

How to improve results after an ASCT? The role of PET

The impact of ASCT in the long-term outcome of patients with relapsed/refractory HL is not the same in all subgroups of patients. Several authors have retrospectively analyzed prognostic factors at first relapse with independent impact on the results of ASCT. In this sense, different authors found time to relapse (< 12 months vs. ≥ 12 months),[25–27] extranodal disease at relapse,[25,27] advanced stage, and anemia at relapse,[26] B symptoms,[27,28] and refractory disease[28] of clinical importance. More recently, the role of PET has also been analyzed in the autologous transplant setting: in a group of 101 patients with both non-HL and HL, both FDG-PET after two cycles of chemotherapy and clinical risk score were independent prognostic factors for failure-free survival after ASCT.[29] In a similar way, the group from the MD Anderson indicated that pretransplant positive PET/Gallium scans were able to predict poor outcome after ASCT in patients with relapsed/refractory HL.[30]

To improve results after ASCT, both new monoclonal antibodies (SGN-35) and histone deacetylase inhibitors (OLDH568, panobinostat) are being tested in prospective clinical trials as maintenance therapy to prevent relapse in those patients with high-risk relapsed HL being treated with an ASCT.

ASCT in refractory/relapsed Hodgkin's lymphoma

Myeloablative conditioning and allo-SCT in HL

The first reports on allogeneic stem cell transplantation (allo-SCT) in patients with HL appeared in the mid-eighties.[31,32] Two larger registry-based studies published in 1996 gave disappointing results. Gajewski *et al*. analyzed 100 HL patients allografted from HLA-identical siblings and reported to the International Bone Marrow Transplant Registry (IBMTR).[33] The 3-year-rates for OS, DFS, and the probability of relapse were 21%, 15%, and 65%, respectively. The major problems after transplantation were persistent or recurrent disease or respiratory complications, which accounted for 35% to 51% of deaths. A case-matched analysis including 45 allografts and 45 autografts reported to the EBMT was performed by Milpied *et al*.[34] They did not find significant differences in actuarial probabilities of OS, PFS, and relapse rates between allo-SCT and ASCT (25%, 15%, 61% vs. 37%, 24%, 61%, respectively). The actuarial TRM at 4 years was significantly higher for allografts than for autografts (48% vs. 27%, p=0.04). Acute GvHD greater than or equal to grade II was associated with a significantly lower risk of relapse, but also with a lower survival rate.

A number of reports confirmed the registry data: allo-SCT resulted in lower relapse rates but significantly higher toxicity with no improvement over ASCT when PFS or OS were considered.[35–37] Although the poor results after myeloablative conditioning could at least partly be explained by the very poor-risk features of many individuals included in these early studies, the high procedure-related morbidity and mortality prevented the widespread use of allo-SCT.

Reduced intensity conditioning and allo-SCT in HL

Since the first clinical experiences that suggested that

allo-SCT after a nonmyeloablative conditioning (RIC/allo-SCT) might represent an interesting alternative to classical allo-SCT, a number of reports have addressed the question whether RIC/allo-SCT might also work for patients with HL. Although the overall number of patients with HL treated with allo-SCT has remained low in comparison with other hematological malignancies, the percentage of patients with refractory and relapsed HL who received a RIC/allo-SCT has been growing steadily in Europe over the last 5 years (Figure 2).

The largest cohort of patients treated with RIC/allo-SCT in HL was recently reported by the LWP of the EBMT[38] (n = 285). Median time from diagnosis to allo-SCT was 41 (4–332) months. Patients had received an average of four lines of prior therapy[1–8] and 288 patients (77%) had failed one or two ASCT. At the time of allo-SCT, 47 patients (17%) were in CR, 123 patients (43%) had chemosensitive disease, and 115 patients (40%) had chemoresistant disease or untested relapse. One hundred and seventy two patients (63%) were allografted from a matched sibling donor (MRD), 94 (33%) from a matched unrelated donor (MUD), and 19 from a mismatched donor (7%). Grade II-IV acute GvHD (aGVHD) was reported in 27% of patients, chronic GvHD (cGVHD) in 40% of patients at risk. The 100-day TRM was 12% but increased to 20% at 12 months, and to 22% at 3 years; it was significantly worse for patients with chemoresistant disease. Two-year PFS was 29% and again significantly worse for patients with chemoresistant disease ($p < 0.001$). The development of chronic GVHD was associated with a higher TRM and a trend to a lower relapse rate. In a landmark analysis, the development of either acute or chronic GVHD by 9 months post transplant was associated with a significantly lower relapse rate.

The MD Anderson Cancer Center recently updated their experience[39] in 58 patients with relapsed or refractory HL who underwent a RIC/allo-SCT from a MRD (n = 25) or a MUD (n = 33). Forty-eight (83%) patients had received a prior ASCT. Disease status at RIC/allo-SCT was sensitive relapse (n = 30) or refractory relapse (n = 28). The conditioning regimen employed was fludarabine (125–130 mg/m^2 over 4–5 days), melphalan (140 mg/m^2 IV over 2 days) (FM), and antithymocyte globulin (thymoglobulin 6 mg/kg over 3 days) was added for the 14 most recent MUD transplants. Cumulative 100-day and 2-year TRM were 7% and 15%, respectively. The cumulative incidence (CI) of grade II-IV acute GVHD was 28%. The CI of chronic GVHD at any time was 74%. Fourteen patients (24%) received a total of 25 (range 1–5) donor leukocyte infusions (DLIs) for disease progression/relapse. Five of them (35%) received CT as well, and nine (64%) developed acute GVHD after the DLI. Projected 2-year OS and PFS were 64% and 32%, with 2-year projected disease progression at 55%. There was no statistically significant difference between MRD and MUD transplants with regard to OS, PFS, and disease progression. There was a trend for the response status prior to allo-SCT to favorably impact PFS ($p=0.07$) and disease progression ($p=0.049$), but not OS ($p=0.4$). Partial responders and patients with stable/ refractory disease fared similarly with regards to OS and PFS.

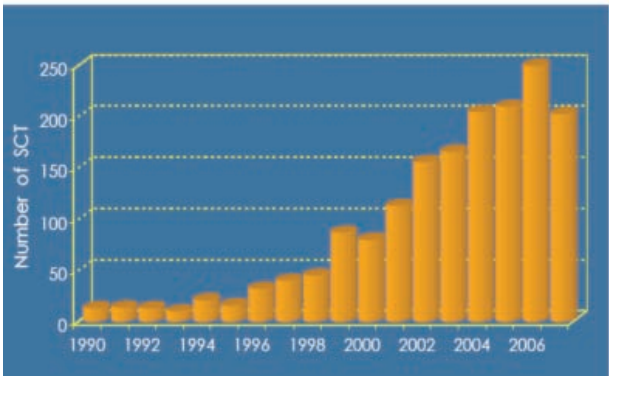

Figure 2. Number of allogeneic stem cell transplantation for relapsed/refractory Hodgkin's lymphoma per year (EBMT Database from 1990–2007).

Forty patients with relapsed or refractory HL treated with the combination of fludarabine (150 mg/m^2) and melphalan (140 mg/m^2) have recently been presented by the Spanish group.[40] GvHD prophylaxis consisted of cyclosporine A (CsA) and methotrexate (MTX). Twenty-one patients (53%) had received more than two lines of CT, 23 patients (58%) had been irradiated, and 29 patients (73%) had failed a previous ASCT. Twenty patients were allografted in resistant relapse and 38 patients received hematopoietic cells from a MRD. One-year TRM was 25%. Acute GvHD developed in 18 patients (45%) and cGvHD in 17 (45%) of the 31 evaluable patients. Extensive cGvHD was associated with a trend to a lower relapse rate (71% vs. 44% at 24 months, $p=0.07$). The response rate 3 months after RIC/allo-SCT was 67%. Eleven patients received donor lymphocyte infusions (DLIs) for relapse or persistent disease. Six patients (54%) responded. OS and PFS were 48% and 32% at 2 years, respectively. Refractoriness to CT was the only adverse prognostic factor for both OS and PFS.

Investigators from Seattle reported their results in relapsed/refractory HL patients.[41] Thirty-eight patients had a MRD, 24 a MUD, and 28 a HLA-haploidentical related donor. The patients received 2 Gy total body irradiation (TBI) alone (n = 17) or in combination with fludarabine (90 mg/m^2) and immunosuppression consisted of micophenolate mofetil (MMF) and CsA. All patients were heavily pre-treated with a median of five prior regimens administered. Ninety-two percent of the patients had failed a previous ASCT. Prior to RIC/allo-SCT, 22 patients were in CR, 30 in PR, 9 had relapsed disease, and 29 had refractory disease. The overall incidence of grade II-IV aGvHD was 50%. The incidence of extensive cGvHD was 55% at 1 year. TRM was significantly lower in those patients being allografted from a HLA-haploidentical donor. Relapse risk was also lower in haploidentical recipients.

Peggs *et al.* explored the effects of *in vivo* T-cell depletion with alemtuzumab followed by fludarabine (150 mg/m^2) and melphalan (140 mg/m^2) in multiply relapsed patients; 90% of them had failed a previous autograft.[42] At transplant, 8 patients were in CR, 25 patients were in PR, 1 patient was in untested relapse, and 15 patients had refractory disease. Thirty-one patients were allografted

from a MRD and 18 from MUDs. All patients engrafted, grade II-IV aGvHD occurred in 16% of patients, 14% developed cGvHD before DLIs. Nineteen patients received DLIs for progression (n = 16) or mixed chimerism (n = 3). Nine patients (56%) showed a response, which was significantly associated with acute and/or extensive chronic GvHD. Non-relapse mortality was 16% at 730 days. Projected 4-year OS and PFS were 56% and 39%, respectively. Clinical characteristics and outcome of the mentioned studies are summarized (Table 1).

Finally, the final results of a multicenter phase II prospective study on the role of RIC/Allo-SCT were presented at ASH2010.[43] Ninety-two HL patients with an HLA identical sibling or a MUD were treated with two courses of salvage chemotherapy. Seventy-eight patients (85%) who showed at least stable disease were eligible to receive a RIC/Allo-SCT; all 14 patients with chemorefractory disease died from progressive lymphoma. Most allografted patients had failed a prior autologous transplantation (86%); 50 patients were allografted with chemosensitive and 28 with resistant disease; MUDs were used in 23 patients. Fludarabine (150 mg/m^2 iv) and melphalan (140 mg/m^2 iv) were used as conditioning regimen, and cyclosporine A plus methotrexate as graft-versus-host disease prophylaxis. Non-relapse mortality was 8% at 100-days and 15% at 1-year. Relapse was the major cause of failure. PFS was 48% at 1-year and 24% at 4-years. Chronic GVHD was associated with a lower relapse incidence and a better PFS (Figure 3). Patients allografted in CR had a significantly better outcome. OS was 71% at 1-year and 43% at 4-years.

No definitive information is available with respect to the best conditioning protocol or the impact of T-cell depletion in this setting. If one accepts that attempting an effective graft-versus-HL reaction may require several months, preventing early progression by administering a vigorous conditioning regimen remains an essential goal still to accomplish. In this sense, the combination of a more intensive preparative regimen, the BEAM protocol together with a profound T-cell depletion with alemtuzumab as aGVHD prophylaxis has demonstrated to be associated with sustained donor engraftment, a high response rate, minimal toxicity (NRM 7.6%), and a low incidence of GVHD.[44] The two analyses presented by the Lymphoma WP of the EBMT also strengthen this argument. The use of TBI-based RIC protocols significantly increased disease progression after RIC/allo-SCT in Robinson's analysis[38] and TBI-based conditioning regimens also emerged as an adverse prognostic factor for disease progression after transplant, PFS and OS in the recently performed comparative analysis between conventional and RIC protocols.[45]

Comparison of myeloablative and reduced-intensity conditioning prior to allo-SCT in relapsed and refractory HL

The LWP has performed the only analysis reported so far which compares outcomes after reduced-intensity or myeloablative conditioning in patients with HL.[45] Ninety-seven patients with HL were allografted after RIC and 93 patients were allografted after a conventional regimen. A previous ASCT was more frequent in the RIC/allo-SCT group (59% vs. 41%, p=0.03) as was the use of peripheral blood stem cells (82% vs. 56% p < 0.001). Non-relapse mortality was significantly decreased in the RIC/allo-SCT group (HR 2.43 (95%CI 1.48–3.98), p < 0.001). PFS and OS were also better in the reduced intensity group (HR 1.28 (95% CI 0.92–1.78), p = 0.1 and HR 1.62 (95% CI 1.15–2.28), p = 0.005). The development of chronic GVHD significantly decreased the incidence of relapse after transplantation, which translated into a better PFS and OS. This analysis indicates that RIC/allo-SCT is able to significantly reduce TRM after transplantation and improves the long-term outcome of relapsed and refractory patients treated with an allograft.

Graft-versus-Hodgkin effect

The significant reduction of the TRM observed in the RIC/allo-SCT has been able to put in evidence the existence of a graft-versus-HL effect. Direct evidence of a graft-vs-HL effect comes from two main sources: the demonstration that the development of acute or chronic GVHD after allo-SCT is associated to a lower relapse rate, and the clinical information coming from DLIs. Relapse rate is significantly lower in those patients developing GVHD after transplantation. This fact was already indicated by single center analysis, including a low number of patients,[39,40,42,43] but also by larger retro-

Author, year	N. patients	Sensitive vs refractory	Matched sib / MUD / haplo	Condition. regimen	NRM	RR	PFS	OS
Robinson, 2009	285	213 / 72	172 / 94 / 0	Varied	19.5% (1 yr)	59% (3yr)	25% (3yr)	43% (3yr)
Anderlini, 2008	58	30 / 28	25 / 33 / 0	Flu-Mel	15% (2 yr)	55% (2 yr)	32% (2yr)	64% (2yr)
Burroughs, 2008	90	61 / 28	38 / 24 / 28	Flu-TBI	21% / 8% / 9% (2yr)	53% / 63% / 40% (2yr)	23% / 29% / 51% (2yr)	53% / 58% / 58% (2yr)
Peggs, 2005	49	34 / 16	31 / 18	Flu-Mel-Campath	16.3% (730 days)	NA	32.4% (4yr)	55.7% (4yr)
Alvarez, 2006	40	20 / 20	38 / 2	Flu-Mel	25% (1 yr)	NA	32% (2yr)	48% (2yr)

Table 1. Reduced intensity allogeneic stem cell transplantation for relapsed/refractory Hodgkin's lymphoma. Summary of the outcomes of the different published trials.

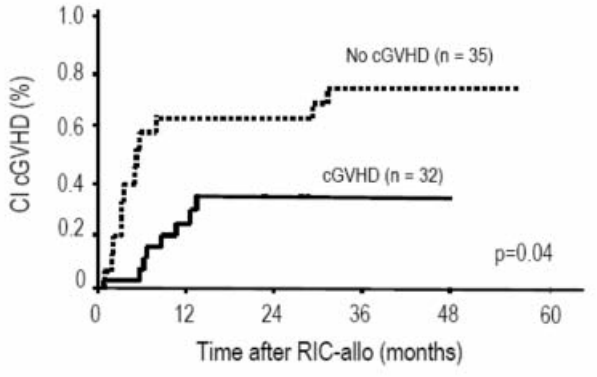

Figure 3. Impact of the development of chronic graft versus host disease on relapse relate after allogeneic stem cell transplantation. Final results of the HDR-Allo Trial (Sureda *et al*., unpublished results).

spective analyses, such as those performed by the Lymphoma WP of the EBMT. The development of either acute or chronic GVHD by 9 months after transplant was associated with a lower relapse rate in Robinson's analysis.[38] The development of acute GVHD alone did not reduce the relapse rate but was associated with a significantly higher TRM and a lower PFS and OS. In contrast, the development of chronic GVHD alone was associated with a trend to lower relapse rate, a significantly higher TRM, but had no impact on OS or PFS. In the comparative analysis between conventional allo-SCT and RIC/allo-SCT, the development of chronic GVHD after transplantation significantly reduced relapse rate after transplant and improved PFS in those patients presenting with this complication.[45]

The most direct evidence for a graft versus malignancy effect comes from observations relating to disease responses to DLIs. Peggs *et al*. have previously reported clinical responses in 9 of 16 patients receiving DLIs for measurable disease post RIC/allo-SCT.[42] Other small series have reported response rates of 44–54% following DLI administration.[39,40] In Robinson's analysis,[38] many DLIs were administered following adjunctive therapy but disease responses were reported in 30% of patients receiving DLI alone. Patients responding to DLIs had a higher incidence of GVHD post DLI than those not responding. These data confirm the presence of a clinically effective graft versus Hodgkin's effect. However, the efficacy of DLIs is likely to depend upon the bulk of disease at the time of administration and the optimal use of DLI requires further refinement. Preemptive, dose escalating, or PET scan guided strategies may improve the overall efficacy of DLIs.[46,47]

How to improve the results of allo-SCT?

Relapse rate is the major cause of failure for those patients with relapsed/refractory HL being considered candidates for such an approach. There are several possible ways to address this issue: better patient selection and chemosensitivity of the tumor. Disease status at the time of allo-SCT is the most important prognostic factor for the long-term outcome of this procedure. Only those patients in CR or very good PR should be considered adequate candidates for an allo-SCT at least with the current protocols. In this sense, new salvage strategies to try to put patients into a better response should be sought. Pre-transplant PET does not seem to have a prognostic impact on either OS or PFS after allo-SCT but PET was able to diagnose relapse after allo-SCT earlier than conventional computed tomography.[46,48] In this sense, the role of PET should be further explored in this setting.

Modulation of the intensity of the conditioning regimen can also result in lower relapse rate after allo-SCT. Low-dose TBI containing regimens seem to be associated with a high relapse rate in the RIC-allo setting.[38,45] New drugs with potential antitumoral activity. in front of HL but with a safe profile are being currently tested by different groups. The intensity of conditioning regimen can be eventually augmented without significantly modifying Non-Relapse Mortality (NRM), taking into consideration the population of patients that are considered candidates for an allo-SCT and the fact that GVL effect is not as potent as in low-grade lymphoproliferative disorders.

Finally, the "so called" maintenance strategy currently being explored in the ASCT setting can also be analyzed in the allogeneic one.

Conclusions

HL is a highly curable hematological malignancy with the current available first line combination therapy. Nevertheless, a fraction of these patients are primary refractory to first line therapy or eventually relapse after having achieved a CR. Radiotherapy alone is usually not considered a curative therapeutic option for those patients with relapsed disease. In this setting, ASCT is the standard of care. Results of ASCT in primary refractory patients, as well as in those presenting with adverse prognostic features at relapse have room for improvement and unfortunately, a significant proportion of patients do still relapse after the autologous procedure. Allo-SCT is now considered a therapeutic option for those patients relapsing after an ASCT. Nevertheless, it is still associated with a high relapse rate after the procedure, and its potential role in earlier phases of the disease deserves further studies.

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Immune pathophysiology of primary immune thrombocytopenia

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A B S T R A C T

Primary immune thrombocytopenia (ITP) is characterized by antibody mediated destruction of platelets and suppression of megakaryocyte and platelet development. The underlying defect leading to autoantibody production is unknown, and it is likely that both genetic and environmental factors are involved. Platelet-specific autoantibodies are often directed against a restricted number of "dominant" epitopes of GPIIbIIIa, or less frequently of GPIbIX or other platelet glycoproteins.

The T cell compartment is now known to play a crucial role in ITP. Cytotoxic T cells may be involved in platelet destruction and suppression of megakaryopoiesis. Many models of autoimmune disease have a Th1 bias, which is also seen in ITP, and which is reversed upon treatment. Furthermore, a reduction in suppressor T-regulatory cells may predispose to the emergence of autoantibodies in response to exogenous antigens.

Finally, an ITP-like presentation occurs in the setting of chronic infections, such as HIV, HCV, and *Helicobacter pylori*. Antibodies that cross-react with platelets have been identified in patients who develop thrombocytopenia in association with these infections, suggesting that molecular mimicry and epitope spread may be a common pathway to antiplatelet antibody development in patients with ITP as well.

Introduction

Primary immune thrombocytopenia (ITP) is an acquired autoimmune disorder characterized by isolated thrombocytopenia in the absence of conditions known to cause thrombocytopenia, such as infections, other autoimmune disorders, drugs, and so on.[1]

In this review, we shall discuss the current understanding of the pathophysiology of ITP.

Abnormalities of B and T cells

Harrington's seminal experiment provided the first evidence that thrombocytopenia in ITP is caused by a plasma-derived factor,[2] later identified as antiplatelet antibodies.[3,4] The most commonly identified antigenic target of these autoantibodies is platelet glycoproteins (GP). Autoantibodies are often directed against a restricted number of "dominant" epitopes of GPIIbIIIa, or less frequently of GPIbIX or other platelet glycoproteins.[5] A number of ITP patients have antibodies directed to multiple platelet antigens.[6] Antibodies against GP IIb/IIIa show clonal restriction in light-chain use,[7] and antibodies derived from phage-display libraries show selective usage of a single Ig heavy-chain variable region gene ($VH_{3\text{-}30}$).[8] Sequencing of the antigen-combining regions of these antibodies suggests that they originate from a limited number of B-cell clones by antigen-driven affinity selection and somatic mutation.[8] It should be noted, however, that autoantibodies are not detectable in up to 50% of ITP patients,[6,9] and that remission in ITP can occur despite the continued presence of platelet autoantibodies.[10] Reasons for these findings may include technical factors (current monoclonal-based assays only detect antibodies with known specificity, typically GPIIb-IIIa and GPIb-IX; variable sensitivity of the assays), removal of autoantibodies by megakaryocytes, and the presence of alternative mechanisms of the thrombocytopenia.

As a matter of fact, several lines of evidence also link T cells to the pathogenic process in ITP. Platelet-reactive T cells have been found in the blood of patients with this disorder, with the major target antigen being GP IIb/IIIa.[11] In these patients, T cells stimulate the synthesis of antibody after exposure to fragments of GP IIb/IIIa but not after exposure to native proteins.[12] The derivation of these cryptic epitopes *in vivo* and the reason for sustained T-cell activation are unknown. It has been hypothesized that cryptic epitopes, normally not exposed in a self-antigen, may become exposed and recognized by the immune system under certain circumstances, for example, an infection.[13]

The cytokine profile in the peripheral blood of patients with ITP is consistent with a Th1 (proinflammatory) response,[14] a pattern seen in most organ-specific autoimmune diseases. These results were supported by flow cytometry studies, showing an

increased Th1/Th2 ratio in ITP patients compared with controls.[15] In keeping with these findings, investigation of whole blood gene expression profile using DNA microarrays identified an ITP-specific signature, which also included interferon (IFN)-induced genes, such as GBP2 and IFIT2.[16] Pathway analysis demonstrated that IFN signaling, death receptor, and protein ubiquitination pathways were associated with ITP.

Other studies have shown that patients with chronic ITP often exhibit expansion of oligoclonal T-cells[15,17] and the presence of cytotoxic T cells against autologous platelets.[18] In fact, T cells from patients with ITP show increased expression of cytotoxic genes, such as tumor necrosis factor- alpha, perforin, and granzyme A and granzyme B.[18,19] Interestingly, several members of the killer cell immunoglobulin-like receptor (KIR) family were upregulated in patients with active disease, and CD3+ lymphocytes expressing KIRs were greater in number in ITP patients in remission than in patients with active ITP or normal controls patients.[18] Since KIRs downregulate cytotoxic T-lymphocyte (CTL) and natural killer cell responses by binding to MHC class I molecules, thereby preventing lysis of target cells, it may be speculated that cytotoxic T cells play a part in at least some patients with ITP.

The emergence of antiplatelet autoantibodies and antiplatelet cytotoxic T cells is a consequence of a loss of the immunological tolerance for self antigens. Filion *et al*. have shown that autoreactive T cells directed against GPIIb/IIIa are present in the peripheral blood of all healthy individuals,[20] implying that peripheral tolerance mechanisms are crucial to prevent autoreactive T cells from becoming activated. Several other T cell abnormalities have emerged from the investigation of immune regulation in ITP patients. Among these, CD4+CD25+ regulatory T cells have an impaired suppressive activity when compared with healthy subjects.[21] Also, CD3+ T lymphocytes from patients with active ITP present an altered expression of genes associated with apoptosis and are significantly more resistant to dexamethasone-induced suppression compared with normal lymphocytes.[18,22]

As far as B cells are concerned, the expansion of autoreactive clones is suppressed in the bone marrow. If some B cells escape this suppression or deletion, peripheral mechanisms, most importantly the functional balance between activating and inhibitory Fc receptors (FcR), may also be launched to maintain tolerance.[23]

Antigen-presenting cells in ITP

Like all immunoglobulin (Ig) isotype-switched IgG antibody responses, autoreactive IgG against a protein antigen is initiated by activated T helper cells that recognize specific peptide sequences on major histocompatibility complex class II-positive antigen-presenting cells (APCs). The role of APCs for the loss of tolerance in ITP remains unclear, but dendritic cells from patients with ITP have upregulated costimulatory molecules enhancing autoreactive T- and B-cell responses against platelets.[24] A model has been advanced in which APCs are crucial in generating a number of new or cryptic epitopes from platelet glycoproteins.[25] In this model, APCs expressing these novel peptides, and along with costimulatory molecules, induce the activation of T cells that recognize these additional platelet antigens. Thus, this acquired recognition of new self-determinants, or epitope spreading, may play an important role in the initiation and perpetuation of ITP. T-cell clones that react with cryptic epitopes may escape the negative selection in the thymus when self-determinants are present at a sub-threshold concentration.

Infection-associated ITP and the role of molecular mimicry

Thrombocytopenia may accompany or follow a variety of infections from which ITP must be differentiated. In adults, the most prevalent infections associated with thrombocytopenia are those from hepatitis C virus (HCV), human immunodeficiency virus (HIV), and *Helicobacter pylori* (*H. pylori*).[26] In typical cases, the thrombocytopenia presents with an insidious onset, has no tendency to remit spontaneously (although its severity may parallel the stage of the infectious disease), and may closely mimic chronic ITP. Response to infection may generate antibodies that cross-react with platelet antigens, most notably GP IIb-IIIa or immune complexes that bind to platelet Fc receptors.[27–30]

Many patients with HIV-associated thrombocytopenia have autoantibodies that recognize a restricted peptide sequence (GPIIIa49-66) in platelet membrane GPIIIa, and can be recovered from patient plasma in the form of immune complexes consisting of autoantibody and platelet fragments.[27,28] Recently, Zhang *et al*. found that sera from patients coinfected with HCV and HIV reacted with four peptides present in nonconserved regions of the HCV core envelope 1 protein.[29] Antibodies raised against one of these peptides (PHC09) caused severe thrombocytopenia when injected into wild-type mice whose GPIIIa is more than 80% identical to that of humans. Immunization of wild-type mice with HCV core envelope protein 1 had no effect on platelet count. However, NZB/W F1 mice, a strain in which immune surveillance is defective, produced antibodies specific for PHC09 and became thrombocytopenic. The titer of PHC09-specific antibody in patients coinfected with HCV, and HIV correlated with both the incidence of thrombocytopenia and its severity. The authors conclude that humans and immunodeficient mice immunized with HCV core envelope protein 1 often produce antibodies that recognize GPIIIa49-66 through molecular mimicry and are capable of causing clinically significant thrombocytopenia.

With regards to ITP and *H. pylori* infection, platelet-cross-reactivity has been shown between platelet-associated immunoglobulins and bacterial components, in particular cytotoxin-associated gene (Cag) A protein[30] and urease B.[31]

In support of the molecular mimicry theory, microbial reduction/eradication leads to remission in a substantial fraction of infected patients. In the case of *H. pylori*, questions remain regarding why there appears to be marked variation in response rates among patients from different geographic areas.[32]

Genetic factors

Little is known of the genetic factors that may contribute to the predisposition to develop ITP or influence the natural history of ITP and response to treatment. A pilot study in 37 children with chronic ITP investigated

common variants in the regulatory regions of cytokines (TNF, LTA, IL1RN, IL1A, IL1B, IL4, IL6, and IL10) and structural variants of the low affinity Fcγ receptors (FCGR2A, FCGR3A, and FCGR3B).[33] Two combinations of genotypes (TNF and FCGR3A; P = 0.0003, and LTA and FCGR3B; P = 0.011) were significantly associated with ITP compared with healthy controls. The NA1/NA1 genotype of the FCGR3B locus was observed in 30% of patients compared with 10% of controls and may be particularly relevant to ITP, as NA1 has a higher affinity than NA2 to IgG. The heterozygous V/F genotype of the FCGR3A locus was also more frequent in patients than in controls (62% vs. 41%). These results suggest that immune complex handling may play a role in the pathophysiology of ITP, and that variant FcγR genes with decreased activity may provide partial protection against ITP.

With regards to cytokines, the transcriptionally more active allele of TNF (allele 2 of –308) and the closely linked LTA allele 1 were both less common in children with ITP than in healthy controls. No clear hypotheses to account for these findings have been advanced.

In an earlier study, deletions of Humhv3005, a developmentally regulated Ig variable (V) gene, and/or highly homologous VH genes were found more frequently in ITP patients (14 of 44, 31.8%) than in healthy controls (7/88, 8%, p = 0.002).[34]

Finally, associations with HLA-DRw2[35] and HLA-DRB1*0410[36] have been reported, although the role played by these MHC molecules remains obscure.

Mechanisms leading to thrombocytopenia

Ex vivo studies have shown that the spleen is the primary site of antibody production,[37,38] whereas platelet kinetic studies have shown that the spleen is also the dominant organ for the clearance of IgG-coated platelets.[39,40] In a minority of patients, hepatic clearance predominates.

Human macrophages express several Fc receptors that bind IgG specifically.[41] Functionally, there are two different classes of Fc receptors: the activation and the inhibitory receptors, which transmit their signals via immunoreceptor tyrosine-based activation (ITAM) or inhibitory motifs (ITIM), respectively. Clinical data, along with information gained from animal models, suggest that the FcγRI, the high affinity receptor, does not play a relevant role in ITP.[42,43] On the other hand, evidence has accumulated to indicate that the low-affinity receptors FcγRIIA and FcγRIIIA are primarily responsible for removal of opsonized platelets.[44] Engagement of FcγRIIA on the surface of human macrophages by anti-GPIIb/IIIa-coated platelets triggers intracellular signaling through the tyrosine kinase Syk that leads to engulfment of the opsonized platelets.

The presence of antibodies against GP Ib/IX has been associated with resistance to intravenous immunoglobulin therapy both in a mouse model[45] and in retrospective series of ITP patients.[46] These findings suggest the possibility of direct cytotoxicity or complement fixation as a mechanism of platelet destruction rather than antibody-dependent, Fc receptor-mediated phagocytosis by macrophages.

Interestingly, platelet kinetic studies using indium-111 (^{111}In)-labeled autologous platelets have shown considerable heterogeneity in platelet turnover in patients with chronic ITP.[39,40,47,48] While the platelet lifespan is often markedly decreased in most patients, in some it is only mildly reduced; furthermore, platelet turnover (a measure of platelet production) is frequently subnormal. Overall, approximately 40% of patients with ITP were found to have a reduced platelet turnover.[39,40]

If platelet destruction were the only mechanism to cause thrombocytopenia, then platelet production would be expected to increase and offset low platelet counts. It, therefore, was proposed that thrombocytopenia may result not only from platelet destruction, but also from antibody-mediated damage to megakaryocytes. Evidence to support this hypothesis has accumulated over time.

McMillan *et al*. reported that IgG produced by cells (grown *in vitro*) from the spleens of patients with ITP would bind to megakaryocytes, whereas IgG produced by cells from the spleens of healthy controls did not bind to megakaryocytes.[49] A few years later, other investigators demonstrated that antibodies against platelet antigens would bind to megakaryocytes as well.[50,51] More recent *in vitro* experiments have further defined the role of autoantibodies in patients with ITP. Two studies in particular, by Chang *et al*.[52] and McMillan *et al*.[53] support the view that autoantibodies in ITP suppress megakaryocyte production and maturation and platelet release.

Electron microscopy studies have clarified some aspects of the autoantibody-induced damage in bone marrow megakaryocytes from patients with ITP. Extensive megakaryocytic abnormalities were consistently present in a significant percentage of all stages of ITP megakaryocyte.[54,55] In the most recent of these studies, Houwerzijl *et al*. described the features characteristic of nonclassic apoptosis, including mitochondrial swelling with cytoplasmic vacuolization, distention of demarcation membranes, and condensation of nuclear chromatin.[55] Para-apoptotic changes could be induced in megakaryocytes derived from CD34+ cells grown in ITP plasma, suggesting that autoantibodies may initiate the cascade of programmed cell death. In addition, megakaryocytes may be surrounded by neutrophils and macrophages, suggesting an inflammatory response against these cells.

A role for direct T cell–mediated cytotoxicity against platelets has been demonstrated *in vitro*.[18] Whether this effect occurs *in vivo* and its relative importance in determining platelet destruction has not been elucidated. There is also evidence that ITP is associated with accumulation and activation of T cells in the bone marrow that occurs through increased VLA-4 and CX3CR1 expression.[56] It has been advanced that these activated T cells may mediate the destruction of platelets in the bone marrow.[56]

Thrombocytopenia associated with infectious diseases is characterized by antibody-mediated platelet destruction. However, platelet production may be impaired by infection of megakaryocytes (HCV and HIV), decreased production of thrombopoietin (HCV), and splenic sequestration of platelets secondary to portal hypertension (HCV).[26]

A unique feature of antibodies specific for GP49-66, frequently found in patients with HIV and HCV infec-

tions, is their ability to induce reactive oxygen species through activation of 12-lipoxygenase and NADPH oxidase, leading to complement-independent platelet fragmentation.[57] This mechanism has not been described for antiplatelet antibodies with other epitope specificities.

Pathophysiology of secondary ITP

Secondary forms of immune thrombocytopenia are legion and include those associated with autoimmune and lymphoproliferative disorders, acute and chronic infections, and certain drugs. Secondary ITP differs in specific aspects of pathobiology from primary ITP. We will confine our discussion to Systemic Lupus Erythematous (SLE) and lymphoproliferative disorders.

SLE is a multisystem disorder with wide-ranging clinical and laboratory manifestations. Of these, thrombocytopenia is a more common finding, with platelet counts less than 100×10^9/L found in 7–30% of patients.[58] However, less than 3% of patients have counts below 20×10^9/L, which is associated with a significant risk of bleeding and usually requires treatment.[59] Several genes correlated with ITP have been shown to be associated with expression signatures in systemic lupus erythematosis,[60] indicating an overlap between the two autoimmune disorders. Purported mechanisms leading to thrombocytopenia in SLE include anti-GPIIb/IIIa antibody-mediated platelet destruction and inhibition of megakaryopoiesis by antibodies directed

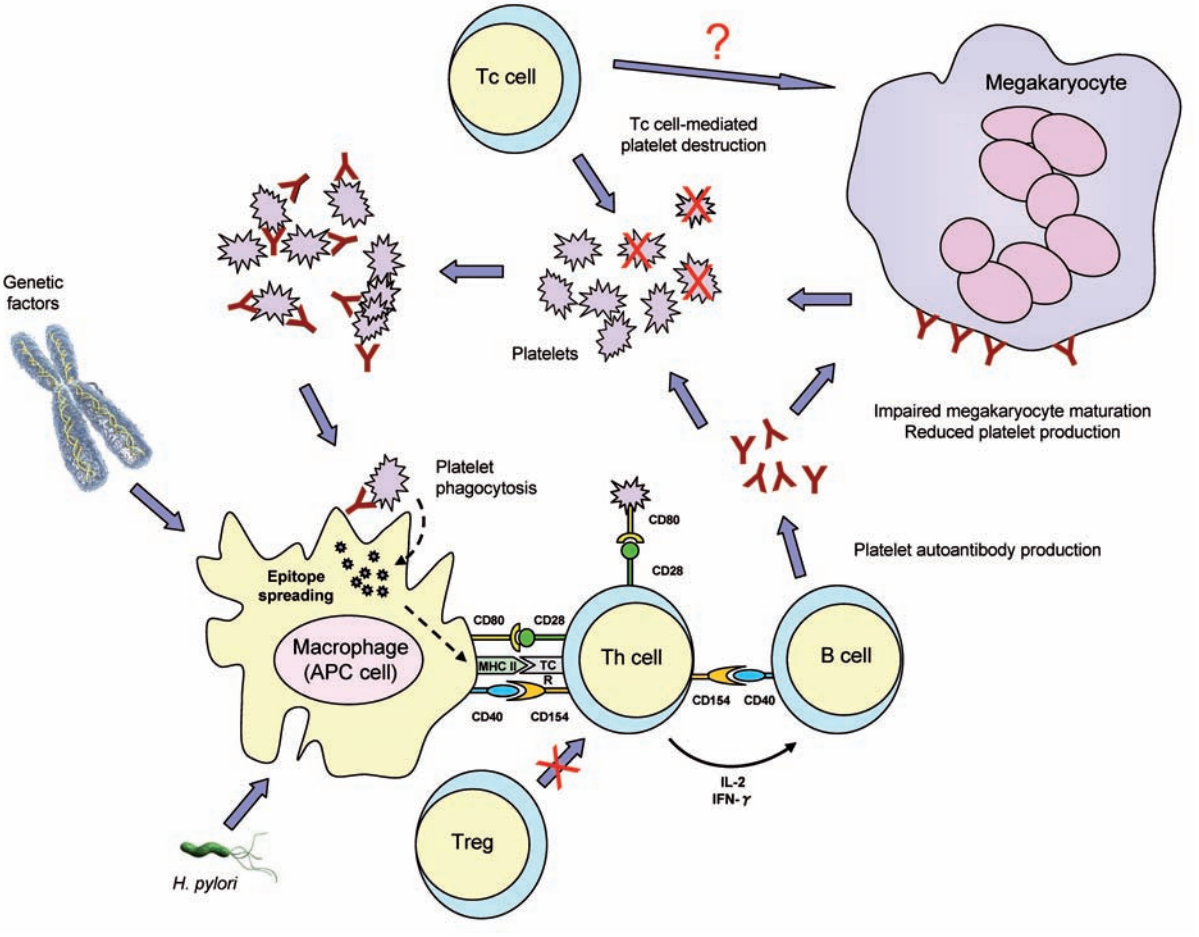

Figure 1. Simplified representation of the pathophysiology of ITP. The primary mechanism for the loss of tolerance in ITP remains unknown, although in some cases, infections may play a causative role. The emergence of antiplatelet autoantibodies remains the central pathogenetic mechanism. Platelet glycoproteins are cleaved to peptides by macrophages or another antigen-presenting cell (APC) and expressed on the APC cell surface via MHC class II molecules. APCs are crucial in generating a number of new or cryptic epitopes ("epitope spreading"). Genetic factors may be crucial in how the immune complexes are processed in APCs and expressed on MHC class II molecules. The T-cell receptor (TCR) of the Th cell can then bind the peptide-MHC complex and signal activation that upregulates CD154 (CD40 ligand) to interact with CD40 on the APC and cause additional costimulatory interactions to occur. An additional co-stimulatory signal can originate from the binding of the CD80 molecule, overexpressed on the cell membrane of ITP platelets, with CD28 expressed on Th cells. The activated Th cell produces cytokines (interleukin-2 and interferon-γ) that promote B-cell differentiation and autoantibody production. Tregs normally inhibit Th cell activity and proliferation, but their function in ITP is impaired. Autoantibodies opsonize platelets, which are taken up and destroyed by macrophages predominantly in the spleen. They also bind bone marrow megakaryocytes, thereby impairing megakaryocyte maturation and platelet production. An alternative pathway of platelet destruction is by autoreactive cytotoxic T cells, although the relevance of this mechanism *in vivo* is not known. Tc cells in the bone marrow might also inhibit megakaryopoiesis and thrombopoiesis, although this has not been formally demonstrated. The role of infections such as *H. pylori* has not been completely elucidated, but cross-reactivity has been shown between bacterial antigens and platelet glycoproteins.

against the TPO receptor (cMpl).[61] Predictably, the antibody present dictates the clinical features: patients with anti-GPIIb/IIIa antibodies have normal or increased bone marrow megakaryocyte density and, similar to patients with immune thrombocytopenic purpura (ITP), show a good response to conventional immunosuppression. Those with anti-TPO receptor antibodies demonstrate megakaryocytic hypoplasia and have a poor response to steroids and intravenous immunoglobulin.[61]

Patients with some lymphoproliferative disorders, particularly those with chronic lymphocytic leukemia (CLL) and CD8 T-lymphocyte large granular lymphocytic leukemia (LGL), appear to have an increased incidence of immune thrombocytopenia. ITP occurs in approximately 5% of patients with CLL when stringent diagnostic criteria are applied.[62] The development of ITP is significantly associated with unmutated IgVH, a positive direct antiglobulin test, and the development of autoimmune hemolytic anemia. Patients with CLL and IT have poorer survival than other patients with CLL, and this effect appears to be independent from common clinical prognostic variables.[62] Severe thrombocytopenia occurs in about 1% of patients with LGL, and has been associated with clonal suppression of megakaryopoiesis.[63,64]

Conclusions

The pathophysiology of ITP is much more complex than once believed; we can depict a simplified view of our current understanding in this area (Figure 1). While abnormalities of both the B cell and the T cell compartments have been identified, the mechanisms of the failure of the immune tolerance remain poorly understood. Recent studies have focused on the specificity of the antiplatelet immune response in patients with ITP and showed that GPIIIa is the most important target of the autoantibodies. GPIIIa is known to contain important B- and T-cell determinants, and seven immunodominant epitopes. Identification of GPIIIa sequences that are recognized by autoreactive Th cells from most patients with ITP is the first step towards specific peptide immunotherapy to re-induce Th tolerance.

There is now greater understanding of the mechanisms of thrombocytopenia in ITP, which involve both increased platelet destruction and, in a significant proportion of cases, impaired platelet production. In fact, stimulation of platelet production with thrombopoietin receptor agonists has been a recent successful therapeutic application deriving from this concept.

Many important issues still need to be adequately addressed in future research. Major gaps remain the understanding of the role played by T cell and B cell subtypes, as well as the role of factors modulating the clinical phenotype of the disease, including response to treatment and spontaneous remissions.

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Thrombopoietic agents in immune thrombocytopenia

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A B S T R A C T

Thrombopoietic agents have created a major paradigm shift in the management of Immune Thrombocytopenia (ITP). As part of their development, far more randomized, placebo-controlled trial data have been generated with these agents than with all other treatments of ITP combined; five studies have compared these agents with placebos (with standard of care and rescue therapy as needed in certain studies). Furthermore, with at least one additional study, patients have been treated continuously for more than 5 years so that long-term efficacy and safety data is emerging. The purpose of this review is to focus on what we know about these agents, including their biology and their clinical profile; what can be expected from them and what cannot be expected from them; and what are the toxicities of real concern, as well as theoretical toxicities. Finally, there will be a brief section at the end on potential future uses.

Introduction and background

Hematopoietic growth factors have revolutionized supportive therapy for malignancies, bone marrow failure syndromes, kidney failure, and other causes of blood cell deficiencies. The discovery of growth colony stimulation factors allowed the escalation of chemotherapy beyond what would normally be possible. Erythropoietin was cloned and entered clinical trials in the second half of the 1980s.[1] G-CSF was cloned and went into clinical trial several years later.[2,3] These compounds clarified the until-then theoretical concept that there would be growth factors capable of being lineage specific that would have important effects in people. Meanwhile, animal and human studies, in parallel to those with EPO and G-CSF, clarified that thrombopoietin (TPO) existed. Identification of a receptor that had the cytokine receptor motif suggested several means by which thrombopoietin could be identified and isolated. One technique involved taking a 3T3 cell line, inserting the c-mpl receptor with the cytokine receptor motif, engineering the 3T3 cell line so that it was dependent upon stimulation via this receptor, and then screening a cDNA library from a cell secreting c-mpl ligand.[4] Another technique used the c-mpl receptor in an affinity purification procedure.[5,6] There was intense interest in this, and several groups simultaneously cloned the ligand molecule for the receptor: thrombopoietin. The initial cloning of thrombopoietin in 1994 led to the development of two therapeutic agents: one was a recombinant full-length TPO molecule, and the other was a recombinant protein, encompassing the cmpl-binding domain of TPO attached to a polyethylene glycol moiety. Many trials (at least 15 or 20) were completed with the two agents; efficacy in chemotherapy-induced thrombocytopenia was the primary target, resulting in proof of efficacy.[7]

Disappointingly, while these agents were successful at raising platelet counts in healthy volunteers, they did not show efficacy in patients receiving myeloablative chemotherapy. In contrast, unequivocal efficacy was seen with these agents in patients undergoing non-myeloablative chemotherapy for solid tumors, although the number of platelet transfusions avoided would be minimal. One study explored the possibility that use of a TPO-receptor agonist would help with maintaining dosing intensity without having to hold or reduce one or more doses of chemotherapy. Soon these agents were discontinued from clinical use as a result of antibodies to them that cross-reacted with native thrombopoietin, resulting in persistent severe thrombocytopenia.[7]

Second generation agents were subsequently developed after random screening of thousands of compounds identified specific ones that were potent stimulators of c-mpl, the TPO-receptor. They first entered trial in ITP in 2002, and at least six agents have been in clinical trial thus far (Table 1).

Thrombopoietin and thrombopoietin-mimetic agents

Megakaryocytes are driven to maturation and platelet production through signaling via the c-mpl receptor. Thrombopoietin was identified as the primary c-mpl agonist; it binds to and activates the c-mpl receptor, which is found on hematopoietic stem cells, megakaryocytes, and platelets, and is the primary stimulus for megakaryocyte development and platelet production.[8] Binding of

Table 1. Platelet production is suboptimal in ITP patients.

Autoantibodies inhibit Mk growth and promote apoptosis[31]
Autologous ^{111}In-platelet studies show platelet production ≤ normal in 2/3 pts[32]
Decreased absolute platelet retics[33]
TPO levels normal in 75% of ITP patients[34]
Damaged or Dysfunctional Mk in marrow[35]

TPO to its receptor either causes formation of a dimer or releases inhibition of a preformed inactive dimer. Activation results in signaling through several intracellular pathways. Jak2 and Stat5 pathways are activated, which drive cell cycling. The Akt pathway is also activated, resulting in an anti-apoptotic and pro-survival effect. Mutations in c-mpl generally lead to loss of bone marrow megakaryocytes and low platelets, a genetic disorder called amegakaryocytic thrombocytopenia, which predisposes to bone marrow failure.[9,10] Gain of function mutations in TPO are activating, leading to higher levels of TPO, and, as a result, high platelets counts (or thrombocytosis).[11]

Thrombopoietin mimetics

Romiplostim aka AMG531 aka Nplate: By itself the single peptide binds to cmpl but does not activate it well. However, when two of the peptides are joined with a linker, the activity is near that of TPO. Because the peptides are rapidly cleared from the circulation, they have been attached to the C-terminus of an IgG Fc fragment. A pair of peptides, separated by a short linker, is attached to each Fc chain, resulting in a complete Fc fragment with a total of four peptides attached to one end. The result is a peptibody with multiple cmpl binding peptides, which can survive in circulation and can activate the native cmpl receptor with an efficacy near TPO.[12] Romiplostim is a competitive inhibitor of TPO, and thus presumably binds directly to the TPO binding site on cmpl. Romiplostim is believed to be cleared in the reticuloendothelial system and recirculated via FcRn.

Eltrombopag aka Promacta aka Revolade: This small molecule activates c-mpl only in humans and chimpanzees. A single amino acid (histidine or lysine) in the transmembrane domain of c-mpl affects the binding of eltrombopag; it is not a competitive inhibitor of TPO. Thus, it appears not to bind to the TPO binding site but this has not been formally proven. Eltrombopag is excreted unchanged through the kidney.[12]

Table 2. Status of thrombopoietic agonists in active ITP clinical trial.

- AMG531 --- pivotal studies completed --- licensed in US August 25th, 2008
- Eltrombopag --- pivotal studies completed --- licensed in US November 19th, 2008
- E5501 --- initial study in ITP completed
- LGB4665 --- initial study in ITP completed (molecule purchased by GSK)
- S888 (Shionogi) --- in trial
- 3SBIO --- trials in progress

Thrombopoietin mimetics and immune thrombocytopenia

Immune thrombocytopenia (ITP) generally occurs through increased destruction of platelets via autoantibody mediated clearance in the reticuloendothelial system of the spleen and liver. However, there is also a significant component of defective production in the bone marrow (Table 1). Thus, administration of exogenous TPO may specifically correct a portion of the pathophysiology of ITP (inappropriately low TPO levels), in addition to whatever the effects of supraphysiologic TPO activity might accomplish in elevating the platelet count.

Romiplostim clinical trials

Thus far in published information, over 300 ITP patients have received at least one dose of Romiplostim.

Two phase 1/2 studies were published in 2006.[13,14] Both were multicenter dose and schedule-finding studies: one was performed in the United States (US), as part one of a two-part study, and the other was performed in Europe. Patients were eligible for the phase 1 and 2 trials if they had failed at least one conventional ITP therapy and had not received other therapies in the past 4 weeks. Subjects could remain on maintenance steroids at a constant dose. In the US study, subjects in study part one received 0.2, 0.5, 1, 3, 6, or 10 μg/kg of body weight subcutaneously on day 1 and then were retreated on day 15. The European study was identical except that the doses of Romiplostim were fixed at 30, 100, 300, and 500 μg. Overall, 17 of 28 subjects who received 3, 6, or 10 μg/kg in the US study, and 8 of 11 subjects who received more than 1 μg/kg in the European study had significant responses to Romiplostim. Most subjects on both trials reported adverse events: the most common was headache. No major adverse events were reported, but four subjects experienced transient post-treatment worsening of thrombocytopenia. There were no thrombotic events in any subjects receiving Romiplostim.

The US study then continued on to a second part, which was a 6-week double blind, placebo-controlled trial of Romiplostim to evaluate the safety and to determine a weekly dose that would maintain platelets in the desired range. Of note, the first patient to receive a 6 μg/kg dose had a follow-up platelet count over $1000x10^9$/L and it was decided to discontinue the 6 μg/kg arm. Similarly, the first subject in the European study to receive the 500 μg dose developed a platelet count over $1000x10^9$/L, and subsequently this dose was discontinued. Thus, both studies identified an upper limit to dosing. Analysis of the data suggested that doses of at least 1 μg/kg were active; suggesting that dosing by weight was a more practical approach.

A pair of parallel randomized placebo-controlled trials were undertaken to determine the efficacy and safety of long-term use of Romiplostim in splenectomized and non-splenectomized patients.[15] Each trial intended to compare 42 patients on Romiplostim to 21 patients receiving placebo. The studies were designed such that the "dose" of the study drug was to be titrated according to the subject's platelet response and then concomitant medications, for example, prednisone, could be titrated as well. Rescue medications were allowed for very low

platelet counts and/or bleeding. The primary study endpoint was a durable response, defined by a platelet count of more than $50x10^9$/L and twice baseline for 6 out of the last 8 weeks. Both splenectomized and non-splenectomized patients had overall responses to Romiplostim (4 weeks of 24 with platelets > $50x10^9$/L) with 79% and 88% response rates, respectively. Less rescue medications were used on the active treatment arm, and there were increased rates of discontinuation or reduction of concomitant medications. As before, the most common adverse event was headache. There were three deaths during the study in the placebo arm. One subject from the treatment arm died of intracranial hemorrhage soon after completion of the study. One splenectomized patient developed increased bone marrow reticulin while receiving Romiplostim, which resolved after cessation of the drug. One elderly subject with extensive thrombotic history developed a popliteal thrombosis on the treatment arm: the platelet count was $11x10^9$/L at the time. The clot was treated and the patient remained on the study.

Results from a long term, open label study continued to support the efficacy and safety of Romiplostim.[16] From 2004 to 2010, 292 patients had been followed for up to 5 years. Romiplostim increased the platelet counts to over $50x10^9$/L in 95% of patients. Of these responders, their platelet counts were in the target range on 67% of visits. More than half of the patients maintained platelet counts greater than $50x10^9$/L for 90% of visits. Study subjects had the option to self-administer at home, and two-thirds of patients did so successfully. Most patients were maintained on a constant dose. Headache was the most common adverse event. There were serious adverse events related to Romiplostim in 13 patients: bone marrow reticulin was elevated in 8 patients, 12 patients had severe bleeding, and 7 patients had thrombotic events. Three patients died during the study, but none of the deaths was deemed related to Romiplostim therapy. Two patients developed neutralizing antibodies against Romiplostim that disappeared after cessation of the drug; these antibodies did not cross react with native TPO.

Finally, a phase 3 comparison study of Romiplostim involved head-to-head comparison with standard of care in a multi-center, randomized, open label, 52-week efficacy study.[17] Non-splenectomized adults were treated with weekly subcutaneous Romiplostim (157 patients) or the institutional standard of care (77 patients). Subjects receiving Romiplostim had fewer treatment failures than those receiving standard of care (11% vs. 30%, respectively) as well as fewer splenectomies (9% vs. 36%, respectively. Patients receiving Romiplostim also had lower rates of bleeding and fewer blood transfusions. Using the SF36 assessment scales, the Romiplostim arm had improved quality of life compared with standard of care. Adverse events were similar to previous studies, with headache as the most common adverse event. No patients had to cease Romiplostim use due to an adverse event. There were two deaths in the standard of care arm and one death in the Romiplostim arm: none were felt to be related to therapy. This study suggested that Romiplostim therapy in adults with chronic ITP prior to splenectomy could delay splenectomy as compared with standard of care. Long term follow up of these patients has not been reported.

A 12-week randomized, placebo-controlled pediatric phase 2/3 trial was performed to determine the safety, dosing, and efficacy of Romiplostim in children with chronic ITP.[18] Fifteen of 17 subjects responded to Romiplostim by achieving a platelet count greater than $50x10^9$/L for 2 weeks in a row; none of the five placebo patients did this. Only one patient had an (unrelated) serious adverse event, a flu-like illness followed by sepsis.

Eltrombopag clinical trials

The first phase II clinical trial of Eltrombopag in chronic (then duration of ITP of 6 months) ITP with platelet counts less than $30x10^9$/L was a randomized, double blind, placebo-controlled study comparing placebo with 30 mg, 50 mg, or 75 mg of Eltrombopag daily for 6 weeks.[19] Randomization was stratified by splenectomy status, concomitant ITP medication use, and baseline platelet count below $15x10^9$/L. Primary endpoint was platelet count greater than $50x10^9$/L at day 43. This endpoint was achieved by 81% of patients in the 75 mg arm and by 70%, 28%, and 11% of those in the 50 mg, 30 mg, and placebo arms, respectively. Median platelet counts by the end of six weeks were 16, 26, 128, and $183x10^9$/L in the placebo, 30 mg, 50 mg, and 75 mg arms, respectively. Adverse events were similar in all groups. However, one patient developed serious liver abnormalities, and a black box warning was added to the package insert when eltrombopag was licensed. Interim data analysis after 117 subjects were enrolled met the stopping criteria for effectiveness; the study was closed due to eltrombopag showing clear efficacy at the 50 and 75 mg doses.

A phase III confirmation study included 114 patients randomized to either 50 mg or placebo.[20] Patients assigned to the 50 mg dose could increase their dose to 75 mg if they had not achieved $50x10^9$/L by 22 days of the 43 day trial. The design was otherwise parallel to the phase II trial above. Response was achieved by 59% on active drug compared with 19% on placebo, a highly significant difference. In addition, a small number of patients increased their platelet count into the 30–$50x10^9$/L range, a clinically significant improvement. There was no increased incidence of serious or severe AEs on the active drug arm.

A phase III, randomized, double-blind, placebo-controlled study compared the efficacy of standard of care therapy with standard of care plus eltrombopag (RAISE) over 6 months of therapy,[21] paralleling the Romiplostim pivotal trials. Subjects were started at a dose of 50 mg/day and then the dose was adjusted to keep the platelet count above $50x10^9$/L, up to a maximum of 75 mg/kg/day or down to 25 mg/day or even less. Subjects were also stratified according to baseline platelet count less than $15x10^9$/L, concurrent ITP therapy, and splenectomy status. The primary endpoint was the odds of response to eltrombopag versus placebo.

Of the 135 subjects receiving eltrombopag, 79% responded, compared with 28% response to placebo, a more than eight-fold odds increase. Most patients were taking 75 mg at the end of the 6 month study. In addition, 59% of patients receiving eltrombopag reduced their concomitant therapies, compared with 32% of

patients receiving placebo. Three patients receiving eltrombopag had thromboembolic events compared with none in the placebo group.

These three trials clearly demonstrated that eltrombopag was effective in increasing the platelet count in patients with ITP, that it was tolerable, and that it appeared to be safe. Subjects who participated in these (and one other) clinical trials were eligible to take part in a long term dose-finding and safety monitoring study. The EXTEND study followed 299 patients previously participating in eltrombopag studies for up to 3 years on eltrombopag.[22] Eighty-seven percent of these patients achieved platelet counts greater than $50x10^9$/L. The number of patients reporting any bleeding decreased from 56% at start of study to 20% after 2 years of treatment. There were no differences in response between the stratification groups.

The most common adverse event was headache in 29% of subjects. Serious adverse events occurred in 26% of subjects and included 21 thromboembolic events in 16 subjects (13%): 8 were DVT and 4 were MIs. No patients in the placebo groups had thromboembolic events: however, there were no placebo patients on the EXTEND trial and thus the overall time on active drug was more than 10-fold longer than the time on placebo. Thirty patients had elevated liver enzymes during the study, which resolved after cessation of the drug; most could safely resume treatment. Bone marrows were examined in over 135 patients and did not show signs of excessive reticulin deposition or collagen fibrosis except in quite infrequent cases.[23]

Efficacy and potential risks

There are potential risks associated with TPO and TPO mimetics and these can be divided into several categories. One risk category is the potential to affect hematopoietic stem cells, such as by driving proliferation of a malignant clone or by depleting the stem cell compartment. Because hematopoietic stem cells express the cmpl receptor, the TPO mimetics could stimulate cells outside the megakaryocyte lineage. Thus far, no effect on lineages other than megakaryocyte has been reported. Similarly, the concern that TPO mimetics might increase proliferation of leukemia stem cells has not been observed in ITP. Leukemic blasts have circulated in MDS patients treated with Romiplostim; most regressed with discontinuation of therapy.[24,25] Current usage involves combining Romiplostim with other agents, such as Vidaza.[26]

Another concern is the potential for the stimulated megakaryocytes to deposit excessive extracellular matrix in bone marrow, leading to myelofibrosis. Surveillance bone marrow biopsies showed increased reticulin in a few patients receiving Romiplostim, but no evidence of collagen deposition.[27] Similarly, no significant bone marrow reticulin and no collagen deposition were reported from surveillance marrow biopsies of patients receiving eltrombopag.[20] The effect of long term exposure to TPO mimetics on bone marrow will only be revealed by careful monitoring of patients over years of therapy. Both agents are involved in studies in which marrows are done at entry and then at defined intervals after treatment to prospectively assess changes.

ITP is thought to be a hypercoagulable state, despite thrombocytopenia. A sudden increase in platelet count from TPO mimetic therapy might predispose patients to thrombosis. Patients with atherosclerosis or coronary artery disease in particular may have an elevated risk of thrombosis in the setting of rising or supranormal platelet counts. In fact, no increase in thromboembolic events has been reported in study subjects receiving Romiplostim or eltrombopag, compared with control subjects in any of the six large randomized controlled trials, despite the fact that the platelet counts are much higher on active drug than on placebo. It is important to note that the thromboembolic events that did occur were generally not at the highest platelet count on study.

The second generation TPO mimetic agents have been designed specifically to avoid inciting antigenicity against endogenous thrombopoietin. This possibility has been closely monitored and thus far two patients have developed antibodies against Romiplostim, which resolved after withdrawal of the drug. No patient has developed antibodies against thrombopoietin.

Other indications

Eltrombopag has been evaluated in several studies with hepatitis C. One has been reported with positive results allowing thrombocytopenic patients to persist with peg-interferon-ribavirin treatment towards eradicating their hepatitis C infection.[28]

Romiplostim in combination with Vidaza has been effective in allowing platelet transfusion dependent MPD patients to increase their platelet counts and become transfusion independent.[25]

A recent study has demonstrated the efficacy of eltrombopag in over 80% of 15 patients with MYH9-RD (aka May Hegglin) requiring treatment.[29] A study reported in abstract form has shown efficacy in patients with the XLT form of Wiskott Aldrich syndrome.[30]

Areas of exploration in the future include use in the NICU to increase the platelet counts and decrease the bleeding risk of ill preterm infants, to increase the platelet count in healthy platelet donors thus increasing the yield of collection, and in chemotherapy induced thrombocytopenia. Whether any of these will be actively pursued and whether they pan out remains to be seen.

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The diagnosis and management of chronic immune thrombocytopenia in adults

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A B S T R A C T

Primary immune thrombocytopenia (ITP) is a complex autoimmune disorder involving antibody- and cell-mediated destruction of platelets and suppression of platelet production. The definition of ITP requires an isolated thrombocytopenia with a platelet count below 100×10^9/L, and with no other obvious cause or clinically evident secondary form of immune thrombocytopenia. There is no "gold standard" test for ITP, but the diagnosis is based on exclusion.

ITP is a minor disease for many patients; however, the natural history is variable and unpredictable. Many patients who are in otherwise good health can be left untreated, especially if their platelet counts are close to or above 20×10^9/L. The overall mortality rate for ITP is less than 1%, and the morbidity and mortality associated with treatment can be worse than the disease.

While there is general agreement over treatment of the newly presenting adult with ITP, few evidence-based studies direct therapy in the relapsed and refractory patient. There is an understanding that the patient should be treated for their clinical state rather than their platelet count but not what second line treatment should be used and in what order. The options will be considered in this review.

Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune disorder typified by a platelet count below the normal range. Clinical manifestations are related to the severity of thrombocytopenia and include purpura and bleeding episodes, which can be potentially life-threatening and require emergency treatment. In the past, "acute ITP" has been used to describe a self-limited form of the disease (e.g., secondary to viral illness) and "chronic ITP" identified ITP lasting for more than 6 months. This definition was felt to be unsatisfactory, and a more coherent staging scheme was felt necessary in order to compare studies and define the disease.

The terminology was agreed and definitions published in 2009 by Rodeghiero *et al.* on behalf of an International Working Group.[1] The abbreviation ITP now stands for immune thrombocytopenia and not immune thrombocytopenic purpura. It is defined as a platelet count of under 100×10^9/L without any other cause of thrombocytopenia. A new staging scheme was also proposed, which defines the following stages of ITP:

- Newly diagnosed ITP: months 0–3 after presentation
- Persistent ITP: months 4–12
- Chronic ITP: greater than 12 months
- Refractory ITP: patient has failed splenectomy
- Severe ITP: patient has had major clinical bleeding.

In the absence of reliable predictive clinical or laboratory tests, variables parameters of disease duration, the term "newly diagnosed ITP" has been adopted for all cases at diagnosis. A new category, called "persistent ITP," was introduced for patients with ITP to define the period lasting between 3 and 12 months from diagnosis. This category includes patients not achieving spontaneous remission or not maintaining their response after stopping treatment between 3 and 12 months from diagnosis. The term "chronic ITP" is used for patients with ITP lasting for more than 12 months.

Acute abrupt-onset ITP, with duration of less than 6 months, occurs mainly in children, often following viral infection or immunization, with boys and girls being equally affected. Chronic ITP is typically seen in adults, more often affecting women of childbearing age. In Europe, the incidence of ITP in adults has been estimated to be between 1 and 4 per 100,000 persons.[2–4] When a cut-off platelet count of less than 50 x 10^9/L was applied, the annual incidence of ITP was estimated to be 2.25 per 100,000 in Denmark[2] and 1.6 per 100,000 in the UK.[4]

In up to 30% of patients with immune thrombocytopenia, the cause may be secondary to infection (HIV, HCV, CMV, or EBV), drugs, another autoimmune disorder (e.g., SLE) or in association with a malignancy (malignant lymphoma or chronic lymphocytic leukemia; CLL).

The natural history of ITP is variable and unpredictable. For patients who are in otherwise good health, many can be left untreated, especially if their platelet counts are close to or above 20×10^9/L. The overall mortality rate for ITP is less than 1%, and the morbid-

ity and mortality associated with treatment – often related to infections – can be worse than the disease. Thus, platelet count and risk for bleeding events should be considered before treatment of ITP is initiated. A guideline describing the consensus of management in Europe was published in 2003.[5] These guidelines followed those of the American Society of Hematology in 1996.[6] It was generally felt that these needed updating due, in part, to the introduction of new classes of therapeutic agents and a greater understanding of the disease pathophysiology. The International Consensus Report on the diagnosis and treatment of ITP were published in 2010.[7] The writing committee reviewed over 1000 manuscripts written in the last 20 years to try to establish working guidelines, evidence based where possible, that could subsequently be used, in conjunction with the new terminology, to review practice and provide future audited treatment advice. ASH have subsequently updated their guidelines with broadly similar recommendations. The main difference is the recommendation that splenectomy remains the main second line treatment of chronic ITP, which in the light of newer options and patient preference, is increasingly controversial.[8]

Diagnosis

There is no "gold standard" test for ITP, but the diagnosis is one based on exclusion of other causes of isolated thrombocytopenia. The essential investigations include history and physical examination, full blood count, and examination of the blood smear. A bone marrow examination is now considered to be necessary in only a few selected groups of patients: those over 60 years of age, those who fail to respond to therapy or have relapsed after therapy, or non-responding patients being considered for splenectomy, and some of these remain debatable. All adult patients should be screened for HIV and hepatitis C, and in appropriate countries, for *H. pylori*.

Some tests should be considered in special circumstances. In patients who might go on to receive intravenous immunoglobulin, an underlying immunoglobulin deficiency should be considered and the IgG level checked. This is particularly important in children to exclude Common Variable Immune deficiency, but should also be considered in adults. In patients being considered for anti-RhD, the Rh group and direct antiglobulin test (DAT) should also be evaluated. Hepatitis B should also be screened for in those who may receive the anti-CD20 monoclonal antibody rituximab.

Testing for glycoprotein-specific antibodies may confirm the diagnosis but the tests lack sensitivity and are rarely used routinely. Testing for anti-phospholipid antibodies and other auto-antibodies is not useful diagnostically but may be important to exclude associated conditions.

It is important to exclude secondary thrombocytopenia, and the following should be considered prior to diagnosing primary ITP: In addition to the viral infections mentioned above other immune or autoimmune disorders (including SLE), malignancy (especially non-Hodgkin lymphoma), liver disease, alcohol abuse, medications (prescription or non-prescription), environmental toxins, quinine exposure, primary bone marrow diseases, and recent transfusions or immunizations should all be considered and where possible, excluded.

There is much controversy over the incidence and importance of immunizations, which are infrequently but clearly associated with thrombocytopenia. Measles, mumps, rubella (MMR) is typical, and it is estimated that one in 40,000 children given MMR will develop severe thrombocytopenia about 1–2 weeks post vaccination. It is associated with platelet-reactive anti-glycoprotein antibodies, and is self limited in 80% of patients. In the remaining 20%, thrombocytopenia persists but is responsive to ITP specific therapy, and recurrence is rare.[9]

Inherited thrombocytopenias should be considered in patients who have a life-long history of thrombocytopenia or suggestive family history.

Anti-platelet and other auto-antibodies and their role in the pathogenesis of ITP

In early 1980 it was noted that sera or eluates of platelets from patients with ITP would bind to normal platelets but only about one-quarter would bind to the platelets of patients with Glanzmann's thrombasthenia.[10] It was speculated that ITP patients produced auto-antibodies against either platelet glycoprotein (GP) IIb or GPIIIa since thrombasthenic patients lack these proteins. Since that time, several laboratories have provided direct evidence for the presence of auto-antibodies against GPIIb/IIIA and other platelet antigens in ITP.[11–14]

In the meantime, platelet kinetic studies using radiolabeling methods had reignited the debate about the mechanisms of thrombocytopenia in ITP. These studies showed shortened platelet survival, and high rates of platelet production ranging from 4 to 9 times normal in patients with ITP.[15–17] With the introduction of ^{111}In, the small numbers of autologous platelets in ITP could be adequately labeled, and it became evident that there was considerable heterogeneity in platelet turnover between patients, with a substantial proportion having platelet production within normal limits even though the mean cell life of platelets was significantly reduced compared with that in healthy subjects.[18–20] If platelet destruction were the only mechanism to cause thrombocytopenia, then platelet production would be expected to increase to offset low platelet counts. It was, therefore, proposed once again that thrombocytopenia may result not only from platelet destruction, but also from antibody-mediated damage to megakaryocytes. Evidence to support this hypothesis has accumulated over time.

The infections most closely linked with ITP are HIV, HCV, and *H. pylori*.[21–23] It has been demonstrated, at least *in vitro*, that patients may have antibodies that bind to antigens on these organisms and that cross react with an epitope of GPIIIa or other unspecified epitopes on the platelet surface suggesting molecular mimicry and epitope spread.

As has been seen in the exclusion of secondary causes of thrombocytopenia, an ITP-like presentation occurs in

diverse other clinical contexts. These are often seen in conditions also affecting aspects of the immune system and durable complete remission are rare (SLE, ALPS - autoimmune lymphoproliferative syndrome, Evans syndrome, anti-phospholipid syndrome) or uncommon (CLL, common variable immune deficiency, post-transplantation/immunosuppression). Even patients who present with primary ITP often express or develop antibodies to phospholipids, thyroid antigens, nuclear antigens, and RBCs,[24] and some develop clinical manifestations associated with these antibodies.

Even among patients that have no overt signs of secondary forms of the syndrome, ITP is still heterogeneous, and a varying proportion of patients express other autoantibodies. Anti-nuclear antibodies are found in approximately 20% of patients with ITP, but less than 2% develop systemic lupus.[9,24] The incidence of antiphospholipid antibodies is approximately 40–50%, reaching 70% in some series.[9,24] Available evidence is conflicting as to whether the presence of antiphospholipid antibodies predisposes to thrombosis after successful treatment. Thus, there are many clinical scenarios where it is not at all clear whether or not the patient has primary or secondary ITP.[25]

These include:

- positive serology but no clinical evidence of HCV or *H. pylori* with failure to respond to antimicrobial therapies
- positive ANA, but not lupus
- anti-phospholipid binding protein antibodies, but no history of thrombosis or pregnancy loss
- antimicrosomal or antithyrogobulin antibodies, but normal TSH
- positive DAT, but no haemolytic anemia
- paraprotein or a T cell clone on flow cytometry, which is becoming increasingly apparent in older patients with ITP, but no clinical evidence of a clonal disorder
- low IgG, but no history of recurrent infection.

Management of immune thrombocytopenia

In general, the treatment should be tailored to the individual patient; there is no standard therapy that all patients ought to undergo. Factors to be considered include whether the patient is bleeding, other medical problems (including diabetes), activity and lifestyle, individual tolerance of side effects, patient preferences and concerns, and finally the platelet count. Treatment should not be based solely on platelet counts, but considered with the totality of the patient's medical issues and preferences. Treatment is not indicated in patients with a platelet count above 50 x 10^9/L, except in the following situations:

- platelet dysfunction or another haemostatic defect
- trauma or surgery
- comorbidities for bleeding, e.g., dialysis
- mandatory requirement for anticoagulant therapy
- profession or lifestyle that exposes the individual to trauma.

In chronic ITP, the goal of treatment is to achieve a platelet count that prevents major bleeding, rather than to raise platelet count to the normal range. Management is tailored to the individual patient and in general, patients with platelet counts greater than 30 x 10^9/L do not require treatment unless they are undergoing procedures likely to result in blood loss (e.g., surgery, dental extraction or parturition).[5,6]

Most of the available treatments for ITP are aimed primarily at attenuating excessive platelet destruction. Corticosteroids (e.g., prednisolone) are the standard first-line treatment. They are cheap and effective in approximately two-thirds of patients, although most will relapse.

Prednisolone is usually given at a dose of 0.5–2 mg/kg for 2–4 weeks, and if there was no response, then the therapy should be stopped and alternates considered. Recent data indicates that Dexamethasone at a dose of 40 mg daily for 4 days every 4 weeks for 1–4 cycles can lead to sustained responses,[26] but many patients find the side-effects intolerable, and unless used in a clinical trial, evidence is not strong enough to recommend this as the major form of first line therapy for most patients. In addition to the well-recognized short term effects of hyperactivity, anxiety, and appetite stimulation, there are well-recognized long-term side effects of steroids, including infections, hypertension, glaucoma, cushingoid appearance, hypertension, diabetes mellitus, osteoporosis, and the occasional development of avascular necrosis of the hip, and their use should be strictly controlled and time limited.

Intravenous (IV) immunoglobulin is usually reserved for "rescue" treatment of acute bleeding episodes and for patients who are refractory to steroids, or require unacceptably high doses of these agents to maintain a safe platelet count. Anti-D is no longer available in Europe, but is still used in North America despite the risk of DIC and the associated increase in mortality. It remains an option in patients who are Rh(D) positive and who are not splenectomized. It is an appropriate initial therapy in patients who are not candidates for corticosteroids (e.g., insulin-dependent diabetes) or who are acutely bleeding and require a rapid elevation in platelet count.

Vinca alkaloids are also a useful, but short-lived, treatment for ITP. In a number of small studies, short-term elevation in platelet counts is seen in nearly 50% of patients, especially those who have not responded to corticosteroids.

Patients who fail to respond to such first-line therapies or whose conditions relapse face the options of undergoing treatment with second-line drug therapy or splenectomy; however, there is a lack of evidence based clinical data to confirm the efficacy and safety of any of these second-line treatments. In some patients with chronic disease, it is worth looking for, and treating *H. pylori* see previous comment infection which may be all that is necessary. A recent article by Psaila and Bussel has reviewed the current treatment options in refractory disease.[27]

A number of different immunosuppressive agents have been used in ITP. In general, the evidence comes from controlled clinical studies, without randomization, or from descriptive studies. The evidence for azathioprine and cyclophosphamide falls into this category but the consensus is that these drugs remain an important option for treating ITP patients. An increased risk of leukemia has not been reported in ITP patients exposed

to azathioprine, and is particularly useful if they have associated haemolytic anemia. There is reasonable Grade B evidence for the use of cyclosporin A, mycophenolate, and rituximab as alternate immunosuppressive options. Mycophenolate produces a response in about a quarter to a half of all patients and is well tolerated by the majority of patients.[28] It should be remembered, however, that suppression of the immune system can predispose patients to infection, which is a major cause of death in ITP patients.[29,30] The long term use of immune suppressive agents, particularly in patients who have failed splenectomy, is an especial risk.

Rituximab

In 2001, Stasi *et al*. reported for the first time in an observational study the efficacy of rituximab in adults with chronic ITP.[31] A number of other studies have confirmed the efficacy of rituximab in patients with ITP. In 2007 Arnold *et al*. published a meta-analysis combining some of these studies including at least five patients treated.[32] The overall response rate (platelet count >50×10^9/L) correct was estimated to be 60%, with an overall complete response rate (platelet count >15×10^9/L) correct of approximately 46%. The study by Cooper *et al*. in 2004[33] showed that rituximab could be equally effective both pre-splenectomy and post-splenectomy. The better the initial response was, the longer the response was. The only factor correlated with a lower likelihood of response was the duration of ITP. The multicentre prospective French study of Godeau *et al*.[34] in chronic ITP (over 6 months' duration) showed similar results and after 2 years of follow-up, the overall response rate was 33%.

The study by Zaja in Italy[35] was a prospective, randomized comparison between a single course of dexamethasone 40 mg/m^2 correct for four consecutive days and dexamethasone followed by rituximab 375 mg/m^2 weekly for 4 weeks. The study in previously untreated patients showed a rate of sustained response (platelet count >50×10^9/L at 6 months) of 63% in the dexamethasone plus rituximab arm compared with only 36% in the dexamethasone arm. After a median follow-up of 20 months, approximately 75% of the initial responders in both arms had a lasting response.

All these studies used rituximab at a conventional dose of 375 mg/m^2. Other studies from Italy and the UK[36,37] suggest that a lower dosage of rituximab (i.e., 100 mg weekly for 4 weeks) can be sufficient to achieve a response in the ITP setting.

No serious adverse events were reported in the initial studies in ITP. However, infusion reactions are not infrequent, and both serum sickness and hypogammaglobulinemia have been reported.[34] More recently, there has been concern about the possible induction of progressive multifocal leucoencephalopathy (PML), an opportunistic infection of the brain, which is fatal in almost 100% of cases. A review of adverse events associated with rituximab[38] reported 57 cases of PML in patients treated with rituximab; the majority of these patients had been treated for a lymphoma, whereas only a few had autoimmune disorders, including two with ITP. Most of these patients had received not only rituximab but also chemotherapy or long-term corticosteroids and/or immunosuppressives. The role of rituximab, therefore, is not clearly established at this time. In the past 5 years, a registry has been set up by rheumatologists in France looking at the safety of rituximab in rheumatoid arthritis outside a clinical trial setting.[39] This registry has included more than 1000 patients, some of whom have received up to eight courses of rituximab over years. The overall incidence of infection was slightly higher than the one reported in clinical trials was, reflecting that some patients treated with rituximab would have not been not included in clinical trials because of comorbidities, but not a single case of PML has been reported. Overall, 5% of patients acquired secondary hypogammaglobulinemia.

Splenectomy

Splenectomy has proved an effective therapy in the management of primary ITP, with a systematic review of 135 reported series from 1966 to 2004 finding a complete response (CR), defined by a platelet count of at least 150 x 10^9/L and 30 days or longer on no treatment, in 66% of patients.[40] However, the procedure is not without risk, including intra-abdominal hemorrhage,[41] thromboembolic events,[42] and overwhelming post-splenectomy infections (OPSI).[43] Although the risks of these complications in patients with primary ITP is estimated to be low,[44] when coupled with the risk of failure to achieve CR in one-third of patients, limited data on long-term relapse risk, and increasing awareness of the pathogenic heterogeneity of the disease,[45–47] they provide sufficient impetus to investigate potential pre-operative predictive variables of response.

A number of such clinical predictors have been suggested, including i) response to corticosteroid and IVIg therapies; ii) platelet turnover; iii) platelet lifespan; iv) patient age; v) duration of disease; vi) platelet-bound immunoglobulin; and vii) site of platelet destruction as determined by radionuclide labeling techniques. In a systematic review of the effectiveness of splenectomy among adult patients with primary ITP, Kojouri *et al*. report inconclusive evidence in support of this last variable.[40]

However, past investigations into platelet sequestration studies have adopted heterogeneous methods and varied widely with regard to power. Utilizing patients with primary ITP who underwent ^{111}In-labelled, autologous platelet sequestration studies at Barts and The London NHS Trust between 1994 and 2008, we evaluated the effectiveness of sequestration site in predicting short, medium, and long-term surgical failure through multivariable logistic regression modeling. In total 256 patients with primary ITP underwent scanning, with 91 (35.5%) proceeding to splenectomy. Logistic regression revealed adjusted, statistically significant odds ratios (ORs) for surgical failure of 7.47 (95% CI, 1.89–29.43) at 1–3 months post-splenectomy, 4.85 (95% CI, 1.04–22.54) at 6–12 months post-splenectomy, and 5.39 (95% CI, 1.34–21.65) at last follow-up (median: 3.8 years [range: 0.5–13.1 years]) in patients with mixed or hepatic versus purely or predominantly splenic platelet sequestration. These findings support adoption of a cautious approach to splenectomy in patients exhibiting mixed or hepatic sequestration.[48]

Thrompopoietin receptor agonists

Initially ITP was thought to be a disease of increased platelet destruction, which is why current standard treatments are either of a non-specific immunosuppressant nature or targeted solely at decreasing platelet destruction. Recent evidence suggests that suboptimal platelet production by suppression of megakaryocyte production and maturation may also have a role in ITP. Therefore, it was postulated that treatment aimed at increasing platelet production may provide an opportunity to improve outcomes in patients with this chronic disease.[49]

Megakaryopoiesis and platelet production are governed by signaling through the Mpl receptor and the ligand for this receptor, known as thrombopoietin (TPO), has a pivotal role in the regulation of platelet production. Following the cloning of the molecule, two recombinant thrombopoietin molecules were developed: recombinant human thrombopoietin (rhTPO) and pegylated human recombinant megakaryocyte growth and development factor (PEG-rHuMGDF). These agents underwent extensive clinical testing for a range of thrombocytopenic disorders. The clinical development of these recombinant growth factors was halted in 1998 with reports that patients receiving PEG-rHuMGDF (which has significant sequence homology with endogenous TPO) developed severe thrombocytopenia

Table 1. Investigation of suspected ITP.

Basic evaluation	Tests in selected cases	Tests of unproven benefit
Patient history	Antiphospholipid antibody (including ACL and Lupus)	Glycoprotein-Specific Ab
Family history	Anti thyroid antibody and thyroid function	Thrombopoitein Assay
Physical examination	Pregnancy test	Reticulated platelet count
Full blood count	Antinuclear antibodies	Indirect PaIgG
Peripheral blood film	Viral PCR for parvovirus and CMV	Bleeding time
Blood group (Rh) and reticulocyte count		Platelet survival
DAGT		Serum complement levels
Quantitative Ig measurement		
Bone marrow examination*		
H. pylori		
HIV and HCV		

Table 2. Summary of treatment options.

Clinical situation	Therapy option
First line (initial treatment for newly diagnosed ITP)	– Anti-D – Corticosteroids: dexamethasone, methylprednisolone, prednis(ol)one – IVIg
Second line	– Azathioprine – Cyclosporin A – Cyclophosphamide – Danazol – Dapsone – Mycophenolate mofetil – Rituximab – Splenectomy – TPO-receptor agonists – Vinca alkaloids
Treatment for refractory ITP patients (patients failing first and second-line therapies)	**Category A:** **Treatment options with sufficient data** – TPO-receptor agonists **Category B:** **Treatment options with minimal data and considered to have potential for considerable toxicity** – Campath-1H – Combination of first- and second-line therapies – Combination chemotherapy – Haemopoietic stem cell transplantation (HSCT)

Modified from[7]

stemming from antibodies against PEG-rHuMgDF that cross-reacted with endogenous TPO (eTPO), neutralizing its biologic activity.

Despite safety concerns raised by autoantibody cross-reactivity, the success of the first-generation thrombopoietic growth factors in stimulating platelet production led to the development of a second generation of thrombopoietic growth factors that had no sequence homology with native TPO. Clinical trials with these, the TPO peptide mimetic AMG 531 (also known as Nplate or Romiplostim, Amgen, Thousand Oaks, California) and the nonpeptide mimetic eltrombopag (also known as Promacta, GlaxoSmithKline, Research Triangle Park, North Carolina) have both resulted in dose-dependent increases in platelets in healthy subjects and in significant increases in platelets in patients with chronic ITP.[50]

The extensive clinical studies have shown that platelet responses are seen in approximately 80%, a much greater percentage than in other second line studies and the response is maintained while the drugs continue to be administered. They are almost as effective in splenectomized patients as in the nonsplenectomized ones.[51] Recent phase III studies have confirmed the efficacy and safety following long-term usage of both of the currently available products.[52,53] The licenses for these agents vary throughout the world. In some (USA and Canada) the license covers pre-splenectomy use whereas in Europe, this is only covered for patients in whom the surgery is contraindicated.

These agents appear to be well tolerated without the formation of autoantibodies that were seen in the studies with the first generation of thrombopoietins. Increases in marrow reticulin have been reported, but these appear to be a reversible phenomenon and not associated with formation of collagen fibrosis. The incidence of increase in marrow reticulin is unknown but is likely to be higher in patients treated with high doses, which should therefore be used with caution. There appears to be no increased incidence of thrombotic events in patients who achieve normal platelet counts compared with those receiving placebo, however, thrombotic events have been reported in patients with other risk factors of cardiovascular disease, and a recent report has shown an increased of venous and arterial thrombo-embolism in any patient with ITP, suggesting that ITP is a pro-thrombotic condition.[54]

New developments

With the increasing understanding of molecular pathways in ITP and of the aetiology of the disease, more targeted and immune based therapies are under study. Ongoing clinical trials in ITP involve antibodies against the Fc receptor, such as MDX-33, a humanized anti-FcγRI monoclonal, and GMA-161, a humanized anti-FcγRIII monoclonal.[55] Investigation of inhibition of FcR signaling mechanisms is currently under investigation with R788,[56] a small molecule prodrug of the biologically active R406. This is a potent and relatively selective orally available inhibitor of Syk (spleen tyrosine kinase). There are also a number of anti-CD20 monoclonal antibodies attempting to duplicate and improve on the results shown with Rituximab first described by Stasi in 2001.[31] Future treatment options have been recently reviewed.[57]

Conclusion

While there is general agreement over treatment of the newly presenting adult with ITP, few evidence based studies direct therapy in the relapsed and refractory patient. There is an understanding that the patient should be treated for their clinical state rather than their platelet count but not what second line treatment should be used and in what order. In order to develop a rational approach, an international group produced a consensus report on investigation and management giving (where possible) evidence based advice on treatment pathways. It is hoped that by following such an approach, treatment in the future can be audited and authoritative guidelines developed[7] and the place of the newer treatments understood.

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Relevant pathogenetic pathways in diffuse large B cell lymphoma

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A B S T R A C T

Diffuse large B cell lymphoma (DLBCL), the most common lymphoid malignancy in adults, is an aggressive disease characterized by a marked morphologic, phenotypic and clinical heterogeneity. Although durable remissions can be achieved in a substantial proportion of cases by using multi-regimen treatment approaches, DLBCL remains incurable in over 30% of patients, underscoring the need for an improved understanding of its biology. Over the past decade, significant progress has been made in unraveling the pathogenetic basis of this heterogeneity through the development of high-resolution technologies which allow the assessment of genomic and transcriptional changes. These studies revealed the existence of multiple DLBCL phenotypic subtypes, which are often associated with distinct clinical behavior. Notably, individual subtypes also segregate with largely distinct genomic alterations that target key cellular pathways in lymphoid maturation and differentiation, and may influence treatment outcome.

This review summarizes current knowledge about the molecular mechanisms that are responsible for DLBCL development, and describes how recent advances in the genomic characterization of these tumors have provided new insights into the biology of this disease. Newly identified genetic lesions lead to alterations in cellular pathways that represent potential attractive targets for improved diagnosis and therapy.

Introduction

Diffuse large B-cell lymphoma (DLBCL) represents the most prevalent aggressive B cell non-Hodgkin's lymphoma (B-NHLs) in adulthood, accounting for ~40% of all new diagnoses and including cases that arise de novo as well as cases that result from the clinical evolution of various, less aggressive B-NHL types (i.e., follicular lymphoma and chronic lymphocytic leukemia).[1,2] Despite significant advances in both diagnosis and treatment, DLBCL remains an important clinical challenge since at least one third of patients are not cured by currently available therapeutic regimens, including combination immune-chemotherapy. Such lack of success stems in part from the remarkable heterogeneity of this disease, which – unlike many other B-NHL types – can be observed at the morphologic, phenotypic, genetic and clinical level. Indeed, gene expression profile studies over the past decade have demonstrated the existence of several molecularly distinct DLBCL subtypes that reflect the origin from B cells at various stages of differentiation or the coordinated expression of comprehensive transcriptional signatures. These subgroups not only differ in the expression of specific gene signatures, but also seem to rely on separate oncogenic mechanisms and are predictive of different overall survival rates, thus providing a molecular framework for the development of rationally targeted therapeutic approaches.

The germinal center reaction and lymphomagenesis

A key concept for the understanding of DLBCL pathogenesis is the relationship between these tumors and the unique DNA modification events that take place in normal B cells as they progress through various differentiation stages in order to shape their antibody repertoire and to enable the production of highly efficient neutralizing antibodies. Especially relevant is the biology of the germinal center (GC) reaction, the hallmark of T cell dependent immune responses and also the target structure from which most B-NHL, including DLBCL, are thought to be derived.

GCs form in secondary lymphoid organs upon encounter of a naïve B cell with its cognate antigen, in the context of T-cell dependent co-stimulation. This event triggers a process of rapid and intense cell proliferation (doubling time, <12 hours) along with clonal expansion,[3] leading to the formation of a histologically well-defined structure.[3-5] Within the GC, the antibody genes of the B lymphocyte are modified through the process of somatic hypermutation (SHM) and class switch recombination (CSR), two DNA remodeling events aimed at favoring the emergence of cells that produce antibodies with increased antigen affinity and are capable of distinct effector functions. While representing mechanistically quite distinct processes, both SHM and CSR depend on

the activity of a single enzyme, activation-induced cytidine deaminase (AID).

The specialized microenvironment of the GC can be divided into two anatomically distinct areas: the dark zone, populated by rapidly dividing centroblasts (CB), and the light zone, which is composed of smaller non-dividing centrocytes (CC) admixed with a reticulum of follicular dendritic cells (FDC). CBs are characterized by elevated expression of BCL6,[6,7] a potent transcriptional repressor[8] that acts as a GC master regulator and is also a frequent target of genetic lesions in DLBCL. BCL6 exerts its biological function by repressing or modulating the expression of a broad set of genes, including those involved in B cell receptor (BCR) and CD40 signaling;[9,10] T cell mediated B cell activation;[9] induction of apoptosis;[9,11] sensing and response to DNA damage;[12-15] negative regulation of cell cycle progression;[13,16] a multitude of cytokine and chemokine signaling pathways;[9,11] and plasma cell differentiation, the latter via suppression of the PRDM1/BLIMP1 master regulator[16-19] (Figure 1). This transcriptional program suggests that BCL6 plays a critical role in the GC by sustaining the proliferative status of CBs while allowing the execution of DNA remodeling events (SHM and CSR) without eliciting DNA damage responses, and by preventing their premature activation and exit from the GC prior to the selection for the survival of cells producing high affinity antibodies.

CBs are then believed to stop proliferating and differentiate into CCs in the light zone, where they are re-challenged by the antigen presented by FDCs via interaction with follicular T helper cells.[4,5] CCs expressing a BCR with reduced affinity for the antigen or a self-reactive BCR are eliminated by apoptosis, while few cells with high-affinity antigen receptors are selected to differentiate into plasma cells or memory B cells through stimulation by a variety of signals, including engagement of their BCR by the antigen and activation of the CD40 receptor by the CD40 ligand (CD40L). These two stimuli represent known activators of NF-κB responses, and lead to downregulation of BCL6 in part by inducing expression of the NF-κB target IRF4, a key regulator of plasmacytic differentiation and a direct BCL6 transcriptional repressor.[4,20,21] Downregulation of BCL6, in turn, restores a variety of cellular programs including those governed by PRDM1, thus enabling the B cells to develop into an antibody-secreting plasma blast (Figure 1).

The definition of two distinct phases during the GC reaction and the identification of the transcriptional programs that are associated with these two phases in B cells are important for two reasons. First, they reflect stages of B cell differentiation and function that can be recognized to some extent in the two most common subtypes of DLBCL, as defined based on cell-of-origin (COO) classification (see below). Second, two central mechanisms of genetic lesion in DLBCL – namely chromosomal translocations and aberrant somatic hypermutation (ASHM) – result from mistakes in the machinery that normally diversifies the Ig genes during B lymphocyte differentiation, further supporting the GC origin of this disease.

The DLBCL cell of origin

Over the past decade, the advent of genome-wide expression profile technologies has allowed the identifi-

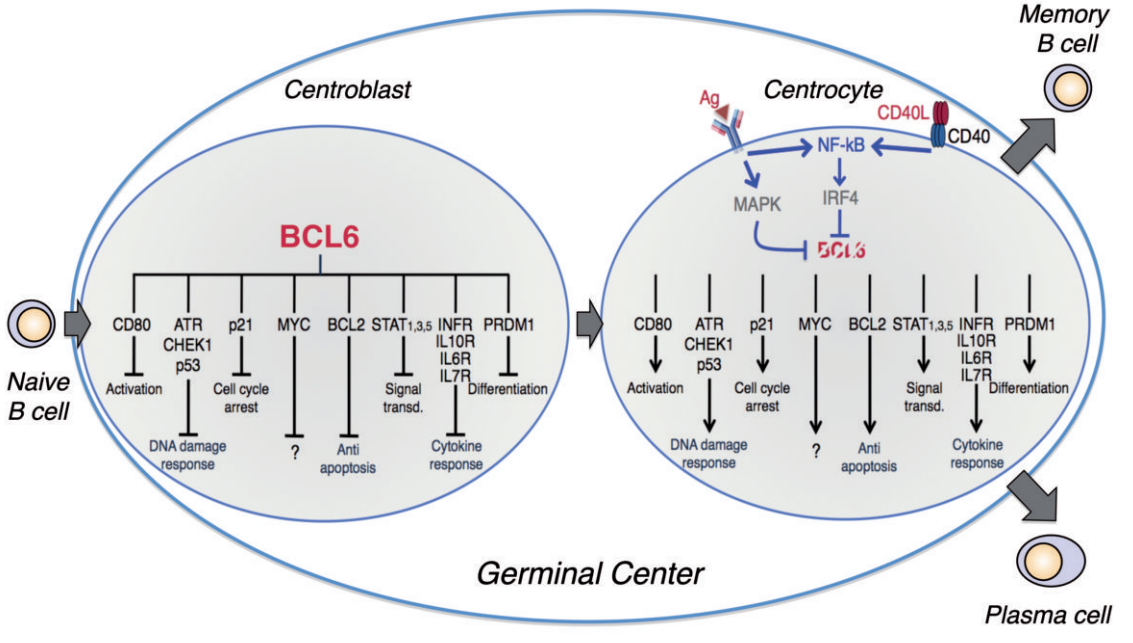

Figure 1. Biological role of BCL6 in the germinal center. Schematics of mature B cell development, with the two main stages of B cell differentiation during the GC reaction. In proliferating centroblasts, BCL6 controls a broad transcriptional program (only representative targets shown), in part to prevent premature response to signals that may cause exit from the GC before these cells have completed the remodeling of their Ig genes in order to produce high-affinity antibodies. BCL6 expression is downregulated in the light zone by a number of signals, including BCR cross-linking and engagement of CD40 by CD40L, thus restoring cellular programs that are required to proceed through terminal B cell differentiation.

cation of molecularly distinct subgroups of DLBCL, which reflect either the derivation from different stages of normal B cell differentiation or the coordinated expression of comprehensive consensus clusters (CCC). Based on similarities with the transcriptional signature of their putative cell of origin (COO), DLBCL have been divided into at least three distinct subgroups, termed germinal center B cell-like (GCB) DLBCL, activated B cell-like (ABC) DLBCL, and primary mediastinal large B cell lymphoma (PMBCL).[22-25] The remaining 15-30% of cases remain unclassified.[22,23]

GCB-DLBCLs appear to derive from a proliferating CB, as their expression profile is enriched in genes that are specific for GC B cells, including BCL6.[22] These tumors also carry highly mutated Ig genes that show evidence of ongoing SHM[26] and have undergone CSR in most cases.[22]

The ABC-DLBCL subgroup displays a transcriptional signature related to plasmablastic B cells, presumably blocked during plasma cell differentiation, and to mitogenically activated peripheral blood B cells.[22] Their gene expression profile suggests that the putative cell of origin has received signals to downregulate the GC specific program and to upregulate genes that are required for terminal B cell differentiation, such as IRF4 or XBP1; however, this cell has acquired genetic lesions that disrupt this pathway, namely BLIMP1 loss or BCL6 translocations, and is therefore precluded from exiting the GC (see next section).[21,27,28] ABC-DLBCLs are also characterized by constitutive activation of the NF-κB, BCR, and JAK/STAT signaling pathways. ABC-DLBCLs do not have ongoing SHM[39] and only rarely show evidence of legitimate CSR, because of abnormalities in the regulation of this process.[26,29]

The third subtype of DLBCL, PMBCL, is observed most commonly in young female adults and is postulated to arise from thymic B cells.[24,25] This tumor typically involves the mediastinum and shares significant histological, molecular and clinical features with nodular sclerosis Hodgkin's lymphoma (HL), including the presence of an immune/inflammatory cell infiltrate with a distinctive cytokine profile, the decreased expression of BCR signaling pathway components, and constitutive NF-κB activation.[24,25]

Stratification of DLBCL cases according to gene expression profiles has prognostic value, since patients diagnosed with a GCB-DLBCL display a better overall survival rate compared to ABC-DLBCL.[30] However, these categories are not yet incorporated into clinical practice. A separate classification schema identified three discrete subsets reflecting the coordinated expression of comprehensive consensus clusters (CCC), defined by the expression of genes involved in oxidative phosphorylation (OXP), B-cell receptor/proliferation (BCR), and tumor microenvironment/host inflammatory response (HR).[31] Although highly reproducible, the COO defined and CCC defined molecular subtypes do not overlap, indicating that these two classification schemas capture different aspects of DLBCL heterogeneity, and further underscoring the complexity of this disease.

Mechanisms of genetic lesion in DLBCL

The processes of SHM and CSR are absolutely critical for the execution of effective immune responses, and individuals that are unable to perform these functions suffer from severe forms of immunodeficiencies.[32-34] However, these same processes pose a constant risk to the genome of the B lymphocyte due to their ability to introduce DNA strand breaks. Moreover, these reactions take place in an environment – the GC – wherein B cells are replicating at remarkably fast rates and DNA damage checkpoints are silenced as a result of the BCL6 transrepressor activity. Therefore, not surprisingly, many of the structural alterations that contribute to DLBCL development derive from errors occurring during one of these two reactions. Formal experimental proof for this model has been provided by studies showing that removal of AID in mice can prevent the formation of MYC-IgH rearrangements and the development of DLBCL in lymphoma prone mouse models.[35,36]

Common mechanisms of genetic lesion in DLBCL include chromosomal translocations leading to deregulated expression of proto-oncogenes, gene amplifications, genomic deletions, and point mutations.[37,38] Additionally, DLBCL are susceptible to aberrant somatic hypermutation (ASHM), a mechanism of genomic instability due to a malfunction in the physiologic SHM process.[39] In GC B cells, SHM is restricted to the *Ig* locus and a few other genes, including BCL6,[40-42] in part because, although AID can bind to multiple DNA sequences,[43,44] mutations at off-target genes are normally repaired with high accuracy.[45] On the contrary, nearly half of DLBCL cases display multiple somatic mutations in as many as 10% of the transcribed genes, including the well known proto-oncogenes PIM1 and MYC.[39] These lesions are typically distributed within promoter-proximal sequences (i.e. the hypermutable domain in the Ig locus)[42] and, depending on the genomic configuration of the target gene, may affect untranslated as well as coding regions, possibly altering gene transcriptional regulation, or modifying key structural/functional properties.[39] While a comprehensive characterization of the potentially extensive genetic damage caused by ASHM is still missing, this mechanism may contribute to the heterogeneity of DLBCL via the alteration of diverse cellular pathways in different cases.[46] Interestingly, preferential targeting of individual genes by ASHM has been observed in GCB- versus ABC-DLBCLs, with mutations of MYC and BCL2 being almost exclusively observed in the former one, and mutations of PIM1 being significantly higher in frequency in ABC-DLBCL (unpublished).

Genomic lesions disrupt key pathways in DLBCL

The heterogeneity of DLBCL is mirrored in the catalogue of genetic lesions that are associated with its pathogenesis. Notably, several of these abnormalities are not randomly distributed, but appear to be preferentially or exclusively associated with individual COO-defined DLBCL phenotypic subtypes, indicating the involvement of distinct oncogenic pathways (Table 1).

GCB-DLBCL

Genetic lesions that are specific to GCB-DLBCL include the t(14;18) translocation, which deregulates the expression of the anti-apoptotic BCL2 protein in 35-

Table 1. Most common genetic lesions and relevant pathways in DLBCL.

DLBCL subtype	Genetic Lesion	Frequency	Functional Consequences	Gene Function/Affected Pathway
GCB-DLBCL	BCL2 Tx	30-40%	Transcriptional deregulation	Inhibitor of apoptosis
	MYC Tx	10%	Transcriptional deregulation	Proliferation and growth
	BCL6 Tx	15%	Transcriptional deregulation	Negative regulation of DNA damage response, B cell activation, differentiation
	EZH2 M	22%	Gain of function	Methyltransferase
	CREBBP/EP300 M/D	40%	Loss of function	Acetyltransferase; regulation of BCL6 and p53 function
ABC-DLBCL	BCL2 amplification	30%	Increased gene dosage	Inhibitor of apoptosis
	BCL6 Tx	25%	Transcriptional deregulation	Negative regulation of DNA damage response, B cell activation, differentiation
	PRDM1 M/D	25%	Loss of function	Regulation of terminal B-cell differentiation
	TNFAIP3 M/D	20%	Loss of function	Negative regulation of NF-κB signaling
	MYD88 M	29%	Gain of function	Activation of NF-κB, JAK-STAT, and TLR signaling
	CD79B M	20%	Gain of function	Activation of NF-κB and BCR signaling
	CARD11 M	9%	Gain of function	Activation of NF-κB signaling
	CREBBP/EP300 M/D	15%	Loss of function	Acetyltransferase; regulation of BCL6 and p53 function
PMBCL	JAK2 amplification	50%	Increased gene dosage	Activation of JAK-STAT pathway
	PDL1, PDL2 amplification	50%	Increased gene dosage	Regulation of Immunomodulatory responses

GCB, germinal center B-cell-like; ABC, activated B-cell-like; PMBCL, primary mediastinal B cell lymphoma; Tx, chromosomal translocation M, mutation; D, deletion.

45% of cases,[46] in part by allowing it to escape BCL6-mediated transcriptional repression.[11,47] In 10-14% of cases, chromosomal rearrangements involving the MYC oncogene cause its ectopic expression by placing its coding exons under the influence of strong regulatory elements derived from the *Ig* locus, and in some cases by removing the sequences that are required for binding and repression by BCL6.[48-50]

Mutations in histone methyltransferases

Somatic heterozygous mutations of the polycomb-group oncogene EZH2 have been reported in ~22% of GCB-DLBCL patients.[51] These mutations result in the replacement of a single evolutionary conserved tyrosine residue (Tyr641) within the SET domain of the EZH2 protein, a histone methyltransferase responsible for trimethylating Lys27 of histone H3 (H3K27). Mutant EZH2 alleles were recently shown to associate with increased levels of H3K27me3 through a mechanism that involves altered catalytic specificity of the mutant EZH2 enzyme for its substrates.[52,53]

Alterations of acetyltransferase genes

Recent studies have revealed the presence of mutations and/or deletions inactivating the acetyltransferase genes CREBBP and, less frequently, EP300, in nearly 40% of all DLBCL cases, with preference for the GCB-DLBCL subtype.[54] CREBBP and EP300 lesions are mostly detected in heterozygosity, suggesting a haploinsufficient tumor suppressor role. In fact, CREBBP mutations were shown to impair the ability of this enzyme to acetylate known substrates, such as BCL6 and p53, leading to constitutive activation of the oncoprotein and to decreased p53 tumor suppressor function.[54] Since the balance between the activities of these two genes is critical for the regulation of DNA damage responses during Ig gene remodeling in the GC,[12,13] one consequence of BCL6 activity overriding p53 would be an increased tolerance for DNA damage in the context of impaired apoptotic and cell cycle arrest responses. Given the broad involvement of histone acetyltransferases (HAT) in gene transcriptional regulation, additional studies will be required to dissect the entire set of cellular targets/pathways that are critically affected by HAT reduction in lymphoma. Importantly, the identification of mutations in CREBBP and EP300 may have direct therapeutic implications in view of current attempts to use histone deacetylase inhibitors as anti-cancer drugs.

Somatic mutations affecting negative autoregulatory elements in the BCL6 promoter

Mutations in the 5' sequences of the BCL6 gene are detected in up to 75% of DLBCL cases[40,55,56] and reflect the activity of the physiologic SHM mechanism that operates in GC B cells.[40,41] However, a subset of mutations have been found specifically in tumor cells, while being absent in normal GC cells.[57] These mutations disrupt an autoregulatory circuit by which the BCL6 protein controls its own transcription, thereby causing its deregulated expression.[57,58] In a smaller subset of cases, nucleotide substitutions in the BCL6 non-coding exon 1/intron 1 sequences prevent CD40-induced IRF4-mediated downregulation of BCL6 expression in post-GC B cells.[21] Since the full extent of mutations deregulating BCL6 expression has not been characterized, the fraction of DLBCL cases carrying BCL6 abnormalities cannot be determined.

Other lesions

Mutations and deletions of the TP53 tumor suppressor gene are mostly detected in cases deriving from the transformation of FL, and therefore more often associated with chromosomal translocations of BCL2[59] and with a GCB-DLBCL phenotype. Other genetic lesions that have been reported preferentially in GCB-DLBCLs include deletions of the tumor suppressor PTEN (~10%

of cases)[46] and amplifications of the region encompassing the mir-17-92 microRNA cluster on chromosome 13, which has also been shown to suppress PTEN.[60]

ABC-DLBCL

Several genetic abnormalities are observed almost exclusively in ABC-DLBCL, including amplifications of the BCL2 locus on 18q24;[61,62] mutations within the NF-κB (CARD11, TNFAIP3/A20),[63,64] BCR (CD79A/B)[65] and JAK/STAT (MYD88) signaling pathways; inactivating mutations and deletions of PRDM1;[27,28,66] and deletion or lack of expression of the CDKN2A tumor suppressor gene. In addition, chromosomal translocations deregulating the BCL6 oncogene are found more frequently in this subtype. Mutations of ATM have also been reported in a small subset of cases.[67,68]

NF-κB pathway lesions

A prominent feature of ABC-DLBCL is the constitutive activation of the NF-κB signaling pathway, as revealed by the selective enrichment in NF-κB target genes expression, and by the requirement of NF-κB for the proliferation and survival of ABC-, but not GCB-DLBCL cell lines.[85] Over the past two years, a number of studies have provided a genetic basis for this phenotypic characteristic by identifying multiple oncogenic alterations that affect positive and negative regulators of NF-κB, specifically in this disease subtype. In up to 30% of the cases, biallelic mutations and/or deletions inactivate the TNFAIP3 gene, which encodes for the negative regulator A20, thus preventing termination of NF-κB responses.[63,86] In ~9% of patients, CARD11 is targeted by oncogenic mutations that cluster within the protein coiled-coil domain and enhance its ability to transactivate NF-κB target genes.[63,64] Less commonly, mutations were found in a variety of other genes encoding for NF-κB components (among others, TRAF2 and TRAF5).[63]

ABC-DLBCL activate NF-κB also via a chronic form of active BCR signaling, which is associated with somatic mutations in the immunoreceptor tyrosine-based activation motif (ITAM) signaling modules of CD79B and CD79A (> 20% of patients).[65] Since BCR signaling can also trigger other pathways, such as MAPK and PI3K, future studies will have to address the relative or coordinate contribution of these pathways to the development of DLBCL (Figure 2). Finally, oncogenically active MYD88 mutations were recently reported in almost one third of all ABC-DLBCLs, where they mostly target an evolutionarily invariant residue within the TIR (Toll/IL1 receptor) domain.[87] Besides activating NF-κB, mutations of MYD88 induce JAK/STAT3 transcriptional responses, also a phenotypic trait of ABC-DLBCL.[88,89] Collectively, lesions converging on the NF-κB pathway account for greater than 50% of all ABC-DLBCL.[63]

PRDM1 inactivation

In up to 25% of ABC-DLBCLs, the PRDM1 gene is inactivated by a variety of genetic lesions, including truncating point mutations, inactivating missense mutations, and/or genomic deletions.[27,28,66] An additional sizeable fraction of cases has lost PRDM1 protein expression due to transcriptional repression by constitutively active, translocated BCL6 alleles.[27,66] The PRDM1 gene encodes for a zinc finger transcriptional repressor that is expressed in a subset of GC B cells undergoing plasma cell differentiation and in all plasma cells,[82,83] and which is essential for terminal B cell differentiation.[84] Thus, PRDM1 functions as a tumor suppressor gene and may favor malignant transformation by blocking post-GC differentiation of B cells. In line with these findings, rearrangements of the BCL6 locus and genetic lesions of PRDM1 are mutually exclusive in ABC-DLBCL, indicating that BCL6 deregulation and PRDM1 inactivation represent alternative oncogenic mechanisms converging

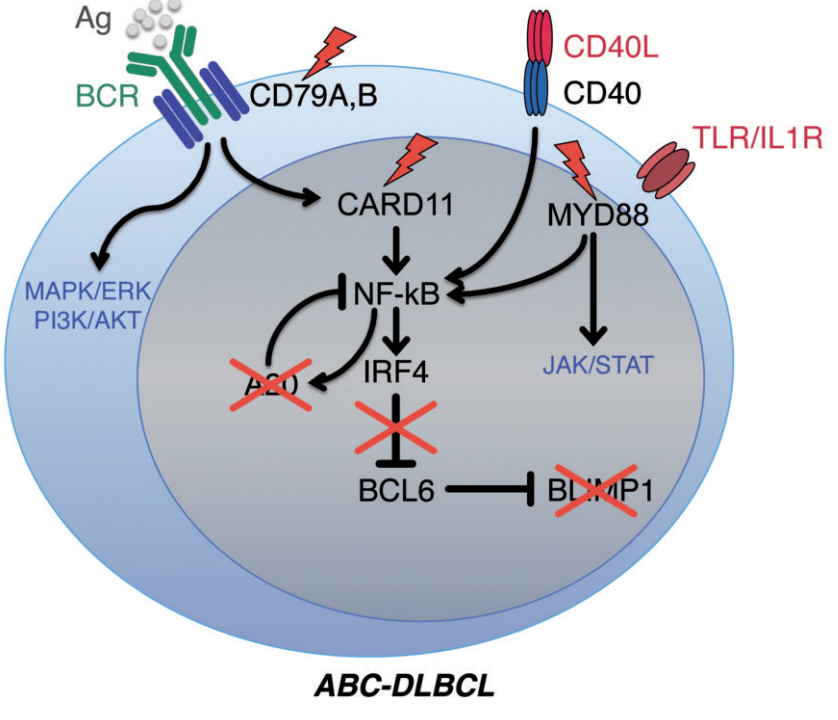

Figure 2. Pathway lesions in ABC-DLBCL. Schematic representation of a germinal center centrocyte, expressing a functional surface BCR, CD40 receptor and Toll-like receptor (TLR). In normal B cells, engagement of these signaling pathways leads to activation of NF-κB and transcription of its targets genes including IRF4 and TNFAIP3/A20. IRF4, in turn, downregulate BCL6 expression, allowing the release of the master plasma cell regulator PRDM1 and the development into a differentiated plasma cell. In DLBCL, a variety of genetic lesions disrupt this circuit at multiple levels predominantly in the ABC-subtype, and contribute to lymphomagenesis by favoring the anti-apoptotic function of NF-κB while blocking terminal B cell differentiation. Crosses indicate inactivating mutations/deletions; lightning bolts denote activating mutations.

on the same pathway (Figure 2). The tumor suppressor role of PRDM1 was recently confirmed *in vivo* by showing that conditional deletion in GC B cells leads to lymphoproliferative diseases with features of the human ABC-DLBCL.[66]

BCL6 translocations

Chromosomal rearrangements of the BCL6 locus represent one of the most common lesions associated with DLBCL,[69-71] being present in up to 35% of all patients, with a two-fold higher frequency in the ABC-DLBCL subtype[72,73] (Table 1). These alterations were shown to be promiscuous in that they involve balanced, reciprocal recombination events between the BCL6 locus and various alternative chromosomal partners in different DLBCL cases.[73-77] As a consequence of the translocation, the intact coding domain of BCL6 is juxtaposed downstream and in the same transcriptional orientation to heterologous sequences derived from the partner chromosome, including the *Ig* loci and at least 20 other chromosomal sites.[78-80] Because of the broader spectrum of activity of these alternative promoters throughout B cell development,[78] the translocation prevents the downregulation of BCL6 expression that is normally associated with differentiation into post-GC cells. Thus, deregulated BCL6 expression may contribute to lymphomagenesis by enforcing the proliferative phenotype that is typical of GC cells, while blocking terminal differentiation. The critical role of BCL6 in initiating lymphomagenesis has been confirmed in a mouse model in which deregulated BCL6 expression promotes the development of DLBCL similar to the human disease.[81]

PMBCL

A genetic hallmark of both PMBCL and nodular sclerosis HL is the amplification of chromosomal region 9q24.1, which is detected in nearly half of these patients.[46,90] Of the many candidate genes that are encompassed by this relatively large interval, those encoding for the JAK2 tyrosine kinase and/or the PDL1 and PDL2 immunomodulatory proteins have been recently shown to be pathogenetically relevant.[46,90,91] Elevated expression levels of these genes may explain in part the unique features of these tumors, which are characterized by a marked inflammatory infiltrate. Additionally, PMBCL shares with HL the presence of genetic lesions affecting NF-κB pathway components, such as TNFAIP3 and the SOCS1 genes.[92-95]

Concluding remarks

Over the past few years, significant research efforts have been focusing on the identification of molecular signatures and genetic alterations that might help in stratifying DLBCL patients with different prognoses and responses to therapy. Targeted resequencing and genomic profiling have led to the discovery of important new genetic lesions, revealing the involvement of several previously unrecognized proteins and pathways that may play critical roles in DLBCL pathogenesis. Some of these (e.g. NF-κB and BCL6) are already being tested as potential targets for clinical application. However, these lesions only affect a fraction of cases, and the full spectrum of genomic aberrations that contribute to lymphoma initiation and progression remains unknown. With recent improvements in sequencing technologies, we now have an unprecedented opportunity to examine the lymphoma cancer genome in a comprehensive and unbiased manner. While it is likely that a broader picture of the DLBCL genome will become available very soon, major efforts will be needed in the future to characterize the functional significance of the identified lesions and their specific role in the pathogenesis of the disease. These findings are expected to have critical implications for the development of new diagnostic tests as well as for the design of treatment approaches that could improve the survival outcome of lymphoma patients with minimal toxicity.

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Diffuse large B cell lymphoma: treatment decisions based on molecular features

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A B S T R A C T

The treatment of diffuse large B cell lymphoma (DLBCL) in the last two decades has heralded an era of increasing exploration of therapies derived from improved biologic understanding of tumors and tumor-host interactions. The focus of drug discovery has moved from identifying classical cytotoxic agents to molecules that target specific pathways involved in signal transduction, apoptosis, and differentiation, among others. These efforts have been greatly aided by insights into the structure of proteins and the ability to design specific inhibitors using small molecules or monoclonal antibodies. The discovery of new signaling pathways through gene expression profiling (GEP) and RNA interference screens that are critical to lymphomagenesis has produced an array of new targets for the treatment of lymphomas. Specifically, GEP has revealed that DLBCL consists of at least three major subtypes: germinal center B cell-like (GCB), activated B cell-like (ABC) and primary mediastinal B cell (PMBL) DLBCL, which derive from B cells at different states of differentiation and with unique molecular pathogenesis. ABC DLBCL shows high expression of target genes of the NF-κB/Rel family of transcription factors, and may benefit from NF-κB inhibition. In GCB DLBCL, studies raise the hypothesis that inhibition of the BCL-6 transcription factor may be therapeutically important, whereas PMBL, like its biological cousin nodular sclerosis Hodgkin's lymphoma, may benefit from dose intensity approaches and inhibition of the Janus Kinases.

Pathological classification

Conceptual therapeutic advances are highly dependent on biologically-based pathological classifications. The National Cancer Institute's Working Formulation, which was in use in the United States until the early 1990's, categorized lymphomas according to their morphology and clinical behavior, and lacked a biological foundation.[1] In contrast, the Kiel Classification, which was used in Europe, was the first to employ a biological foundation.[2] However, it was not until the Revised European-American Lymphoid (REAL) classification, published in 1994, that a clinical-biological foundation was incorporated into the classification of lymphomas.[3] Since then, major genetic and biological insights have been incorporated into the diagnostic criteria codified in the World Health Organization (WHO) classification of tumors of the lymphoid tissues.[4] This evolution in classification is the direct result of insights into the molecular pathogenesis of lymphoma, including the identification of "hallmark" genetic abnormalities, and has led to the discovery of driver pathways and clinical testing of targeted therapy.[5,6]

The classification of diffuse large B cell lymphoma (DLBCL) has been among the greatest beneficiaries of these biological advances. While it has long been recognized that DLBCL is clinically and biologically diverse, it had been difficult to readily subdivide it into distinct disease entities because of overlapping morphology and pathogenetic features.[7] As a result, treatment strategies have primarily depended on clinical features such as stage, age and the International Prognostic Index (IPI) score.[8] Large scale gene expression profiling (GEP) and mutational analysis, however, has led to the recognition of new subtypes of DLBCL. Though still retaining the histological description of a neoplasm of large B lymphoid cells with a diffuse growth pattern, DLBCL can now be subdivided into diseases that arise from B cells at different stages of differentiation with distinctive molecular and clinical characteristics.[4] Thus, when considering treatment, either in the research or clinical setting, it is essential to understand these pathobiological distinctions. This educational review will focus primarily on three distinct subtypes of DLBCL in which molecular, diagnostic and therapeutic advances have been made.

Molecular pathology of germinal center B cell and activated B cell DLBCL

Presently, DLBCL is divided into four major groupings 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) that is 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 follicular lymphoma, CLL, lymphoma and leukemia cell lines, and normal lymphocyte 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

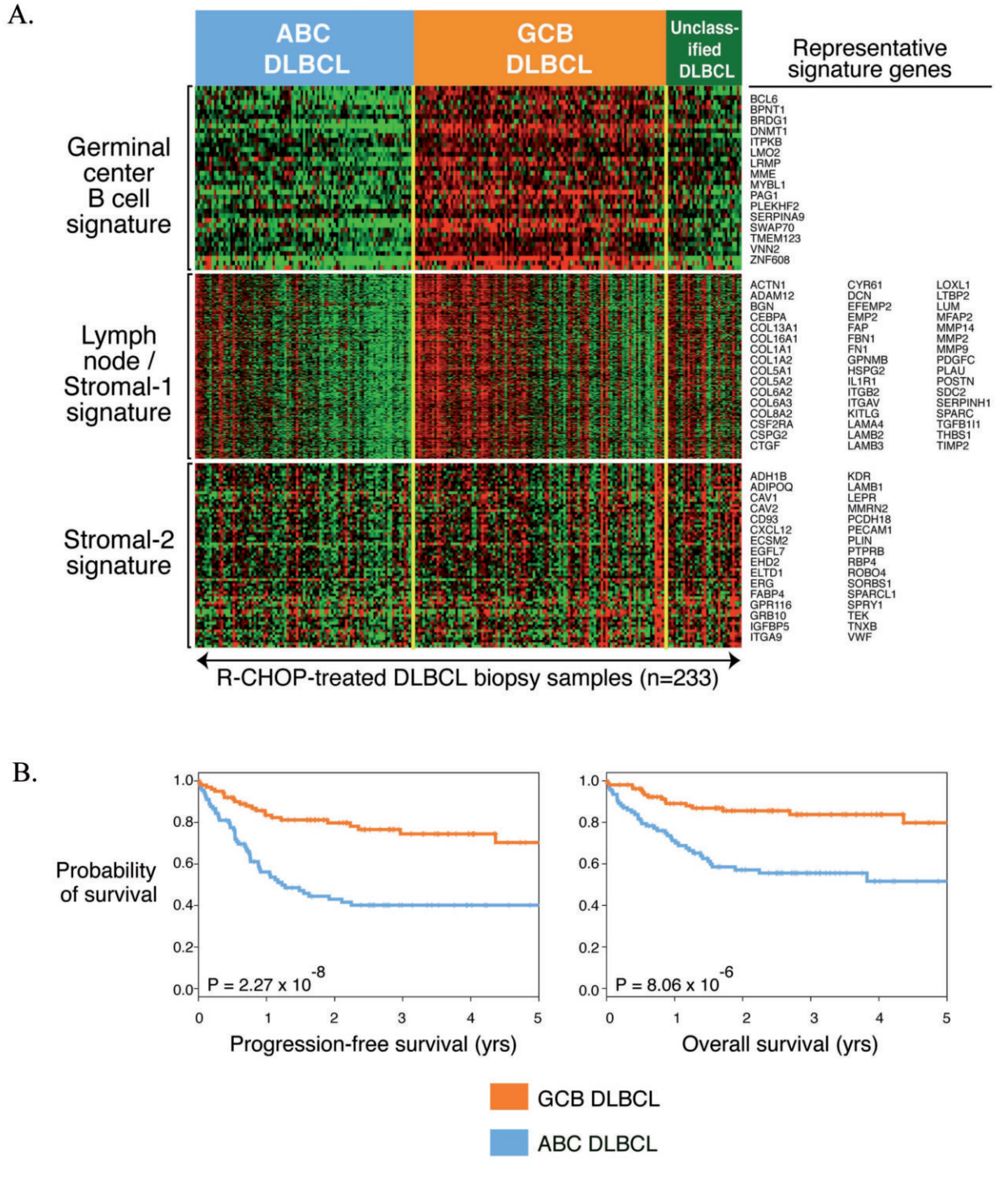


Figure 1. Diagnosis and outcome of DLBCL subtypes by gene expression profiling subtypes. A. Heat map showing expression of genes that discriminate between the GCB and ABC subtypes of DLBCL. Genes associated with the microenvironment, which have prognostic significance, are clustered into stromal 1 and 2 signatures. The stromal-1 signature genes are associated with extracellular matrix deposition and histiocytic infiltration and the stromal-2 signature genes are associated with increased tumor blood vessel density. 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 2 years.

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 check-point 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, as well as numerous new genes (Figure 1A).[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 such as cyclin D2 and CD44. 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] Most ABC DLBCL's had over four-fold higher BCL-2 expression compared to GCB DLBCL's.[11]

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. Genetic analysis has revealed ABC and GCB DLBCL to be pathogenetically distinct. GCB DLBCL is exclusively associated with two recurrent oncogenic events, the t(14;18) translocation involving the BCL-2 gene and amplification of the c-rel locus on chromosome 2p. They also have amplification of the oncogenic mir-17-92 microRNA cluster, deletion of the tumor suppressor PTEN, and frequent abnormalities of BCL-6.[10,18-20] ABC DLBCLs have frequent amplification of the oncogene SPIB, deletion of the INK4a/ARF tumor suppressor locus and trisomy 3. The NF-κB pathway is constitutively activated in most ABC DLBCL cases.[21-23] This has been linked to abnormalities in a variety of upstream proteins, including CARD11, BCL-10 and A20, leading to activation of IκB kinase and NF-κB activation.[21-23] For example, 10% of ABC DLBCL cases have somatic mutations in CARD11, a signaling scaffold protein, that cause it to constitutively engage the NF-κB pathway (Figure 2A and 2B).[22] For the majority of ABC DLBCL cases, NF-κB activation can be observed in the absence of CARD11 or BCL-10 mutations. In these cases, NF-κB activation may be linked to chronic active B cell receptor (BCR) signaling. Using RNA interference screening (RNAi), Staudt *et al.* showed that targeting the BCR pathway component Bruton's tyrosine kinase (Btk) resulted in a significant *in vitro* antiproliferative activity against ABC but not GCB DLBCL. 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.[24] Missense mutations in CD79B protein of the BCR were identified in two cell lines, and in 21% of ABC DLBCL and 3% of GCB DLBCL tumor biopsies.[24] These results suggest that a significant percent of ABC DLBCL may have a heightened BCR antigenic response, leading to abnormal activation of NF-κB.

BCR signaling also activates the PI3K/Akt/mTOR signaling pathway with effects on apoptosis, proliferation and metabolism (Figure 2A). While oncogenic activation of the PI3K pathway has been reported to be associated with gain-of function mutations in the PI3K p110α or p85α isoforms and/or with the loss-of function of the PTEN phosphatase, these are infrequently observed in lymphoid malignancies, where constitutive BCR activation and/or activating cytokines and growth factors in the microenvironment, such as CD30, CD40, BAFF, and RANK, appear to be important.[25,26]

A recent study has also shown ABC DLBCL has a dependence on MYD88, an adaptor protein that mediates TOLL and interleukin (IL)-1 receptor signaling, and oncogenic mutations affecting MYD88.[27] 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 that 29% harbored mutations in MYD88, which were shown to be gain-of-function driver mutations. The L265P mutant promoted cell survival through assembling an IRAK1 and IRAK4 protein complex, which lead to NF-κB signaling, JAK kinase activation of STAT3, and secretion of IL-6, IL-10 and interferon-β. These results indicate that the MYD88 signaling pathway is central to the pathogenesis of ABC DLBCL, and supports the development of inhibitors of this pathway in tumors harboring MYD88 mutations. These results suggest a number of strategies to exploit chronic active BCR signaling in ABC DLBCL (Figure 2A).

If the molecular taxonomy defines true DLBCL subtypes, it should also have prognostic value. An analysis of molecular subtype and outcome following upfront CHOP treatment shows a statistically significant difference in overall survival at five years of 59% in GCB and 31% in ABC subtypes of DLBCL, and these were independent of the IPI risk groups.[11,13] Because this analysis was preformed on biopsies obtained in the pre-rituximab era, a second analysis was performed on 233 biopsies obtained from patients treated with R-CHOP.[10] Similar to the aforementioned results, patients with GCB compared to ABC DLBCL had a more favorable survival, with 3-year OS rates of 84% vs. 56%, respectively ($p<0.001$); expectedly, both GCB and ABC DLBCL performed better compared to the pre-rituximab analysis (Figure 1B). In this analysis, a new "stromal-2" signature was discovered that predicted inferior survival, whereas a stromal-1 signature was found to be favorable (Figure 1A). The stromal-1 signature was associated with extracellular matrix deposition and histiocytic infiltration and the stromal-2 signature associated with increased tumor blood vessel density. Although the stromal-2 signature suggests that anti-angiogenesis treatment may be useful, it is also possible that this sig-

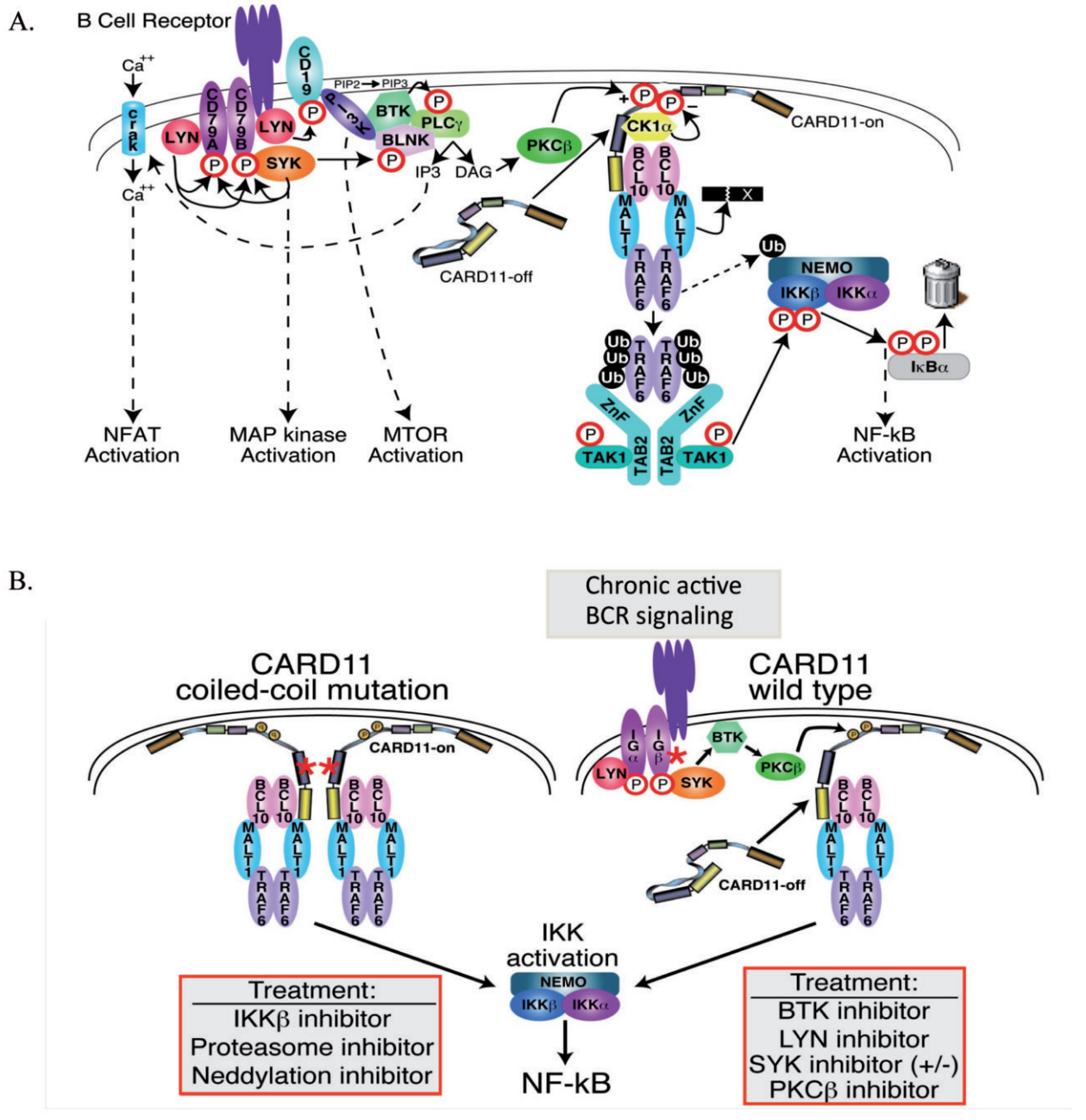


Figure 2. B cell receptor (BCR) signaling pathway and potential targets. A. Signaling through BCR leads to downstream activation of the NFκB transcription factor, which is a driver pathway in ABC DLBCL. Signaling also activates the AKT/MTOR and MAP kinase pathways. B. Targeted therapies in ABC DLBCL to inhibit NFκB are dependent on the presence or absence of activating mutations in CARD11. Tumors with activating mutations are likely to require inhibition of downstream targets (e.g. NFκB activation) whereas those with wild type are likely to be sensitive to both downstream and upstream (e.g. inhibition of BTK or SYK) targets.

nature is secondary to an anaerobic environment and is not a driver event.

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 on frozen tissue remains the "gold standard", it has obvious practical limitations for clinical practice. In its place, investigators have developed immunohistochemical models, which have had variable reproducibility, but nonetheless have successfully distinguished GCB from non-GCB DLBCL in a number of clinical trials.[28-30] 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.

Treatment strategies in germinal center B cell DLBCL

While GCB DLBCL has a better prognosis than ABC DLBCL, upwards of 30% are not cured. The resistance of GCB DLBCL to curative treatment may relate to the effect of BCL-6 on cell growth and survival.[31,32] BCL-6 is an important modulator of B cell development in the germinal center, and its transcriptional silencing is required for exit of the B cell from the germinal center. BCL-6 suppresses genes that are involved in lymphocyte activation, differentiation, cell cycle arrest, and include p21 and p27Kip1, and DNA damage response genes, p53 and ATR.[31] 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.[31,33-36]

These results suggest that BCL-6 is an important target for GCB DLBCL. Inhibitors targeting the BCL-6 BTB domain protein interaction have shown efficacy *in vitro*.[20] 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.[20] An interesting and potentially important observation is the effect of topoisomerase II inhibition on BCL-6 levels. It has been shown that the topoisomerase II inhibitor etoposide leads to down regulation of BCL-6 expression by ubiquitin-mediated protein degradation and possibly through transcriptional inhibition.[37] This may partially 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 checkpoint kinase (Chk2), effects that are inhibited in cells engineered to over-express BCL-6.[38,39] This raises the possibility that inhibition of topoisomerase II may be particularly important in GCB DLBCL. In this regard, the German high-grade lymphoma study group showed that the addition of etoposide to CHOP (CHOEP) significantly improved the event-free survival of younger but not older patients with untreated DLBCL.[40,41] The higher frequency of GCB DLBCL in younger compared to older patients may explain why the benefit of etoposide was only found in the study of patients under 60 years and not over 60 years.[9,40,41]

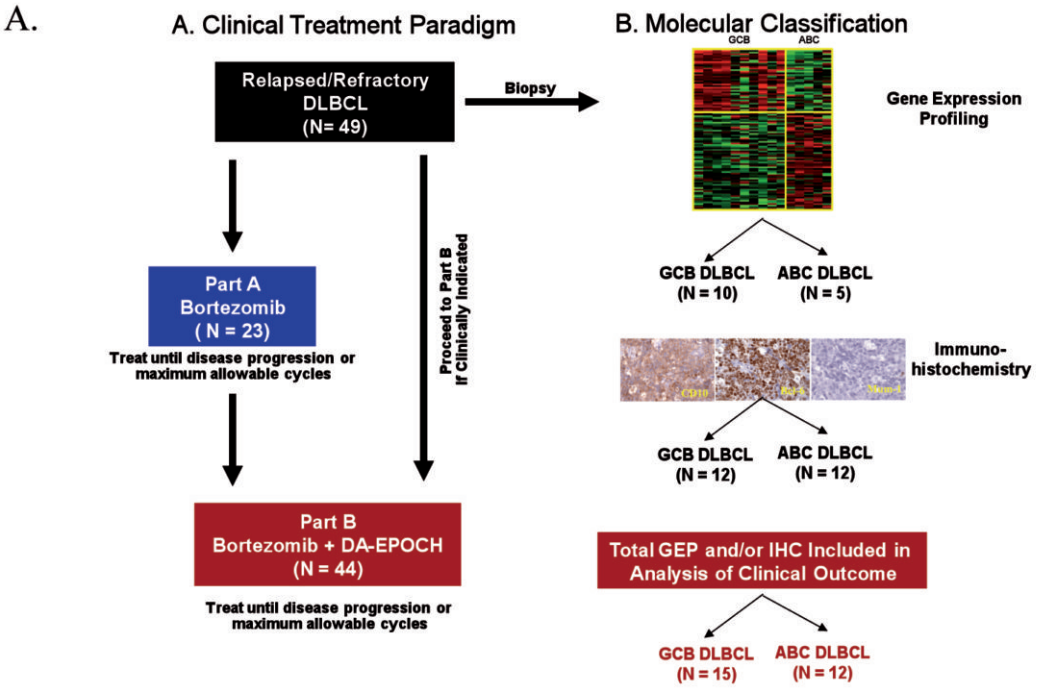
Interestingly, the positive effect of including etoposide in CHOEP was lost when rituximab was added (R-CHOPE).[42] However, this may reflect the overall salutary effect of rituximab on the outcome of DLBCL, including both GCB and ABC DLBCL, and not a specific effect on BCL-6.

The association between topoisomerase II inhibition and inhibition of BCL-6 raises the hypothesis of whether regimens that highly inhibit topoisomerase II would be more effective in GCB DLBCL, even in the setting of rituximab. To help address this hypothesis, we turned to the DA-EPOCH-(R) regimen, which was designed to inhibit topoisomerase II through several strategies: 1. Incorporates two topoisomerase II inhibitors, etoposide and doxorubicin; 2. Optimizes topoisomerase II inhibition through a prolonged 96-hour infusion; 3. Maximizes steady state concentrations through pharmacodynamic dose adjustment.[43] To help address this question, we analyzed the outcome of GCB DLBCL in two trials of DA-EPOCH-R in previously untreated GCB DLBCL. In one trial of 33 patients with HIV+ DLBCL, we observed a 95% EFS at a 5-year median follow-up in those with GCB DLBCL.[44] In another study of 75 patients, performed by the Cancer and Leukemia Group B cooperative group (CALGB), patients with GCB DLBCL achieved a 100% EFS at a 5-year median follow-up (Hsi and Wilson *et al.*, unpublished observations).[45,46] 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.

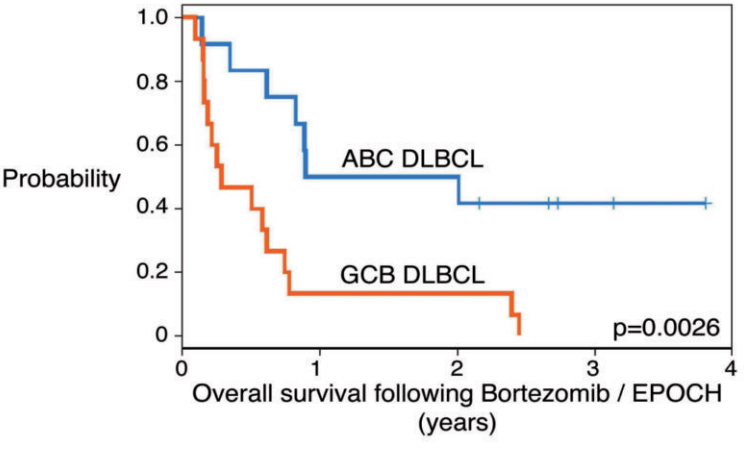
Treatment strategies in activated B cell DLBCL

As discussed above, studies show that ABC DLBCLs are characterized by constitutive activity of NF-κB, which activates genes associated with survival and proliferation and has an inferior clinical outcome. To help assess if NF-κB is a clinically useful target, Dunleavy *et al*. undertook a "proof of principle" clinical study to test whether inhibition of NF-κB might sensitize ABC but not GCB DLBCL to chemotherapy (Figure 3A and 3B).[47,48] Based on *in vitro* evidence that bortezomib, a proteasome inhibitor, blocked degradation of phosphorylated IκBa and consequently inhibited NF-κB activity in ABC DLBCL cell lines (data not shown), bortezomib was combined with DA-EPOCH in patients with relapsed/refractory DLBCL.[49-51] Tumor tissue was analyzed by gene expression profiling and/or immunohistochemistry to identify molecular DLBCL subtypes (figure 3A). As a control, they showed that relapsed/refractory ABC and GCB DLBCL have equally poor survivals following upfront chemotherapy. Bortezomib alone had no activity in DLBCL, but when combined with chemotherapy, it demonstrated a significantly higher response (83% versus 13%; P = 0.0004) and median overall survival (10.8 versus 3.4 months; P = 0.0026) in ABC compared to GCB DLBCL, respectively, as shown in Figure 3B. These results suggest bortezomib enhances the activity of chemotherapy in ABC but not GCB DLBCL, and provide a rational therapeutic approach based on genetically distinct DLBCL subtypes.[52] In another recent study, bortezomib was combined with R-CHOP in patients with previously untreated DLBCL to assess its toxicity and efficacy in ABC DLBCL subtype.[53] In this study of 40 patients with DLBCL, patients achieved a PFS of 64% at 2-years, and there was no difference among patients with GCB and ABC DLBCL, suggesting that bortezomib overcame the adverse prognostic effective of the ABC DLBCL subtype. Based on these studies, a randomized study of R-CHOP ± bortezomib in untreated patients with ABC DLBCL is ongoing (Pyramid Study).

A recent study suggests that lenalidomide, an immune modulatory agent, may also be preferentially effective



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. Clinical treatment paradigm. Patients initially received bortezomib alone at 1.3 mg/m^2 on days 1, 4, 8 and 11 every 21 days (Part A) unless they had disease which the investigators judged to required immediate chemotherapy such as impending or ongoing organ compromise; these patients only received Part B. Patients with progressive disease on Part A received bortezomib with DA-EPOCH (Part B). Of 31 DLBCL cases analyzed by GEP, 16 were excluded due to ineligible subtype by classification or did not receive Part A, leaving 5 ABC and 10 GCB cases eligible for analysis of outcome. Of 24 paraffin embedded tumor biopsies analyzed by immunohistochemistry, 12 each were categorized as GCB and ABC (non-GCB) type.[28] By combining both methods, cases were identified as GCB in 15 and ABC in 12 and included in the analysis of outcome with Part B. B. Response and overall survival of 27 patients with de novo GCB or ABC DLBCL who received DA-EPOCH-B. 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.

in ABC DLBCL (Hernandez *et al.*, 2010 ASCO; Abstract 8038). In a 40 patients phase II study of relapsed/refractory DLBCL, lenalidomide produced had a 29% complete and 53% overall response rate in non-GCB (surrogate of ABC DLBCL) compared to 10% in GCB DLBCL. The median overall survival was also significantly longer in non-GCB (187 days) compared to GCB DLBCL (51 days; P = 0.004). Although the mechanisms of lenalidomide are complex and incompletely understood, these investigators demonstrated that lenalidomide inhibits NFκB in a Raji cell NFκB activity reporter assay, and also inhibits angiogenesis.[54,55] Thus, lenalidomide is another important agent to test in ABC DLBCL.

While it is important to show that inhibition of NF-κB is clinically useful, it is also important to understand and target upstream drive events, which lead to activation of NF-κB. As discussed, chronic BCR signaling and activating mutations of CARD11 and MYD88 lead to NF-κB activation (Figure 2B). These results suggest a number of strategies to exploit chronic active BCR signaling in ABC DLBCL. One such target is Bruton's tyrosine kinase (Btk), where a selective Btk inhibitor, PCI-32765, was shown to be selectively toxic to cell lines with chronic active BCR signaling.[24] Importantly, the position of molecular lesions in the BCR and NF-κB signaling pathways could help guide therapy of ABC DLBCL. For example, ABC DLBCLs with wild type CARD11 and chronic active BCR signaling might respond to a Btk inhibitor, and possibly to inhibitors of Src-family kinases, PKC-β or Syk, in some cases (Figure 2B). In contrast, CARD11-mutant tumors would require agents that target downstream components of the NF-κB pathway. A precise assessment of which ABC DLBCL cases depend on chronic active BCR signaling will require the development of predictive biomarkers and the results of clinical trials involving BCR signaling inhibitors, such as Btk.

Based on these studies, a pilot study of the Btk inhibitor, PCI-32765, has recently begun in patients with relapsed/refractory ABC DLBCL. This study will enroll ten patients with pre-identified DLBCL ABC DLBCL, and tumor biopsies will be obtained pre-treatment and (where possible) 48 hours after the first two doses of PCI-32765 for GEP, to assess effects of PCI-2765 on gene expression, and for sequencing of the BCR, CARD11 and MYD88 proteins. Interestingly, of two patients enrolled, both showed a point mutation in the BCR protein CD79B and had tumor reductions. Furthermore, paired tumor biopsies in one patient analyzed showed inhibition of NF-κB target genes (Staudt and Wilson, unpublished observations).

There are also studies that have targeted the PI3K/AKT/mTOR signaling pathway using mTOR inhibitors in patients with relapsed Hodgkin's and non-Hodgkin's lymphomas (Figure 2A). Although the patients have been heterogeneous, mTOR inhibitors (temsirolimus and everolimus) have induced complete remissions across lymphoma subtypes.[56,57] 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. A recent trial presented at the American Society of Hematology meeting using the PI3K inhibitor CAL-101 also yielded responses in a variety of lymphoid malignancies (Flinn *et al.*, ASH Annual Meeting Abstracts, 2009;114:922). Thus, inhibitors of mTOR and/or upstream targets such as Akt and PI3K need to be evaluated in ABC DLBCL.

Overarching treatment strategies in germinal center and activated B cell DLBCL

The studies we have discussed show that GCB and ABC DLBCL have different driver pathways, which derive from normal pathways associated with their cell of origin. However, they also share potential targets that perform differently according to the subtype of DLBCL. This has been most studied with BCL-2, which is expressed in both GCB and ABC DLBCL. While some older studies found an association between BCL-2 expression and poor outcome in DLBCL, later studies have shown a more complex association.[34,58] The mechanism of BCL-2 over-expression has been related to its prognostic relevance in DLBCL. Gascoyne *et al.* showed that BCL-2 over-expression 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.[58] 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 over-expression is associated with gene amplification or NFκB transcriptional activation.[9,59] In this latter case, BCL-2 expression may simply be a surrogate biomarker for ABC DLBCL, and may not in itself be an important therapeutic target. Of course, this needs to be assessed using high affinity inhibitors of BCL-2, such as navitoclax.[60]

Another potentially important biomarker and target is MYC. High MYC expression has been observed in DLBCL and is associated with the proliferation signature.[9,59] Recent studies have also shown that up to 10% of DLBCL cases harbor MYC translocations, and these are associated with a poor outcome with standard R-CHOP treatment.[61,62] Expectedly, MYC translocation was associated with significantly higher tumor proliferation. Furthermore, MYC translocations were present in both GCB and non-GCB (surrogate of ABC) DLBCL and were adverse in both groups. These studies suggest that newly diagnosed patients with DLBCL should have their tumors analyzed for a MYC translocation and received treatments other than R-CHOP. While the optimal treatment approach is unknown, Dunleavy *et al.* probed for MYC translocations in 59 cases of DLBCL treated with DA-EPOCH-R and found no association between MYC+ and MYC- and EFS at 5-years (83% and 76%, respectively) (Dunleavy *et al.*, 2011, Abstract submitted at the 11th International Conference on Malignant Lymphoma, Lugano, Switzerland).

Molecular pathology of primary mediastinal B cell lymphoma

GEP has also been applied to primary mediastinal B cell lymphoma (PMBL), an important subtype of

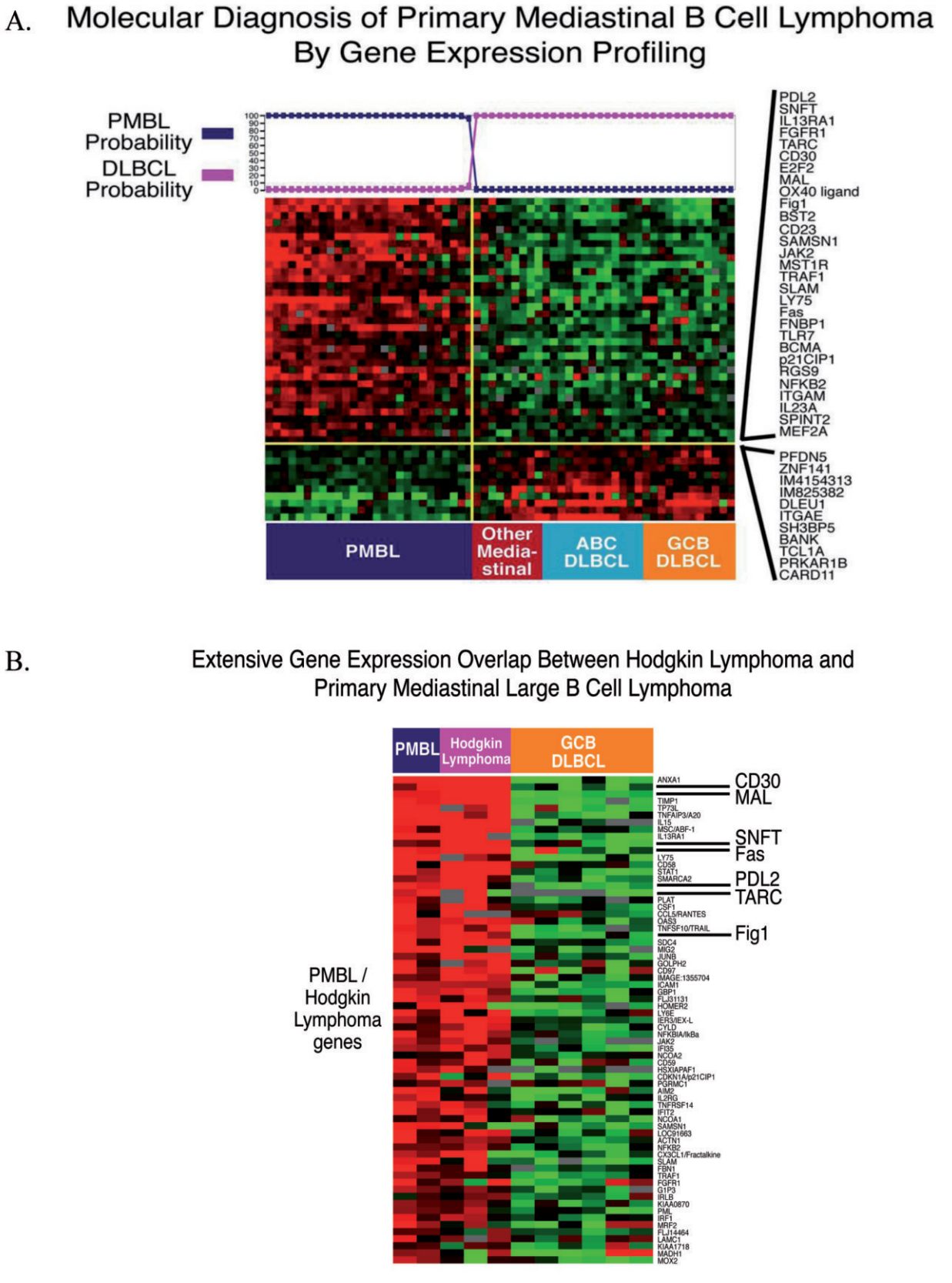


Figure 4. Gene expression profiling of PMBL and comparisons to GCB and ABC DLBCL and Hodgkin's lymphoma. A. Heat map of genes that discriminate PMBL from other mediastinal large B cell lymphomas and ABC and GBC DLBCL. B. Heat map showing overlap in gene expression between PMBL and Hodgkin's lymphomas, including CD30 and PDL2.

DLBCL that mostly occurs in young patients.[63] This subtype is defined by a combination of clinical and pathological features and some cases may have pathological features reminiscent of Hodgkin's lymphoma, all of which can confound an accurate diagnosis.[7,64] Two recent studies using GEP have confirmed the unique biological identity of PMBL and have shown a strong relationship between PMBL and Hodgkin's lymphoma (Figure 4A and 4B).[65,66] Cases of PMBL could be accurately identified by a model using 35 genes that were more highly expressed in PMBL and 11 genes that were more highly expressed in DLBCL.[65] When this model was applied to 46 patients with a diagnosis of PMBL, 76% were classified as PMBL. Of the remaining 11 cases, however, seven and four were classified as belonging to the GCB and ABC DLBCL subtypes, respectively, indicating that, although these latter cases predominantly involved the mediastinum, they were not PMBL. Clinically, cases identified as PMBL by gene expression appeared to have a relatively favorable 5-year survival of 64% compared to 59% and 30%, respectively, for the GCB and ABC DLBCL subtypes.

Over half of PMBL cases and three Hodgkin's lymphoma cell lines had gains/amplifications in a region of chromosome 9p. The amplicon on chromosome 9p includes JAK2, which encodes a tyrosine kinase, and SMARCA2, which encodes a putative chromatin regulator. Functional studies are needed to assess the relative contributions of each of these chromosome 9p genes to the pathogenesis of PMBL.[65] To identify oncogenes in this amplicon, an RNAi screen was performed targeting amplicon genes and identified JAK2 and the histone demethylase, JMJD2C, as essential genes.[67] Inhibition of JAK2 and JMJD2C cooperated in killing these lymphomas by decreasing tyrosine 41 phosphorylation and increasing lysine 9 trimethylation of histone H3, promoting heterochromatin formation. MYC, a major target of JAK2-mediated histone phosphorylation, was silenced after JAK2 and JMJD2C inhibition, with a corresponding increase in repressive chromatin. Thus, JAK2 and JMJD2C cooperative to remodel the PMBL epigenome, and provides rationale for developing JAK2 and JMJD2C inhibitors.

Treatment strategies in primary mediastinal B cell lymphoma

Currently, the treatment standard for PMBL entails immunochemothearpy such as R-CHOP followed by involved field radiation.[68] Unfortunately, the outcome is suboptimal due to treatment failure in approximately 25% of patients despite the recommended use of radiation, which has long-term side effects. This and other studies have suggested that more dose-intense regimens such as MACOP-B or VACOP-B yield a superior outcome compared to CHOP, raising a question of the optimal chemotherapy for PMBL.[68-72] Interestingly, the benefit of dose intensity in PMBL is supported by molecular evidence showing its close molecular relationship with nodular sclerosis Hodgkin's lymphoma, where the value of dose intense regimens such as escalated BEACOPP is well-demonstrated.[73] Based on such evidence that dose intensity is important in PMBL, Dunleavy *et al.* assessed DA-EPOCH-R, a dose intense regimen, without radiotherapy in PMBL.[74,75] In a recent update of 40 patients with untreated PMBL, the EFS and OS were 95% and 100%, respectively, at the median 4-year follow-up. Importantly, only two patients required consolidation radiation treatment and no patients have progressed (Dunleavy *et al*., 2011, Abstract submitted at the 11th International Conference on Malignant Lymphoma, Lugano, Switzerland). 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 PMBL. In this regard, RNAi screens have identified JAK2 as a potentially important target for PMBL. Mutations of JAK2 have been implicated in myeloproliferative disorders, and the selective JAK 1/2 inhibitor, INCB18424 from Incyte corporation, has shown significant activity in these diseases.[76,77] Presently trials are planned to assess inhibitors of the JAK pathway in DLBCL including PMBL, but no clinical data is available at this time.

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Today's treatment of diffuse large B cell lymphomas in adults

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Classification of DLBCL and prognostic stratification

Diffuse Large B cell Lymphomas (DLBCL) are the most common type of non-Hodgkin's lymphoma), with the incidence rising from 2 cases per 100,000 at 20-24 years of age to 112 cases per 100,000 by 80-84 years (Yancik and Ries 2004). In the 2008 WHO Classification of Haematological Malignancies, the category of DLBCL includes a number of disease variants/entities all characterized by being rapidly growing mature B cell tumors with large or relatively large cells (Table 1). With the exception of the Primary CNS DLBCL, all types of DLBCL are broadly treated in a similar way.

Immunophenotyping is an essential diagnostic procedure which allows DLBC lymphomas to be identified and allows DLBC lymphomas to be further divided into germinal centre (GC) type (CD10 + or CD10-, BCL6+ MUM1-) and non GC type (CD10-BCL6- or CD10 – BCL6+ MUM1+) (Hans *et al*. 2004). It had been shown that this GC/non -GC stratification provides valuable prognostic information, but the supporting data mainly related to the pre-rituximab era. The prognostic value of the so-called Hans algorithm is less clear in patients treated with immuno-chemotherapy as opposed to CHOP alone (Nyman *et al*. 2007). Prognostic discrimination can also be achieved with gene expression profiling (GEP) (Alizadeh *et al*. 2000, Rosenwald *et al*. 2002,Rosenwald *et al*. 2003) sub-dividing DLBC lymphoma into GC types, activated B cell (ABC) types and also Primary Mediastinal B cell Lymphoma (PMBL). The prognostic stratification between GC and ABC subtypes remains valid in patients receiving immunochemotherapy (Lenz *et al*. 2008). Further prognostic information can also be obtained by analysis of the reactive stromal signatures (Lenz *et al*. 2008). GEP is technically demanding, however, and robust kits have not entered routine use either for broad based diagnosis or DLBC sub categorisation. Recently a new immuno-histochemistry algorithm has been developed which places less weight on BCL6 staining to identify GC-like lymphomas, than in the Hans algorithm, and additionally uses Germinal Centre B cell Expression Transcript 1 (GCET1) for this purpose, and high level FOXP1 staining to assist in the identification of ABC lymphomas (Choi *et al*. 2009). Importantly, this algorithm has over 90% concordance with the classification derived from GEP.

The GC-like lymphomas probably arise from normal germinal centre B cells and are associated with the t(14;18) translocation, deletion of PTEN, amplification of the microRNA cluster miR-17-92, and p53 mutations (Lenz and Staudt 2010). The ABC Lymphomas are thought to originate from a post-germinal centre B cell and are characterized by activation of the NFkB and JAK kinase signalling pathways and a number of recurrent mutations in the B cell receptor (CD79 genes), CARD11, BCL10 and MALT1(CPM complex). A20, the negative regulator of NFkB signalling, has been identified, which gives rise to these signalling events. BCL2 is usually over-expressed and p16 is often deleted (Lenz and Staudt 2010). Recently it has also been shown that over 30% of ABC lymphomas have a mutation in the MYD88 gene which codes for an adaptor protein that mediates toll and IL-1 receptor signalling (Ngo *et al*. 2010). This also results in activation of NFkB and JAK kinase. The presence of NFkB activation in a subgroup of patients raises the possible value of using NFkB inhibitors in standard therapy and randomized trials are in progress. At the current time there is no evidence that this is beneficial and GEP and mutational screening thus remain experimental investigations.

Prognostic information can also be summarized from a number of clinical features including age, performance status, stage of the disease, number of extranodal disease sites and the LDH level. These parameters are used to form the International Prognostic Index (IPI) (1993) (Table 2), which identifies four risk groups: low, low intermediate, high intermediate and high. An age adjusted (AA) IPI is widely used for stratification and analysis of clinical trials. The data for the IPI again derives from the pre-rituximab era, and when immuno-chemotherapy is used as first line treatment, the IPI appears less discriminatory in some series (Sehn *et al*. 2007) but not in others (Ziepert *et al*. 2010). Sehn and colleagues (2007) suggested a modification to the distribution of the number of risk factors in the different risk categories, and reduced the number of risk groups to three: very good, good and poor. This index, they suggest, remains informative in the post rit-

Table 1. Diffuse Large B cell Lymphoma: variants, subgroups and subtypes, entities.

Diffuse Large B cell Lymphoma not otherwise specified
Diffuse Large B cell Lymphoma subtypes
T cell/ histiocyte rich large B cell lymphoma
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
EBV positive DLBCL of the elderly
Other lymphomas of large B cells
Primary mediastinal large B cell lymphoma
Intravascular large B cell lymphoma
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
ALK positive LBCL
Plasmablastic lymphoma
Large B cell lymphoma arising in HHV8-associated multicentric Castleman's disease
Primary effusion lymphoma
Borderline cases
B cell lymphoma, unclassifiable, with features intermediate between DLBC lymphoma and Burkitt lymphoma
B cell lymphoma, unclassifiable with features intermediate between DLBC lymphoma and classical Hodgkin's lymphoma

From WHO Classification of Tumors of Haematopoietic and Lymphoid tumors. Ed Swerdlow SH et al. Lyon 2008.

Table 2. Charlson Weighted Comorbidity Index (adapted from Charlson *et al.* 1987: J. Chronic Dis 40: 373-83).

Assigned weights for diseases	Condition
1.	Myocardial infarct Congestive heart failure Peripheral vascular disease Cerebro vascular disease Dementia Chronic pulmonary disease Corrective tissue disease Mild liver disease Diabetes without end organ damage
2.	Hemiplegia Moderate or severe renal disease Diabetes with end organ damage Any malignancy
3.	Moderate or severe liver disease
6.	Metastatic solid tumour

Total score obtained by adding up assigned weight for each co-morbidity present.

uximab era. In elderly patients, however, there are no very good risk patients, leaving only two risk categories. For this reason, Advani *et al*. (2010) have added a risk factor of age over 70 yrs in the elderly (over 60's) IPI.

The anatomical staging used in the IPI and its modifications is based on the Ann Arbor staging system, and this mandates the careful taking of the history and performance of a physical examination. It is frequently stated that this should include the examination of Waldeyer's ring, but this requires considerable expertise and is less necessary with modern imaging of the head and neck. The standard imaging procedure is a CT scan of neck, chest, abdomen and pelvis, and MRI scanning is mainly used to better define bony abnormalities or neurological lesions. Whole body PET scanning is now widely used during diagnosis, but this should not be considered as mandatory except perhaps in an apparently localized disease where a curtailed course of chemotherapy and radiotherapy is being considered. It can be argued that a PET scan is highly useful in assessing response to treatment at various stages of the disease and that a baseline investigation at diagnosis is valuable. However, DLBC lymphomas are nearly always PET positive, and the expense of the baseline scan is not readily justifiable. Furthermore, improved response identification is only mandatory if the knowledge obtained can be used to modify treatment and improve outcome. Such data is currently lacking. PET scanning at diagnosis may identify bone marrow deposits and obviate the need for a bone marrow biopsy, a potentially unpleasant procedure, but both false positives and false negatives occur (Carr *et al*. 1998).

Treatment

The modern treatment of DLBC lymphoma has been defined by four major advances in the last seventy years. First, in the 1940s, was the introduction of high voltage radiotherapy which resulted in cures in a small proportion of the patients with localized disease, and radiotherapy still has a role in localized disease. Given alone to Stage IA patients with non-bulky diffuse large cell lymphomas, the cure rate was about 50% with the majority of failures due to progression outside of the radiation field (Vaughan Hudson B *et al*. 1994). With modern imaging including PET scanning, those patients still defined as Stage 1A should therefore have much better results with RT alone, although studies have not been carried out to demonstrate this. Radiotherapy alone may have a role in the frail elderly patient with a localized disease if chemotherapy cannot be tolerated. Standard practice for Stage IA disease is to administer combined modality therapy with reduced duration chemotherapy (typically 3 or 4 courses of CHOP) followed by consolidation RT (Miller *et al*. 2000). The standard dose of RT was traditionally 35-45 Gy but lower doses of RT are probably sufficient (Hoskin *et al*. 2005). In some centers this type of strategy would also be applied to Stage IIA patients without risk factors, but the results appear to be less satisfactory. One study has suggested that radiotherapy is unnecessary, at least in older patients, even after only three cycles of CHOP (Bonnet *et al*. 2007), and a previous French trial suggested that a full course of ACVBP chemotherapy was superior to CHOPx3 followed by radiotherapy in younger patients (Reyes *et al*. 1993), Recent Phase II trials have reported encouraging results when rituximab was added to CHOP plus radiotherapy in localized disease (Persky *et al*. 2008) but whether this is better than CHOP plus rituximab alone without radiotherapy is not known. Radiotherapy may also have a place in the consolidation of initial bulk disease, and partial remissions

if the persisting disease is localized, but the limited data advocating such approaches predates the CT/PET scan era and there is now greater uncertainty. This is well illustrated by Primary Mediatsinal B cell Lymphoma, where the disease is often bulky at presentation and a post-treatment residuum is usual (Boleti and Johnson 2007). Whether or not to give consolidation in a PET negative patient with a residual mass remains controversial.

The next major advance was the development of the CHOP combination chemotherapy regimen (MeKelvey *et al*. 1976), which resulted in long term survivals in about 30% of stage III and IV histologically aggressive lymphomas. Further improvements in outcome occurred over the next two decades, which can be best ascribed to improvements in supportive care. This certainly included better antibiotics and the improved management of neutropenic sepsis, but probably equally important was the greater physician confidence allowing the delivery of more cycles of chemotherapy at full dose without delay. The development of G-CSF has probably contributed to the increase in physician confidence (see below), but in a trial carried out in the UK in the 1990s, the 5 year OS for Stage III/IV patients with an additional poor prognostic factor was nearly 50% following treatment with CHOP alone – about 15% better than 2 decades earlier. This was achieved without G-CSF prophylaxis (Linch *et al*. 2010).

The fourth advance was the addition of the CD20 monoclonal antibody rituximab to chemotherapy. In the seminal GELA LNH 98-5 randomized trial where rituximab 375 mg/m^2/IV was added (or not) to classical CHOP for eight cycles in elderly patients with DLBC, there was a significant improved in outcome associated with the use of rituximab (Coiffier *et al*. 2002). The 5 year PFS was 54% in the R-CHOP patients compared to 30% in CHOP patients (p = 0.00001). The corresponding 5 year OS rates were 58% and 45% respectively (p = 0.0073) (Feugier *et al*. 2005). The benefit was seen with both low and high risk disease as defined by the AA IPI. Broadly confirmatory results were seen in a US Intergroup trial (Habermann *et al*. 2006) and benefits were also demonstrated in younger patients with good prognosis in the MInt trial (Pfreundschuh *et al*. 2008). The value of rituximab was also shown when it was combined with time-intensifed R-CHOP14 (Sonneveld *et al*. 2006, Pfreundshuh *et al*. 2008,). There is no robust randomized trial data for the value of rituximab in younger patients with poor prognostic disease, and such trials are no longer feasible. Population based studies do not, however, suggest that the impact of rituximab will be lost in this group of patients (Sehn *et al*. 2005).

The optimal rituximab regimen has not been determined. The dose of 375 mg/m^2 is somewhat arbitrary and the three weekly frequencies of rituximab infusions in CHOP-R_{21} were designed on logistic rather than pharmacokinetic principles. Indeed Reiser *et al*. (2006) has shown that, even with a CHOP14 schedule, peak CD20 serum levels are not attained until after 5 cycles of therapy, and they are therefore testing a dose-dense rituximab regimen (Poeschel *et al*. 2006) which gives peak CD20 levels from the start of therapy. There is no evidence that maintenance rituximab is of value in DLBCL. In the US, Intergroup trial patients with DLBCL were treated with CHOP and a 2 x 2 randomization for rituximab induction, or not, with the CHOP chemotherapy and for rituximab maintenance therapy or not. Rituximab maintenance resulted in a significant improvement in those patients treated with CHOP alone, but not in those who received R-CHOP as induction therapy (Habermann *et al*. 2006).

Prior to the development of rituximab, research was focused on the addition of more drugs to the CHOP regimen and the shortening of the intervals between each cycle of therapy. The initial encouraging results with multi-agent regimens proved to be a false dawn (Fisher *et al*. 1993), although several of the so-called third generation regimens probably did not deliver higher dose intensity, and in order to give more drugs, the dose of the most efficacious agents was reduced in some regimens. The anthracycline dose is lower in some of the equally effective weekly regimens such as PMitCEBO (Burton *et al*. 2006), than in a full course of CHOP, and might still warrant consideration in some frail patients, where close monitoring is required. The German high grade lymphoma group added etoposide to CHOP and found this to be beneficial in younger patients with good prognosis disease (Pfreundschuh *et al*. 2004a). In older patients of both good and poor prognostic risk, they found that shortening the interval between cycles of CHOP from 21 to 14 days with mandatory G-CSF resulted in improved outcome (Pfreundschuh*, et al*. 2004b). The HOVON group also compared standard CHOP-21 with an intensified 2-weekly CHOP regimen (CHOP-I) in patients with aggressive lymphoma up to the age of 65 years, and in this trial, the minor advantage seen for CHOP-I was not significant (Verdonck *et al*. 2007). Several studies have subsequently compared CHOP14 plus rituximab with CHOP21 plus rituximab, and the early reports suggest that the advantage of the time intensification is no longer maintained (Cunningham *et al*. 2010). In France, a randomized trial also showed an advantage for a more intensive 5 drug regimen (ACVBP), compared to CHOP, in patients with poor-risk aggressive lymphoma between the ages of 61 and 69 years (Tilly *et al*. 2003). The CR rate was similar (58%vs 56%), and despite more treatment related deaths in the ACVBP arm, there was improved EFS (39% vs 29% p=0.005) and OS (46% vs 38% p=0.036). GELA are currently comparing CHOP-R with ACVBP-R in a younger patient population.

Central nervous system CNS prophylaxis

The incidence of CNS progression or relapse in DLBCL is about 5% in most series (Macmillan 2005) and although the seminal trial of R-CHOP did not show a reduction in CNS relapse (Feugier *et al*. 2004), a reduction was apparent in the RICOVER-60 trial (Boehme *et al*. 2009). An analysis of the British Columbia population-based registry suggested a similar reduction in CNS progression or relapse (Villa *et al*. 2010). It is a commonly held belief that if the risk of CNS relapse is sufficiently low, CNS prophylaxis is not justified in all patients, and much attention has been placed on the identification of risk factors for secondary CNS disease (Macmillan A 2005). The risk factors for CNS progres-

sion/relapse are similar to those in the IPI (eg advanced stage, more than one extranodal site and a raised LDH level) and some groups use the IPI to determine who should receive CNS prophylaxis, restricting prophylaxis to high/intermediate and high risk disease. A number of other anatomical sites have been identified as risk factors which include testis, paranasal sinuses, the epidural space and possibly the breast. Hegde and colleagues (2005) have suggested that flow cytometry of the cerebro-spinal fluid (CSF) may enable improved risk stratification. They used sensitive multicolour flow cytometry to detect light chain restricted B cell clones in 51 newly diagnosed patients at risk of CNS disease. One had lymphoma cells detected by standard cytomorphology and a further 10 had small lymphoma clones only detected by flow cytometry. A Spanish co-operative group have recently reported their experience of flow cytometric assessment of the CSF in 67 patients with DLBCL at high risk of CNS disease (Sancho *et al*. 2010). Of the 67 patients, 56 (84%) had negative CSFs by both morphology and flow cytometry, one patient had CNS lymphoma detected by both cytomorphology and flow cytometry, and 10 patients had occult lymphoma in the CSF only detected by flow cytometry.. The most frequently used prophylaxis is intra-thecal methotrexate or cytosine arabinoside, but this is not ideal. Apart from the fact that up to a half of CNS progressions/relapses occur in the context of widespread disseminated disease, CNS relapse is frequently parenchymal and not lepto-meningeal. If intrathecal cytotoxics are to be given, however, the possible use of liposomal ara-C, which has a prolonged half life in the CSF (Glantz *et al*. 1999) and will allow reduced numbers of lumbar punctures, is an attractive option but has not been rigorously tested in randomized trials. In the ABCVP vs CHOP trial mentioned above (Tilly *et al*. 2003) there were significantly fewer isolated CNS relapses in the ACVBP arm with an incidence of only 2.2% with ACVBP compared to 5.8% with CHOP. It should be emphasised that in the ABCVP arm there are not only 4 intrathecal injections of methotrexate but there are also 2 intravenous high dose methotrexate infusions. It is likely that the intravenous methotrexate is key to the low CNS relapse rate. Clearly randomized trials of CNS prophylaxis are necessary but very large trials are required to demonstrate a significant effect on CNS relapse rate.

Transplantation as a component of initial therapy

A number of studies have explored the value of high dose therapy and autologous stem cell transplantation for patients achieving either PR or a CR after initial therapy. The results have been conflicting and a series of meta-analyses concluded that there was no benefit (Simnet *et al*. 2000, Strehl *et al*. 2003, Greb *et al*. 2008). There were three trials, however, that reported a benefit following an autograft (Haioun *et al*. 2000, Milpied *et al*. 2004, Gianni *et al*. 1997), and although this may represent the random chance, it is still possible that differences in the protocols accounted for the favorable results. In both the Haioun and the Milpied study it is noteworthy that an intensified CHOP regimen had been used initially and nearly all the patients were in CR at the time of transplantation. Even if there was a real benefit from consolidation high dose therapy in this situation, it does not mean that this still pertains in the rituximab era, and further trials would be needed. If high dose therapy is of greatest benefit in patients already in CR, then the use of rituximab in induction might increase the proportion of patients in whom high dose therapy would be beneficial, but with the improved results from rituximab, it can be argued that there is less need for an intensive consolidation procedure and it will be more difficult to demonstrate any superiority associated with the high dose therapy. There is currently, therefore, little enthusiasm for autologous transplantation in DLBCL as a component of initial therapy. Similarly there is no role for allogeneic transplantation in first remission.

Treatment of relapse

In those patients either failing to achieve CR or relapsing from CR, who are young and fit enough to receive high dose therapy, the aim must be to induce a remission with second line standard dose regimens and then to proceed to a high dose therapy procedure. In the PARMA trial (Philip T *et al*. 1995), the event-free survival at 5 years after an autograft was 46% compared to 12% in the non-transplanted patients, and the respective overall survivals were 53% and 32%. A large number of second-line regimens have been developed but here has only been one large randomized trial comparing such regimens in DLBCL (Gisselbrecht *et al*. 2010). This showed that the efficacy of R-ICE and R-DHAP were broadly similar. It is standard practice to add rituximab to the second line regimen but whether this is appropriate if the patient has failed while, or soon after, receiving rituximab is debatable. A number of studies showed that it was only advisable to proceed to an autograft if the patient had responded to initial salvage therapy with response being defined by clinical and CT critieria (Philip *et al*. 1987, Gribben *et al*. 1989). With CT/PET scanning now widely available, it appears that autografts are of major benefit only in those patients with no metabolically active disease (Spaepen *et al*. 2003), and further attempts with standard dose therapy should be made to achieve such a state before proceeding to an autograft. The patients failing to achieve a metabolic CR after initial salvage therapy clearly represent a poor prognostic group, and there is enthusiasm for considering these patients for reduced intensity allografts. One study has suggested that the allograft procedure overcomes the poor prognosis associated with a persistently positive PET scan (Lambert *et al*. 2010), but this requires confirmation. It is clear that patients who have received rituximab as part of initial therapy fare less well when they fail that therapy than the group of patients who failed non-rituximab containing regimens (Gisselbrecht *et al*. 2010). This is largely because they have lesser responses to the second line standard dose chemotherapy, and there is no evidence that the outcome of autografting is worse in those who still respond adequately to the second line therapy. Currently the major role of reduced intensity allogeneic transplantation (RIT) is in

those patients who have failed an autograft or in whom an autograft is not possible, and the results from some centers are encouraging. Thomson *et al*. (2009) reported on the use of RIT in 48 consecutive patients with DLBCL (18 transformed from follicular lymphoma), 69% of whom had failed a previous autograft. The overall survival at 4 years was 47%. Less favorable results have been reported from some other centers, and stringency of patient selection is likely to be a major reason for such discrepancies.

The anthracycline problem

It is well established that the total cumulative dose of doxorubicin is the major risk factor for doxorubicin related congestive heart failure (CHF) (Von Hoff, *et al*. 1979), and an upper cumulative limit of 450 mg/m^2 is usually employed. Even at this total dose, cardiac function is compromised in some patients. The risk of CHF increases with age, a history of coronary artery disease, valvular heart disease, diabetes, cigarette smoking, obesity and particularly hypertension (He, *et al*. 2001, Hershman, *et al*. 2008). It is essential that hypertension is well controlled before and during anthracycline therapy. Attempts have been made for over 30 years to develop novel anthracyclines, or derivatives thereof, which have an improved therapeutic window. Epirubicin can be used at cumulative doses nearly double that of doxorubicin without increased cardiotoxicity (Minotti *et al*. 2004), but it has mostly been used at doses considerably lower than two-fold that of doxorubicin. This undoubtedly results in less cardiotoxicity (Smith *et al*. 2010), but uncertainty still remains about its efficacy at those doses. Zinzani and colleagues (1995) substituted doxorubicin 50 mg/m^2 with Idarubicin 10 mg/m^2 in the CHOP regimen and found equal efficacy of CHOP and CIOP with less cardiotoxicity in the Idarubicin containing arm. However when this regimen was tested in a UK trial, CIOP was found to be significantly less efficacious than standard CHOP (Burton *et al*. 2005), and a subsequent study by Trumper *et al*. (2002) suggested that the equivalent dose of Idarubicin, to 50 mg/m^2 of doxorubicin was 14 mg/m^2, at least in terms of myelosuppression. Pixantrone is an aza-anthracenidione structurally similar to mitoxantrone and is the latest anthracycline-like agent to be developed with the aim of minimizing cardiotoxicity without reducing efficacy. Some of the early phase results are encouraging but much larger and more robust trials are still needed (Mukherji and Pettengell 2010).

An alternative strategy is to use doxorubicin incorporated into liposomes. A Cochrane meta-analysis suggested that the liposomal form had similar oncological activity to doxorubicin with a lower rate of clinical and sub-clinical heart failure (van Dalen *et al*. 2008). There have been no phase III trials in lymphoma and liposomal doxorubicin is not licensed for this purpose. There have, however, been some encouraging early phase trials replacing standard adriamycin with a liposomal form in the CHOP regimen (Tsavaris *et al*. 2002, Visani and Isidori 2009) and further studies are clearly justified.

Dexrazoxane is an iron chelator which inhibits hydroxyl radical formation and decreases anthracycline-induced oxidative stress, which is thought to be the major cause of cardiac damage. The Cochrane meta-analysis (van Dalen *et al*. 2008) and a more recent systematic review (Smith *et al*. 2010) indicate that dexaroxane significantly reduces the risk of congestive heart failure associated with anthracycline use. There is still concern however, that the generation of ROS could play a part in anti-tumor activity (Swain and Vici 2004) and there has been reluctance to recommend its use in patients with potentially curable lymphomas. Currently, doxorubicin without a cardio-protectant remains the anthracycline of choice.

Use of G-CSF

G-CSF prophylaxis following chemotherapy reduces the incidence and duration of severe neutropenia and is associated with a reduction in infective episodes. The strongest predictor for haematoxicity is febrile neutropenia in a previous cycle of therapy, but as the greatest risk of infection is with the first cycle of therapy (Lyman and Delgado 2003), G-CSF should be given from the first cycle of chemotherapy. A systematic review and meta-analysis of randomized trials of G-CSF prophylaxis in patients with a variety of different cancers showed a significantly lower early mortality associated with G-CSF use (Kuderer *et al*. 2007) but not a significantly different overall survival. The rationale for G-CSF use has been largely based on pharmaco-economic considerations. Initial cost-minimization assays suggested that G-CSF should be used with any regimen where the risk of febrile neutropenia exceeded 40% (Lyman *et al*. 1993), but when certain indirect costs were taken into account, this threshold was brought down to 20% (Lyman *et al*. 1998). In an analysis of 1246 lymphoma patients treated with CHOP, R-CHOP or CNOP, without early G-CSF, 217 (17%) developed febrile neutropenia, below the 20% threshold (Lyman and Delgado 2003). In a prospective observational study of CHOP recipients (Pettengell*, et al*. 2008), the incidence of febrile neutropenia was 22%. It this seems that CHOP is a marginal regimen form the viewpoint of G-CSF prophylaxis and it should only be used when there is an additional factor for development of febrile neutropenia such as advanced age. Consideration should also be given to the fact that in Europe the cost of G-CSF has plummeted in recent years and a threshold level below the 20% rule may now be appropriate. Pegylated G-CSF, with a prolonged half-life, is an attractive and effective option, but the pharmaco-economic arguments are no longer so compelling.

Treatment of the elderly patient with DLBCL

A recent analysis of cancer registries revealed that the long-term survival of patients with NHL is improving, but for elderly patients the survival in Europe has lagged behind that in the USA (van de Schans *et al*. 2010). There are many possible reasons for this, but one possibility is that it reflects subtle differences in physician attitudes toward the elderly and differences in the expectations of the elderly patients. Every effort must be made to deliver

intensive therapy with curative intent to those elderly patients who can tolerate such therapy, but such a strategy demands greater attention to the evaluation of each individual patient. This must not only happen before chemotherapy starts, but also before each cycle of therapy, with attention to control of hypertension and other concomitant disease, and detection of the signs and symptoms of incipient heart failure or neuropathy. Interim echocardiography should also be performed.

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The biology of myelodysplastic syndromes

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A B S T R A C T

The myelodysplastic syndromes (MDS) are a heterogeneous collection of hematopoietic stem/progenitor malignancies characterized by ineffective hematopoiesis in one or more lineages of the bone marrow, associated with dysplastic changes in fully differentiated cells or their precursors. Diagnosis is dependent on the clinical identification of one or more cytopenias for greater than 6 months associated with dysplasia in one or more of the major myeloid cell lines. The ineffective hematopoiesis is thought to be in part due to an increase in apoptosis of marrow cells balanced by a parallel increase in proliferation. Inhibition of apoptosis is associated with transformation to more aggressive disease. Here we provide an overview of recent advances in the molecular mechanisms of MDS pathogenesis.

Introduction

The myelodysplastic syndromes (MDS) arise in a primitive $CD34^+$ hematopoietic stem/progenitor cell (HSPC), and are among the most common hematopoietic malignancies. MDS is characterized by ineffective hematopoiesis in one or more lineages of the bone marrow, associated with dysplastic changes in fully differentiated cells or their precursors.[1] The median age of diagnosis is over 70 years, although it appears that there is a long lag before patients present to the clinic.[2] There is male predominance of patients with MDS, with a male to female ratio of approximately 1.8.[1] Risk factors include prior chemotherapy or radiation therapy, smoking, and occupational exposure to benzene and agrochemicals. In approximately 20 to 40% of patients, the disorder progresses to acute myelogenous leukemia (AML), while a larger proportion succumb to the consequences of marrow failure.[3,4] The paradoxical finding of normal or increased cellularity in the marrow with cytopenias in the peripheral blood has been attributed to the increased proliferation of hematopoietic cells that is counterbalanced by a simultaneous increase in apoptosis.[5,6]

Cytopenia(s) associated with dysplasia of at least 10% of a specific myeloid lineage, and exclusion of other causes of cytopenia are required for the diagnosis of MDS.[7,8] Given the requirement for exclusion of all other potential causes of cytopenia, and that dysplasia requires a subjective morphological assessment, diagnosis of MDS – in particular lower-risk subtypes – has been challenging.[7,8] Not surprisingly, this difficulty in diagnosis has left understanding of disease pathogenesis murky. It has only been in recent years that a greater understanding of the biology has led to the generation of *in vivo* disease models and the elucidation of how genetic and epigenetic changes lead to the cellular defects found in MDS.

Classification and prognosis of myelodysplastic syndromes

MDS is a remarkably heterogeneous disease and the diagnosis is dependent on the clinical identification of one or more cytopenias for greater than 6 months associated with dysplasia in one or more of the major myeloid cell lines. The revised WHO classification introduced in 2008 separates the myelodysplastic syndromes into seven major classes depending on the number of cytopenias, number of lines affected by dysplasia, blast count, and the proportion of ring sideroblasts.[8] These classes include Refractory cytopenias with unlineage dysplasia (RCUD), Refractory anemia with ring sideroblasts (RARS), Refractory cytopenias with multilineage dysplasia (RCMD), Refractory anemia with excess blasts (RAEB-1, RAEB-2), MDS-unclassified (MDS-U), and MDS associated with isolated del(5q) (previously called 5q- syndrome). In addition, a number of clonal myeloid neoplasms share features of MDS and myeloproliferative neoplasms (MPN), so called overlap syndromes, referred to as MDS/MPN.

Although, the morphologic classification is able to distinguish patients based on their outcome, a simpler clinical scoring system based on the number of cytopenias, blast counts, and cytogenetic abnormalities was initially developed in 1997.[9] The International Prognostic Scoring System (IPSS), although somewhat dated, has proved to have clinical utility in prognostication, but is limited to classifying patients at diagnosis.[9] More recently a WHO classification-based Prognostic Scoring System (WPSS), which stratifies patients into five risk categories,

includes transfusion requirements, as well as cytogenetic findings. The WPSS has the added utility of being applicable at any time during the disease process.[10] Finally, marrow fibrosis and CD34+ cell clusters have also been shown to be adverse prognostic factors.[11]

Cellular defects in myelodysplastic syndromes

MDS arises in a hematopoietic stem/progenitor cell, but the cellular manifestations are protean and depend on the specific molecular alterations that are present. Although, various immune and stromal defects have been described in MDS, all of these may be explained by molecular alterations in a stem or early progenitor cell, either through cell autonomous or non-autonomous mechanisms (Figure 1).[12,13] Despite the fact that MDS manifests as a myeloid malignancy with transformation almost exclusively presenting as a myeloid leukemia, rare cases of acute lymphoblastic leukemias have been reported.[14] The MDS clone has also been shown to involve B cells, NK cells, dendritic cells, and perhaps even T cells.[14-16] Thus, the immune alternations associated with MDS are likely secondary to the stem cell defect rather than being a primary etiologic event. Similarly various stromal elements may derive from the MDS clone, including macrophages and osteoclasts, which may then secrete factors inhibitory to stem cell maintenance or progenitor maturation. As well, various factors released by malignant stem/progenitor cells or their progeny may also explain the increased angiogenesis described in MDS marrows.[17] The crosstalk between the various malignant and normal cellular elements and factors released by these cells make elucidation of the initiating aberrant signals difficult to tease out. Nevertheless, TNF family members and IL-32 have been implicated in the increased apoptosis that is seen in low-risk MDS.[6,18] How marrow cellularity is maintained in the face of increased apoptosis remains a conundrum, but increased proliferation has been suggested to balance out the increase in apoptosis.[6] Alternatively, it may be that the increased apoptosis is seen mainly in the normal hematopoietic elements as a result of the cytokine imbalance.[19] As the molecular defects in MDS are revealed, a better understanding of the biology of the disease will follow.

Cytogenetic abnormalities in myelodysplastic syndromes

Given that the diagnosis requires the exclusion of other hematologic and non-hematologic causes cytopenia, the diagnosis of MDS can be arguably the most difficult of the hematologic malignancies. Standard karyotyping and occasionally fluorescence *in situ* hybridization (FISH) are the only routinely used clinical genetic tests in most settings, and in cases that demonstrate karyotypic abnormalities, the ability to accurately diagnose MDS becomes much more robust.

Deletions and amplifications

In a study comprising over 2,000 patients, 52% showed clonal cytogenetic abnormalities.[20] Karyotypic anomalies in MDS are characterized by large deletions and less commonly amplifications (Figure 2). Interstitial deletion of the long arm of chromosome 5 (del(5q) is the most common abnormality, either in isolation (about half the time) or in concert with other cytogenetic aberrations, and is seen in 30% of patients whose cells harbor a cytogenetic abnormality. In order of descending frequency, -7/del(7q) was observed in 21% of abnormal karyotypes, +8 (16%), -18/18q- (7%), 20q- (7%), -5 (6%), -Y (5%), -17/17p-/iso(17q) (5%), +Mar (5%), +21 (4%), in this large European study.[20] Interestingly, deletions of chromosomes 18/18q, 5, 13/13q, and 21 were rarely seen in isolation.

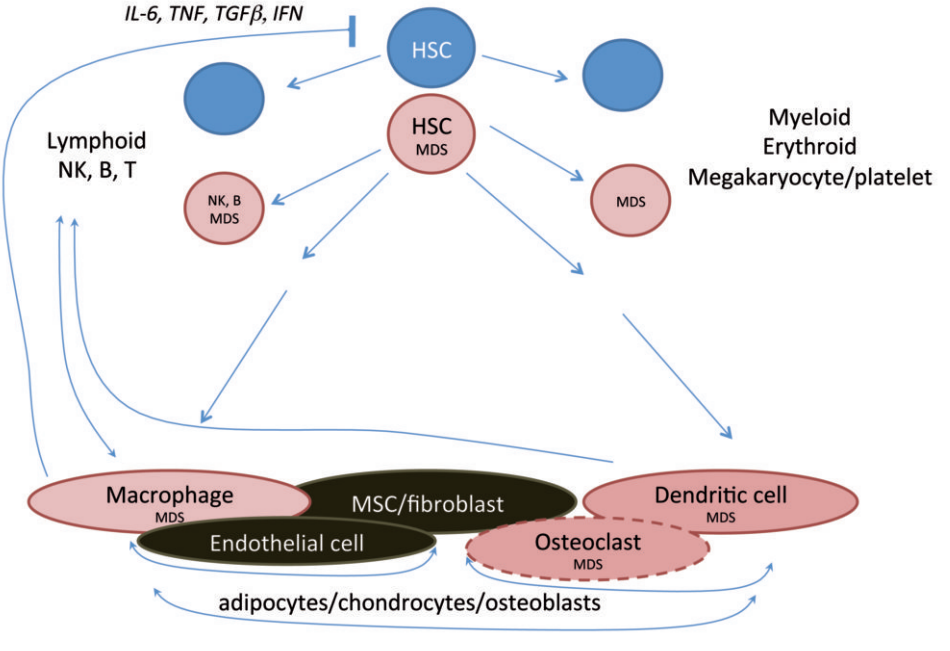

Figure 1. Schema showing the maturation of hematopoietic stem cells (HSC) from normal (blue) and MDS (pink) populations, and the resulting complex cellular interactions and cytokine responses between stroma, immune cells, and myeloid elements.

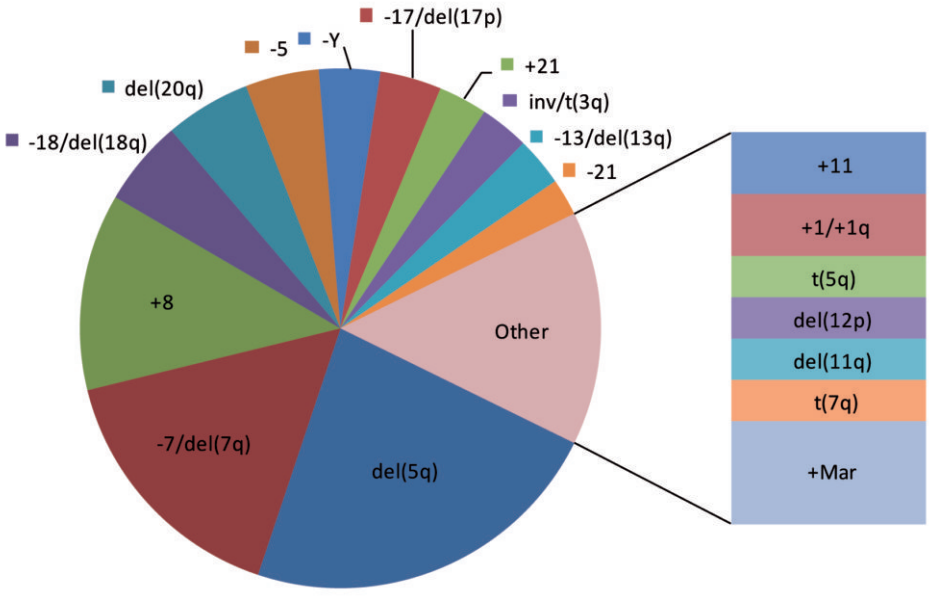

Figure 2. Relative proportions of the different cytogenetic aberrations seen in MDS.[20] This figure only represents the subset of patients (approximately half) that show karyotypic anomalies.

Balanced translocations

In contrast to AML, balanced translocations are seen infrequently in MDS. The most common translocations involve the EVI1 locus at chromosome band 3q26 and result either in an EVI1 chimeric transcript with TEL or AML1, or overexpression of intact EVI1.[21,22] This locus codes for at least three isoforms. The full-length MDS/EVI1 protein has at least partially opposing function to the major EVI-1 isoform.[21,22] Patients whose marrow cells overexpress EVI1 often show marked marrow hyperplasia, dysplastic megakaryocytes, and elevated platelet counts.[21,22] EVI1 is a transcriptional regulator that plays an important role in the proliferation of hematopoietic stem cells, and overexpression in AML may be an independent negative prognosticator.[21,22] Enforced overexpression of EVI1 in mouse marrow cells results in death due to pancytopenia in 10 to 13 months.[23] EVI1 interacts with histone deacetylases (HDACs) and histone methyltransferases, as well as DNA methyltransferases (DNMTs), and increased expression of EVI1 in AML blasts is associated with gene repression through hypermethylation of specific promoters.[24] Two subclusters with increased EVI1 expression have been reported in AML, one of which is associated with rearrangement of band 3q26 and with monosomy 7.[24] Less frequently, translocations involving RUNX1, NUP98, TEL, MEL1, and IER3 are seen in MDS.[25]

Copy number alterations and copy neutral loss of heterozygosity LOH

Recent application of genome-wide technologies, such as array comparative genomic hybridization (aCGH) and single nucleotide polymorphism arrays (SNP-A), have revealed smaller deletions and amplifications of chromosomes not detectable by standard karyotyping.[26-28] Early data suggest that incorporation of these aberrations into clinical algorithms allows better risk stratification than the IPSS score alone, and novel recurrent alterations may provide further insight into the pathogenesis of MDS karyotyping.[26-28] In addition to copy number alterations, SNP-A can also detect copy neutral LOH, also referred to as uniparental disomy, which result from loss of one chromosome or chromosomal region with concomitant duplication of the retained allele. Copy neutral LOH is common in myeloid malignancies.[29] First identified as a mechanism of JAK2V617F homozygosity in myeloproliferative neoplasms, additional homozygous mutations due to copy neutral LOH, for example, *TET2*, *EZH2*, *CBL*, have been identified in MDS.[29]

Molecular alterations associated with large chromosomal aberrations

Identifying the causal genes involved in the pathogenesis of MDS when whole or large tracts of chromosomes are deleted is difficult. However, the task has been assisted both by application of SNP-A, as well as the painstaking characterization – over many years by several investigators – of the proximal and distal breakpoints from groups of patients to define commonly deleted regions (CDRs).

Del(5q)

Perhaps the greatest understanding of MDS has come from the study of the most common genetic anomaly observed in the disease, del(5q).[20] 5q- syndrome, or MDS with isolated del(5q), is characterized by macrocytic anemia, variable neutropenia, normal or elevated platelet counts with dysplastic hypolobated megakaryocytes, and low propensity to develop AML.[30] Two distinct CDRs have been defined on chromosome arm 5q: one at 5q31 and the other at 5q32-33.[31,32] The distal 1.5 Mb CDR is associated with the classic 5q- syndrome, that is, macrocytic anemia, variable neutropenia, and elevated platelet counts associated with dysplastic hypolobulated megakaryocytes.[31] This region encompass-

es 40 protein coding genes and 4 known microRNAs (miR-584, miR-143, miR-145, and miR-378).[31,33] All 40 coding genes have been sequenced and point mutations have not been found in the retained allele, indicating that haploinsufficiency of one or more of these genes within the CDR is responsible for the disease phenotype.[25] Functional studies using an RNA interference screen identified one gene on band 5q32 in the CDR, ribosomal protein S14 (*RPS14*), as a critical haploinsufficiency gene responsible for the erythroid failure.[34] *RPS14* encodes a structural protein of the 40S ribosomal subunit and its deficiency can cause defects in ribosomal biogenesis and translation. Reduced expression of *RPS14* in hematopoietic stem/progenitor cells appears to lead to erythroid cell apoptosis and macrocytosis, consistent with a 5q- syndrome phenotype. This phenotype was rescued *in vitro* by forced expression of *RPS14* in hematopoietic stem/progenitor cells from 5q- syndrome patients.[34] Haploinsufficiency of *RPS14* in mice results in macrocytic anemia and dyserythropoiesis.[35] Interestingly, multiple ribosomal genes are downregulated in $CD34^+$ cells of patients with del(5q) MDS, which is consistent with the impaired erythropoiesis being a result of a ribosomal processing defect.[36] The resulting ribosomal stress activates the p53 pathway in the erythroid progenitors, resulting in cell cycle arrest or apoptosis.[37] Consistent with this finding, crossing mice hemizygous for *RPS14* with p53-deficient mice rescues the progenitor cell defect.[35]

However, *RPS14* haploinsufficiency alone does not explain the megakaryocytic dysplasia and the tendency to thrombocytosis, nor the clonal dominance of del(5q) MDS cells. Examination of non-coding genes at 5q31-5q35 revealed reduced expression of miR-145 and miR-146a in marrow cells from patients with del(5q) MDS.[33] Depletion of these two microRNAs (miRNA) in mice results in variable neutropenia, thrombocytosis, and hypolobated megakaryocytes, with reduced endomitosis in the marrow. Mice transplanted with marrow depleted for miR-145 and miR-146 succumb to a myeloproliferative/leukemic disorder.[38] These two miRNAs target genes involved in the innate immune response pathway, including TIRAP (miR-145) and TRAF6 (miR-146a). Transplantation of TRAF6-transduced bone marrow into wild type mice recapitulated the hematologic phenotype seen with depletion of miR-145/miR-146a, including progression to AML or bone marrow failure, suggesting that ectopic activation of innate immune signaling in the hematopoietic stem/progenitor population is a pathogenic feature of del(5q) MDS.[33]

Depletion of miR-145/miR-146a with activation of innate immune signaling results in NF-κB the activation and upregulation of IL-6, also seen in patients with del(5q) MDS. The platelet and granulocytic defects driven by TRAF6-mediated activation of innate immune signaling and NF-κB are abrogated in mouse marrow cells lacking IL-6, but a similar proportion of mice still develop myeloid neoplasia.[33] Thus, while the paracrine effects of IL-6 likely explain the thrombocytosis and neutropenia, clonal dominance of the MDS cells in the marrow appears to be secondary to cell autonomous effects of miR-145/miR-146a haploinsufficiency and deregulated immune signaling.

Although combined haploinsufficiency of *RPS14*, miR-145, and miR-146 can potentially explain the 5q- syndrome phenotype (Figure 3), other genes on chromo-

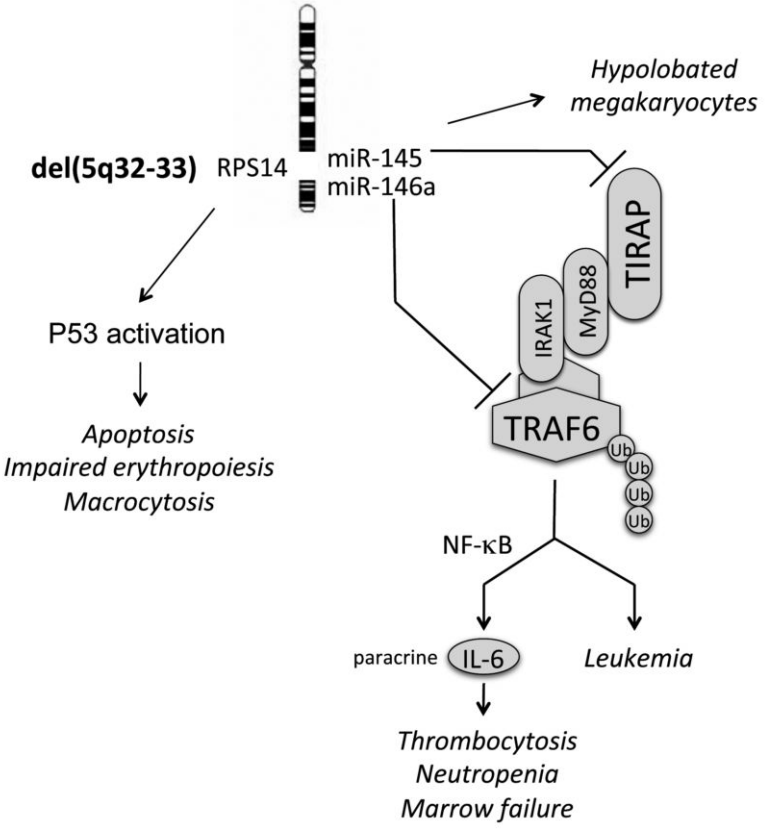

Figure 3. A model depicting how haploinsufficiency of coding and noncoding genes account for the 5q- syndrome phenotype.

some arm 5q have also been implicated in the pathogenesis of del(5q) MDS. These include the tumor suppressor gene SPARC located within the CDR,[39] and others that are located outside band 5q32-33 CDR associated with MDS, including EGR1, CTNNA1, APC, and NPM1.[25]

Recently, the immunomodulatory drug lenalidomide, a structural analogue of thalidomide, has shown high efficacy in del(5q) patients.[40] However, the precise mechanism of action and molecular targets of lenalidomide that account for its selective activity in this subtype of MDS remain largely unknown. Two phosphatase genes, *CDC25C* and *PP2A*, are located on chromosome 5q and have been shown to be inhibited by lenalidomide, and suppression of their expression by shRNA recapitulates the observed susceptibility to lenalidomide seen in del(5q) MDS.[41] Of the 40 coding genes located in the distal CDR, only *SPARC* expression is induced by lenalidomide.[39] In addition, emerging data indicates that lenalidomide increases miR-143 and miR-145 expression in $CD34^+$ del(5q) progenitors, and this induction may be associated with subsequent clinical response to treatment.[42] It remains to be resolved which mechanisms are most important for the effects observed in patients with del(5q) MDS.

Del(7q)

In contrast to the favorable prognosis associated with isolated del(5q), patients with del(7q), copy neutral LOH of 7q, or monosomy 7 have a worse outcome, although recent studies reveal that these patients respond well to demethylating agents, such as 5-azacytidine.[43] At least three regions that appear to define CDRs exist at bands 7q22, 7q31, and 7q36.[44-47] Recent investigations following up on patients with copy neutral LOH or microdeletions at band 7q36 have identified EZH2 as a gene that is mutated in approximately 6 to 7% of patients with MDS, MDS/MPN, or MPN.[48,49] In contrast to what has been described for germinal centre diffuse large B cell lymphomas where activating mutations at Tyr631 are usual, EZH2 mutations found in myeloid malignancies appear to represent loss of function mutations arguing for a tumor suppressive role of EZH2 in myeloid malignancy.[48-51] Although preliminary, it appears that MDS patients with EZH2 mutations have a worse outcome compared with those without EZH2 mutations independent of cytogenetic abnormalities of chromosome 7.[48,49] EZH2 protein was not detectable using cells from the whole marrow of MDS patients, suggesting either an overall low level of expression in primary cells, or perhaps that expression of EZH2 is limited to an underrepresented cell fraction.[48] Interestingly, *EZH2* is frequently targeted at both alleles through copy neutral LOH or microdeletion of one allele in myeloid malignancy, predicting loss of function.[48,49] However, the second allele is only rarely affected in del(7q) or monosomy 7 suggesting additional driver mutations.[48,49] Despite intensive efforts, molecular analysis and *in vivo* models of the other CDRs on chromosome 7q have not uncovered recurrent loss of function mutations.[52] EZH2 functions as a histone H3 lysine 27 (H3K27) methyltransferase, and is associated with transcriptional repression (Figure 4, and see below).[53,54]

Del(20q)

Isolated loss of chromosome arm 20q is associated with good prognosis, but as a sole cytogenetic anomaly is not considered definitive evidence for MDS in the absence of morphologic criteria.[8] A CDR containing 19 genes has been defined, but none have been found to be recurrently mutated.[55] Although the ASXL1 gene has been found to be mutated in MDS, the locus at band 20q11 is centromeric to the CDR.[56,57]

Trisomy 8

Similar to del(20q), isolated trisomy 8 is not considered definitive evidence of MDS as a proportion of cases are due to constitutional trisomy 8 mosaicism.[8]

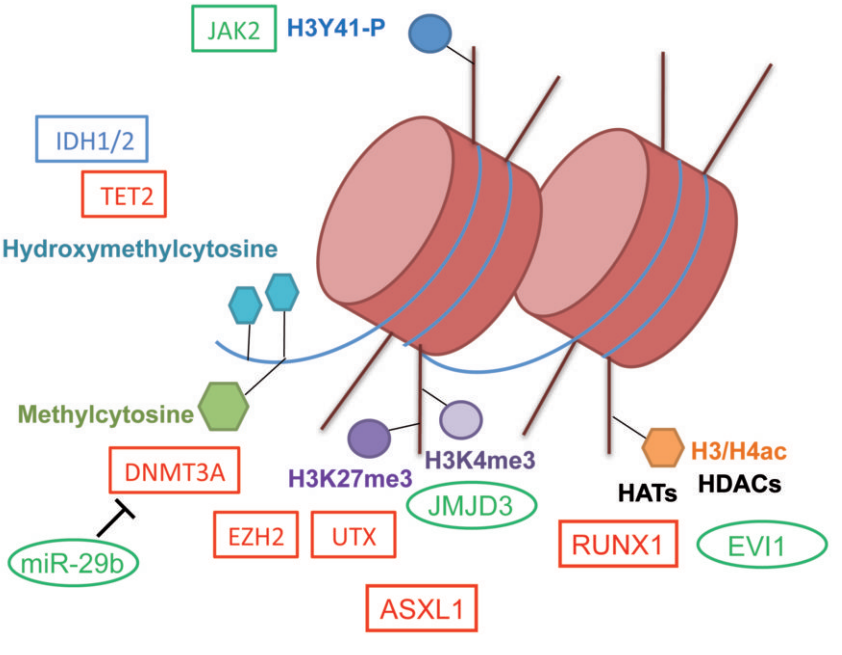

Figure 4. Cartoon showing how various mutations or differential expression can affect chromatin remodeling in MDS. Proteins shown in green represent activating mutations, or are overexpressed in MDS. Molecules in red represent mutations that inhibit function, or show reduced expression in MDS. Blue represents a mutation that alters enzymatic function.

Patients with an isolated trisomy 8 are placed in the intermediate prognostic risk group.[20] A proportion of trisomy 8 patients respond well to immune suppression, although the +8 clone often persists and may actually expand.[13] It has been suggested that the +8 clone is resistant to killing by cytotoxic T cells, and that immune suppression then allows the clone to expand at the expense of normal hematopoietic elements.[13]

Epigenetic changes in myelodysplastic syndromes

Epigenetics is the study of persistent changes in phenotype through mechanisms that do not involve a change in the DNA sequence.[58] Epigenetic changes allow expression patterns to be preserved when cells divide. One of the major ways that cell phenotype is maintained is through the remodeling of chromatin. The mechanisms of chromatin remodeling are complex and dynamic, requiring interactions between transcription factors, noncoding RNAs, and DNA, and histone modifying enzymes.[59-61] However, as a framework, chromatin remodeling can be thought to be accomplished through two main mechanisms: 1. Post-translational modifications of the tails of histone proteins, which changes the physical structure of chromatin. These modifications can act to either compact the DNA around the histones, such that chromatin is in a closed confirmation and transcription is repressed (e.g., H3K27me3, H3K9me3, H3/H4 deacetylation), or to open chromatin to permit transcriptional activation (e.g., H3K4me3, H3K36me3, H3/H4 acetylation); 2. DNA methylation that converts cytosine to 5-methylcytosine usually at CpG sites through DNA methyltransferases (DNMTs).[59-61] Highly methylated regions, particularly at CpG islands residing at promoters, tend to be less transcriptionally active.[58] DNMT1 has preference for hemimethylated DNA and thus is important for maintaining methylation patterns following cell division.[62] DNMT3s, in contrast, have *de novo* methyltransferase activity, and appear to have preferred target CpG sites.[62]

In cancers, there is commonly global hypomethylation of DNA with concomitant hypermethylation at specific sites, usually CpG islands of specific promoters.[63] The hypermethylated genes include the promoters of several bona fide or putative tumor suppressor genes, including CDKN2B, FHIT, and HIC.[64] In MDS and secondary AML, aberrant DNA methylation involving specific chromosomal regions and thousands of genes has been described.[24,65,66] Genes in the WNT and MAPK signaling pathways appear to be prominently involved, and the aberrant methylation pattern is reversed following combination therapy with 5-azacytidine and an HDAC inhibitor.[65] Over recent years, it has become apparent that several recurrent mutations found in cancer target genes are involved in epigenetic regulation. In acute leukemias, the histone acetyltransferase, CBP, has been found to be mutated, and the H3K4 methylase, MLL, is often rearranged. However, it is only recently that mutations affecting epigenetic regulation have been identified in MDS (Figure 4).

Point mutations in genes associated with epigenetic modifying functions

Specific mutation frequencies in MDS vary from study to study and appear to depend on the subtype of MDS or MDS/MPN interrogated. Similarly, given that many of these point mutations are only just beginning to be identified in MDS, the prognostic value of specific mutations can be contentious. In addition, expression levels for some of these factors have also been found to be altered in MDS.

In 2009, several groups independently identified frequent mutations, copy neutral LOH, or microdeletions at chromosome band 4q24, the locus of the *TET2* gene, in MDS, MDS/MPN, AML, secondary AML and CMML.[67-72] It is likely that *TET2* mutations occur in a hematopoietic stem cell or very early progenitor as the mutations are found in both $CD34^+$ stem/progenitor cells and $CD3^+$ T cells.[73] TET2 is an enzyme that converts 5-methylcytosine to 5-hydroxymethylcytosine in DNA, and the mutations associated with myeloid malignancies impair catalytic activity.[74,75] TET2 knockdown in mouse hematopoietic progenitor cells favored monocyte/macrophage differentiation in cultures.[74] While one group has reported global hypomethylation of DNA in the context of *TET2* mutations (which is paradoxical given the putative role of wild-type TET2 in promoting cytosine demethylation), another group has found a specific hypermethylation signature with *TET2* mutation associated with a tendency for gene repression at these sites.[74,75] Further studies are needed to clarify this issue, and to determine the prognostic implications of loss of TET2 function.

The *IDH1* mutation was first identified following whole genome sequencing of the leukemic cells of a patient with normal karyotype AML.[76] Subsequently mutations in *IDH2*, the mitochondrial homologue, were also found.[75,77] Most commonly mutations occur at IDH1-R132 in MDS,[78,79] whereas in AML, IDH2-R140 or IDH2-R172 mutations are also found.[80] Studies suggest that *IDH1* mutations portend a poorer prognosis in MDS and AML, and may play a pathogenic role in disease evolution.[77,79-81] Although the *IDH* genes encode isocitrate dehydrogenases, the IDH1-R132 and the IDH2-R172 active site mutants display a gain of function that results in conversion of α-ketoglutarate to 2-hydroxyglutarate, which accumulates in leukemic cells with the IDH2-R172K mutation.[82] *IDH1* and *IDH2* mutations are mutually exclusive and are always heterozygous as the wild type allele is important for cell proliferation.[82] *IDH1/2* mutations are also mutually exclusive with mutations in *TET2*, and interestingly, the 2-hydroxyglutarate produced by the mutant IDH proteins interferes with the conversion of 5-methylcytosine to 5-hydroxymethylcytosine by TET2.[75] Consistent with this data, these authors found that mutations in *IDH1/IDH2* share an overlapping hypermethylation phenotype with mutant TET2 cells, and expression of mutant *IDH1/2* or TET2 depletion impaired hematopoietic differentiation and increased stem/progenitor cell marker expression, suggesting a common mechanism of leukemogenesis.[75]

As described above, EZH2 (chromosome band 7q36) is the catalytic subunit of the polycomb repressive complex 2 (PRC2), which functions as a histone H3 lysine 27

(H3K27) methyltransferase, and is associated with transcriptional repression.[54] EZH2 also physically interacts with all three DNA methyltransferases (DNMTs).[53] Together these findings would implicate loss of EZH2 in reducing H3K27 trimethylation (and possibly DNA methylation), and thereby potentially activating a subset of pathogenic genes. Of interest, conditional inactivation of *Ezh2* in the mouse hematopoietic system did not have demonstrable effects on myeloid function, although B cell development was defective, thus additional studies will be needed to address its role in myeloid malignancies.[83]

Similar to *IDH1*, a DNA methyltransferase mutation, *DNMT3A*, was identified following whole genome sequencing of the leukemic cells of a patient with normal karyotype AML.[84] In this study, 62 of 281 patients (22%) had *DNMT3A* mutations that were predicted to affect translation, most commonly affecting amino acid R882. These mutations were highly enriched in AML patients with an intermediate-risk cytogenetic profile and were associated with poor outcome. More recently 12/150 patients (8%) with MDS were found to have *DNMT3A* mutations.[85] As *DNMT3A* mutations likely result in loss of function, one would predict that there would be reduced DNA methylation, which would possibly correspond to global hypomethylation. However, EVI1 has been shown to interact with *DNMT3A*, and increased expression of EVI1 favors promoter hypermethylation.[24] Interestingly, expression of miR-29b which directly targets *DNMT3A* (and 3B) mRNA is reduced in a subset of AML and enforced miR-129b expression results in global hypomethylation.[86]

ASXL1 truncation mutations, which are likely loss of function mutations, have recently been reported to be frequent in myeloid malignancy, including MDS.[56,57] *ASXL1* can act in repressor or activator complexes depending on the cellular context and has been found to associated in a histone H2A deubiquitinase complex.[77] *ASXL1* is also a member of a repressive complex containing histone H1.2.[87] *ASXL1* may function as an activator or repressor of retinoic acid receptor signaling, and also regulates HOX genes.[77] *ASXL1* is expressed in most hematopoietic cell types, but mice targeted for the gene only display mild defects in myelopoiesis, do not appear to have hematopoietic stem cell defects, and do not develop myeloid malignancy.[87] Of note, one report suggests that the most commonly reported variant in *ASXL1*, a duplication of G in exon 12 (codon G646W) may not be a somatic alteration, and thus the frequency of *ASXL1* mutations may be significantly lower than originally thought.[88] Further studies are clearly needed.

Several other less well-studied mutations in MDS target epigenetic modifiers, such as *UTX*.[56] As well, differential expression of epigenetic modifiers, such as increased *JMJD3* and H3K27 demethylase, have also been described.[89] The coming years will paint a better picture of how defects in chromatin remodeling contribute to MDS pathogenesis.

Other point mutations observed in myelodysplastic syndromes

While it is not the intent by any means to suggest that all mutations somehow lead to epigenetic modifications, it is important to note that several previously identified mutations that have been shown to function in other pathways are also known to play a role in chromatin remodeling.

TP53 is a tumor suppressor gene located at chromosome band 17p13 that functions to protect the genome against stress-induced damage by regulating various pathways, including apoptosis, cell cycle, senescence, DNA repair, and cell metabolism.[90] *TP53* mutations occur frequently in MDS patients with a complex karyotype, particularly in the presence of del(17p), -5/del(5q), and -7/del(7q), but are usually exclusive of many of the point mutations listed above.[91-93] P53 interacts with histone acetyltransferases and methyltransferases, as well as DNMTs.[94,95] In del(5q) MDS, p53 is activated by the stress induced by impaired ribosomal biogenesis, but although depletion of p53 abrogates the hematopoietic progenitor defect in a mouse model, mutations in *TP53* are associated with transformation to AML in del(5q) MDS.[35,96]

RUNX1 (*AML1*) mutations are commonly found in MDS and minimally differentiated AML, although translocations involving the *RUNX1* locus at chromosome band 21q22 are only seen in AML.[97-99] *RUNX1* mutations are frequently seen in RAEB, as well as therapy- and radiation-related MDS/AML.[97,98] Heterozygous germline mutations of *RUNX1* result in familial platelet disorder characterized by platelet abnormalities and a predisposition to MDS/AML. However, families with normal platelet counts and function have been described.[100] The molecular pathogenesis of *RUNX1*-related myeloid malignancies may be distinct depending on the mutation.[98] N-terminal in-frame mutations are thought to cooperate with -7/del(7q), as well as with activating *RAS* mutations.[97,98] Runx1 is required for initiating definitive embryonic hematopoiesis from the hemogenic endothelium.[101] Although Runx1 can act as a repressor, it mainly functions as a transcriptional activator in association with CBFβ or other partners, by recruiting histone acetyltransferases and methyltransferases.[97,98,102] Evi1 has been shown to interact with Runx1 and inhibit its DNA binding capability.[103]

As with several of the other mutations, *NRAS* and less frequently *KRAS* mutations are more frequently found in higher-risk MDS and MDS/MPN overlap syndromes, and result in activation of the MAP kinase and NF-κB pathways.[104,105] There is also evidence to suggest that DNA-methylation associated repression of tumor suppressors and apoptotic genes are partly regulated by Ras signaling.[106] Ras-activating mutations may not be as frequent as previously thought. Similarly to *RAS*, *JAK2* (chromosome 9p24) and *CBL* (chromosome 11q23) mutations are usually associated with MDS/MPD overlap syndromes.[77] *JAK2*-V617F mutations are usually found in the provisional category of RARS with thrombocytosis (RARS-T).[77] JAK2 is a non-receptor tyrosine kinase that has transforming activity in part through the activation of STAT5. However, recently JAK2 was also found to localize to the nucleus where it can phosphorylate histone H3 on Tyr 41 (H3Y41) in a STAT5-independent manner.[107] Phosphorylation of H3Y41 results in transcriptional derepression at specific genes through the release of the transcriptional repressor heterochro-

matin protein 1α (HP1α), and this may represent an additional transforming mechanism of *JAK2*-V617F.[107] *CBL* is an E3 ubiquitin ligase and mutations are mostly found in chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML).[108]

Conclusions

MDS is a heterogeneous disease, and it would be inappropriate to make categorical statements about MDS pathogenesis other than that there are multiple subtypes not solely defined by morphology. The various subtypes may show opposite activities of specific molecules, resulting in a molecular subtype perhaps linked to the aggressiveness of the disease. Much remains to be learned about which driver mutations are implicated in disease initiation and progression and which are passenger mutations that alter phenotype, but do not contribute to MDS progression. In the following years, elucidation of the various epigenetic and signaling pathways dysregulated in MDS will hopefully lead to more specific therapies and better patient outcomes.

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Controversies in the treatment of myelodysplastic syndromes

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A B S T R A C T

The myelodysplastic syndromes are a group of very complex myeloid disorders that more frequently affect older individuals with comorbidities. Therapy for these patients is therefore complex and should be tailored to both the characteristics of the disease, as well as potentially those of the patient. In general, patients are stratified based on accepted standard criteria, such as International Prognostic Scoring System (IPSS), into lower and higher risk groups. The goal of therapy in patients with lower risk disease is to diminish transfusions requirements, improve quality of life, and potentially delay progression to higher risk disease or acute myelogenous leukemia (AML). In higher risk disease, the goal is to improve survival. A number of therapies are now widely available. These include growth factors, iron chelation, immunosuppresion, lenalidomide, hypomethylating agents, AML-like therapies, and allogeneic stem cell transplantation. In this review, I will try to integrate current knowledge on therapy of MDS and discuss areas of controversy and potential future questions.

Introduction

Over the last decade we have witnessed a revolution in our knowledge of the myelodysplastic syndromes (MDS).[1] This has resulted in the development of new morphological classifications,[2] prognostic scores,[3-6] and effective therapies.[7-9] More recently, we have also witnessed the beginnings of the molecular dissection of the disease.[10-13] The result has been the realization that MDS encompasses a more complex group of disorders than previously anticipated. Indeed recent data from in depth cytogenetic analysis indicate that the level of complexity starts at the cytogenetic level.[14] Furthermore, this group of diseases affects a complex group of patients: the elderly.[15] These patients represent a particular challenge due to the expected high incidence of concomitant comorbidities and less tolerance to intensive forms of therapy. Therefore, treatment should be adapted to the subtype of MDS, the expectations of survival and transformation, specific molecular characteristics, and the physical condition and age of the patient. In this review, I do not plan to provide an exhaustive summary of all treatments available for MDS. Instead, I will try to summarize my approach to the therapy of MDS and highlight areas of controversy in need of further research.

Current "standard of care" options in myelodysplastic syndromes

In the paragraphs below, I provide a succinct review with annotated references to current standard therapies in MDS. This topic was extensively reviewed recently[16] and a proposed algorithm for the therapy of MDS provided (Figure 1).[16]

Current options for patients with lower risk disease

Patients with lower risk disease are those with a low percentage of blasts and normal or intermediate cytogenetics.[3] The degree of transfusion requirements can vary significantly in this subset of patients from none to extensive weekly needs. This has a significant effect on the survival and expectation for the patient.[6,17] Traditionally, patients with lower risk disease have been considered to constitute a group with smoldering disease and relative good prognosis. An analysis by our group of survival in this patient category indicated that survival in lower risk disease can vary significantly and that a significant fraction of patients have dismal prognosis without intervention.(6) Furthermore, we identified the cause of death in these patients as primarily related to the disease and not to disease progression to acute myelogenous leukemia (AML) or due to other comorbidities.[18] These two facts are fundamental for our conceptualization of the disease: they imply the potential need for early therapeutic intervention in patients with lower risk disease but anticipated poor prognosis. This will have to be tested in prospective clinical trials.

At the present time, a number of therapies are widely used in patients with lower risk MDS. Probably the most commonly used front line therapy in lower risk MDS are growth factors. Erythroid growth factors, alone or in combination with myeloid growth factors, are commonly used for patients with anemia.[19] A number of predictive models for response have been devel-

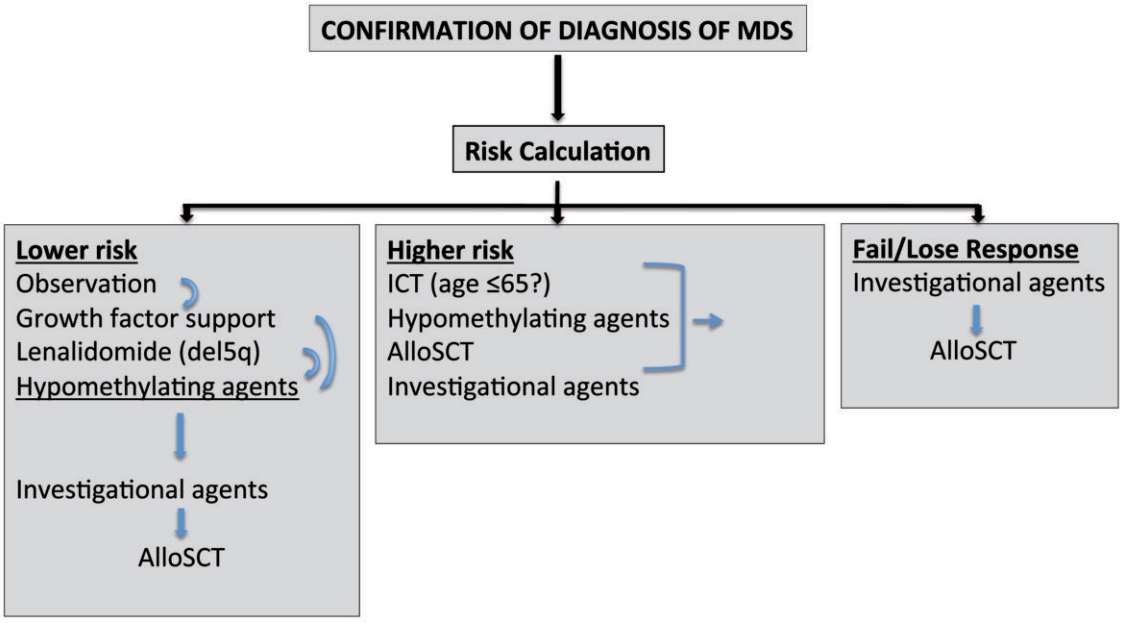

Figure 1. A treatment algorithm for patients with MDS. The first step when evaluating a patient with MDS is diagnoses confirmation. Risk can be calculated using a number of classifications. Traditionally, patients with lower risk disease are those with low or int-1 by IPSS.[3] Patients with higher risk disease are those with int-2 and high risk. A number of options exist for patients with lower risk disease. These range from observation (for those patients transfusion independent with an expected long survival and minimal risk of transformation[3,6]). Growth factor support is usually recommended for patients with anemia early in the course of disease. Lenalidomide is the standard of care for patients with anemia and a deletion of chromosome 5. Results with lenalidomide are better in patients early in the course of their disease, minimally transfused, and no severe thrombocytopenia. As indicated by the arrows, these approaches are sequential. A patient can be initially observed, started on growth factors, and eventually a hypomethylating agent. Investigational agents are considered in patients that have no benefit from standard approaches. AlloSCT is usually reserved for patients that have received all these approaches or are considered to be at high risk of progression or death.[6] Options for patients with higher risk disease are more limited. Observation is never an option for this group of patients. Main options are ICT or a hypomethylating agent. ICT is usually reserved for younger patients that are expected to transition to alloSCT in a short period of time after induction. Results with ICT are better in patients with diploid karyotypes. Most patients are candidates for hypomethylating agent. Younger patients with abnormal karyotypes, particularly those with alterations of chromosome 7, should be considered for a hypomethylating agent instead of ICT. Finally, all candidate patients (younger, available donor) should be considered for alloSCT. Most patients receive some form of therapy before proceeding to alloSCT. The timing of alloSCT for patients that respond to ICT or hypomethylating agent is controversial. Some investigators recommend proceeding with alloSCT as soon as possible, whereas others recommend maintaining use of hypomethylating agents for as long as possible before relapse or transformation. Adapted from Garcia-Manero and Fenaux, J Clin Oncol 2011, 29, 516-23; with permission.

oped, and retrospective analysis suggests that a subset of patients early on the course of the disease, with low serum erythropoietin levels and minimally transfusion dependent may derive a survival benefit.[20] That said, no randomized study has proven the benefit of these interventions in MDS.

Lenalidomide is another agent that targets anemia in MDS. A number of elegant clinical trials[7,21] demonstrated the safety of this agent in patients with anemia and lower risk disease characterized by an alteration of chromosome 5. In this context, lenalidomide results in a reduced need for transfusions in 76% of patients (complete in 67%). Duration of response is long: the median duration had not been reached at the time of initial reporting of this data.[21] That said, the use of lenalidomide, as well as dose and schedule, is dependent on the risk of the disease and cytogenetic characteristics. In lower risk de(5q) MDS, mild thrombocytopenia (platelets less than $100x10^3$ Ku/ml) and prior transfusion requirements have a significant impact on the activity of the drug. Patients with near normal platelet counts with anemia and del(5q) represent less than 5% of patients. It should be noted that lenalidomide is not approved in Europe for this indication due to concerns related to increased risk of transformation to AML. Recently, the presence of persistent malignant stem cells in patients with 5q-MDS treated with lenalidomide has been reported.[22]

The other group of therapeutic agents available for lower risk disease is the azanucleosides, including 5-azacitidine and 5-aza-2'-deoxycitidine (decitabine). Of importance, neither of these two drugs is approved in Europe for lower risk MDS. In the United States (US), both agents are commonly used in lower risk disease, and this is due to fact that although they have not been extensively evaluated in this setting, their labels allow for their use in all patients with MDS (5-azacitidine), or those with int-1 disease or above (decitabine). The dose and schedule of these drugs is not well established in lower risk MDS. A community practice randomized

trial evaluating different doses and schedules of 5-azacitidine in the US indicated that shorter (5 day) schedules may be better tolerated and perhaps more efficacious in patients with lower risk disease.[23]

Current options for patients with higher risk disease

Over the last decade we have witnessed a transformation on therapeutic options for patients with higher risk disease. This was first initiated by the original studies of the CALGB that demonstrated the activity of 5-azacitidine in MDS[24] and the subsequent cross-over randomized trial CALGB 9221. This later study indicated the potential superiority of 5-azacitidine versus supportive care in MDS.[25] This study lead to the approval of 5-azacitidine in the US and to the definitive randomized international trial, led by Fenaux *et al*., that demonstrated the impact of 5-azacitidine on survival compared with conventional care (AZA-001).[8] The results of this study currently represent the standard of care for patients with higher risk disease.

In parallel with the development of 5-azacitidine, decitabine has also been extensively studied in the US and Europe. Initial studies used a 3-day schedule. A randomized clinical trial compared decitabine with supportive care. Overall response rate was 17% versus 0% in the supportive care arm. No significant effect on survival was observed but there was a trend towards longer time to AML transformation.[9] Subsequent trials explored alternative doses and schedules and resulted in the development of the so-called daily x 5 schedule that is now commonly used in the US.[26-28] Despite the significant clinical activity and safety profile of decitabine in these studies, decitabine has failed to improve survival in a randomized European study of patients with higher risk disease.[29]

Other therapeutic options for patients with higher risk MDS may include the use of low doses of cytarabine (ara-C) or the use of intensive AML-like induction therapies.[16] Low dose ara-C is not commonly used in the US. In the randomized trial of 5-azacitidine versus conventional care,[8] outcomes were significantly superior with 5-azacitidine compared with ara-C. On the other hand, the issue of the role of AML-like therapy is more complex. First, this type of strategy is now only used in younger patients fit enough to receive it. This represents a small percent of patients that most likely are also candidates for allogeneic stem cell transplantation (alloSCT). The role and use of high dose AML-like therapy is now controversial due to the results with 5-azacitidine in the AZA-001 trial.[8] That said, AZA-001 study did not prove superiority of 5-azacitidine versus AML therapy.[8] This is discussed below.

Controversies in the treatment of myelodysplastic syndromes

Based on the summary above, it appears that most patients with higher risk disease have a therapeutic option (azanucleoside vs. AML therapy or alloSCT). In the lower risk setting, options may be more limited. Growth factors are active in a small fraction of patients and their efficacy is temporary. Lenalidomide has its best active profile in a restricted group of patients and the rest of patients may be candidates for either 5-azacitidine or decitabine but we are not sure about dose/schedules and what the clinical relevant endpoints are for these agents in these patients. Here, I will address some of these issues in MDS.

Is there are role for iron chelation in MDS?

This is a very controversial issue in MDS. A number of chelating agents are currently available.[30] Data in benign hematology disorders indicate that they are effective in improving the complications of iron deposition in these disorders.[30] Data from randomized clinical trials is not currently available in MDS. A number of consensus groups have suggested guidelines indicating the need to initiate iron chelation once a certain threshold of ferritin levels are achieved.[31] The dogma is that extrapolating from thalassemias, iron chelation may prevent myocardial damage and liver injury in patients with MDS.[32] A Japanese study had indicated that these are common complications in patients with red cell transfusion dependent anemia.[33] Anecdotal studies also have indicated the iron chelation may improve patient outcomes in MDS.[34,35] This data is in contrast with our analysis of cause of death in MDS.[18] In that analysis, the most frequent causes of death were infection and bleeding.[18] Cardiomyopathy or liver cirrhosis were rare in our clinical practice.[18] That said, it is possible that iron accumulation could have a role in the immunosuppresion of patients with MDS and therefore, that chelation could improve outcomes by decreasing the rate of infections in patients with iron deposition. That could potentially explain the positive data reported with the use of chelation in the transplant setting.[36] Second, a study from Spain not only indicated that survival was shorter in patients with increased ferritin levels but that patients also had increased rates of transformation to AML.[37] Therefore, the question is whether iron accumulation can promote leukemia progression in these patients. At the ASH 2010 meeting, Chan *et al*. presented data from an animal model of iron overload, suggesting that iron could promote AML by activating a reactive oxygen species (ROS) response pathway.[38] All these concepts are hypothetical at the present time. An international phase III randomized clinical trial, known as TELESTO, is now open worldwide to attempt to answer some of these questions. At the present, I think that the consensus opinions from NCCN are acceptable.[31]

What is the role of immunosuppresion in MDS?

It is clear from a clinical perspective that a subset of patients with MDS have inflammatory/immune features that could also be observed in patients with aplastic anemia.[39] This would suggest that in a fraction of patients, interventions to induce immunosuppresion may be clinically active. A number of clinical trials from the NIH have indicated that therapy with ATG with or without cyclosporine may be beneficial in patients with MDS.[40] Data from other centers have failed to reproduce these results.[41,42] These studies have suggested that younger patients with MDS benefit more frequently from ATG-based therapy. Despite these discrepancies, the group at the NIH has developed algorithms to predict response to immunosuppressive therapy.[43] These include age, HLA-DR15 status, and prior history of

transfusion requirements. Recently, the group at Moffitt Cancer Center has reported in the potential role of CD4/CD8 ratios as predictors of response as well.[44] Using the NIH algorithm, Sloand *et al*. developed a number of clinical trials using alemtuzumab.[45] The investigators screened 121 patients using the above criteria:[43] 32 (26%) were eligible due to their predicted high expectation of response to immune therapy. Patients were treated with one course of alemtuzumab at a dose of 10 mg daily for 10 days. Results were very impressive, including response rates of 57% to 77% depending of MDS type. Median time to response was 3 months and responses were maintained at 12 months in 60% of patients. This data is now of importance due to two issues. A recent report of a randomized trial failing to demonstrate improvement on survival with ATG based therapy[46] and controversy regarding the optimal source of ATG (horse versus rabbit). Alemtuzumab may constitute a safer alternative to ATG at the present time. Several studies are ongoing with alemtuzumab. This includes a study at MD Anderson Cancer Center (MDACC). My expectation is that appropriate patient selection is critical when using these agents.

What is the proper dose and schedule of azanucleosides in lower risk MDS?

As introduced earlier, both azanucleosides are approved in the US for patients with either low or int-1 disease but there has been little effort on the study of alternative dose and schedules in lower risk disease. Lyons *et al*. used a 5-day schedule of 5-azacitidine instead of the standard 7-day approach in higher risk disease.[23] The data was of interest because not only the 5-day schedule was better tolerated than the 7 day but also because there was a trend towards better response rates. Our group has been interested on this concept. We completed a multicenter phase II trial of decitabine for patients with lower risk MDS.[47] That study was a randomized two arm trial investigating two schedules of the drug: a 3 consecutive day schedule every 28 days versus a weekly x 3 schedule, also every 28 days. The dose was 20 mg/m^2 subcutaneous. The study had a Bayesian design powered to stop early if one of the arms was to underperform. In this study, 54 patients with lower risk MDS were randomized. The ORR was 22% in the daily x 3 arm. Of importance, multilineage transfusion independency was documented in over 60% of the patients. No drug related mortality was documented. This data is now undergoing final analysis but it suggests that very low schedules may be optimal in lower risk MDS. This concept of low exposure to the azanucleoside (hypomethylating) agents is further supported by the experience with the oral derivative of 5-azacitidine.[48,49] A phase I study exploring a 7-day oral schedule of oral 5-azacitidine indicated that this formulation was active and had the capacity to induce global hypomethylation. Toxicity profile was excellent at doses below the defined DLT. That was despite exposures in the range of less than 20% compared with subcutaneous 5-azacitidine. This is of fundamental importance as it suggests that this type of "low concentration approach" could be delivering chronically using different types of schedules with implications not only in MDS but for other leukemias.

Can we improve (or predict) results in higher risk disease with the azanucleosides?

The results of the AZA-001 with 5-azacitidine study are now the standard of care in higher risk disease.[8] That said, it is obvious that we need to continue attempt to improve on them. In that trial (AZA-001), complete remission rates were 17% with 5-azacitidine, and median survival was 24.5 months compared with 15 months in the control group (HR 0.58, p=0.0001). To achieve this type of results, 5-azacitidine has to be administered chronically and it may require several months for maximal response. It is also becoming clear that discontinuation of therapy is associated with resistance (below). It is therefore fundamental to develop strategies to improve on current results with 5-azacitidine. This can be done using two strategies. One is by developing clinical or molecular predictors of response. Selecting patients at high chance of response would result in rapid increase of response rates for selected patients. A number of molecular biomarkers have been analyzed and correlated with response. At the present time, none of the analysis correlating response with induction of either global or gene specific methylation have proven successful.[50-52] Recently, miR29b expression levels have been correlated with response in patients receiving a 10-day schedule of decitabine.[53] Mir29b could have a role in regulating levels of DNA methyltransferase, the target of decitabine. Although this is of interest because this is an easy biomarker to analyze, our group was unable to reproduce these results in a study with 5-azacitidine in combination with valproic acid and ATRA.[51,54] Further studies are needed to confirm the value of miR29b. The other alteration that has been reported to be associated with response to 5-azacitidine is the presence of mutation in TET2. TET2 belongs to a family of genes that control hydroxymethylation.[55] Mutations may result are passive induction of hypomethylation and potentially differential responses to hypomethylating agents. At ASH 2010, a French group reported an association between presence of TET2 mutations and response to 5-azacitidine.[56] This could be a biomarker linking a genetic lesion that controls DNA methylation. This data with TET2 also needs to be confirmed in other studies. It should also be noted that TET2 mutational analysis is complex because it requires sequencing of the whole gene. Finally, a French group has recently proposed a simple clinical model of response to 5-azacitidine.[57] This includes prior therapy, increasing bone marrow blasts, and abnormal cytogenetis.[57] A combination of clinical and molecular models could result in a significant improvement in our ability to target this type of therapy for selected patients.

The other approach to improve results with the azanucleosides is by developing active and safe combinations. The most extensively studied type of combination is that of the combination of a hypomethylating agent with a histone deacetylase inhibitor.[58] A number of studies, both preclinical and phase I/II trials, have indicated that these combinations are safe and potentially more effective than a single agent hypomethylating agent.[58,59] Classic studies include combinations with valproic acid.[50,51,60,61] Two large randomized clinical trials have been performed to date. One performed at MDACC compared decitabine with or without valproic

acid.[62] The second was a large multicenter US study comparing a 10-day schedule of 5-azacitidine with the carbamate HDAC inhibitor MS-275 (entinostat). In the preliminary data presented at ASH in 2009 and 2010, respectively, none of these studies was able to report superiority with the combination arm. Other studies with more potent HDAC inhibitors are ongoing. Another alternative being explored by the MDACC group is that of intercalating azanucleoside with non-cross reactive agents, such as clofarabine. Clofarabine is a nucleoside analogue with clinical activity in MDS.[63]

Does lenalidomide have a role outside anemia in lower risk disease?

At the present time, the best results of lenalidomide are in patients early in the course of the disease with anemia, a chromosome 5 alteration, minimal transfusion requirements, and minimal other cytopenias.[7] In these settings, results with this agent are exceptional, resulting in transfusion independency in over 60% of patients. Results of a randomized study comparing lenalidomide with standard of care were presented at ASH 2009.[64] The question is whether lenalidomide could have a role in other settings in MDS. First, the drug has been used in patients with lower risk disease without alteration of chromosome 5. On the initial study reported by Raza *et al*., transfusion independency was achieved in 26% of patients.[65] Response duration was 41 weeks. Although not insignificant, it is not clear whether these results are better than what could be expected from the use of growth factor support. To test this concept, a randomized clinical trial comparing lenalidomide with standard of care is now ongoing worldwide.

Recently, several studies have evaluated the role of lenalidomide in higher risk disease.[66] The French group reported on 47 patients with higher risk disease treated with standard dose lenalidomide. Seven patients achieved complete remission. Responses were associated with presence of isolated chromosome 5 alteration and platelets over 100x 10^3 Ku/ml.[66] At MDACC, results have not been of this magnitude (Borthakur *et al*., in preparation). Perhaps the activity of lenalidomide could be improved in higher risk MDS by using higher doses. The group at Washington University reported on the safety and activity of lenalidomide at a dose of 50 mg daily in patients with AML.[67] The conclusion of this study was that these doses could be tolerated in older patients with AML and were associated with response rates of 30%. Finally, the other approach is combinations. One such combination is to use lenalidomide with 5-azacitidine. Sekeres *et al*. initially reported on a trial of 5-azacitidine and lenalidomide.[68] This study demonstrated that the combination, using standard doses of 5-azacitidine and lenalidomide, was safe and associated with an ORR of 67%. At MDACC, a clinical trial is evaluating doses of lenalidomide of 50 mg daily x 10 days after 5 days of standard dose 5-azacitidine. Preliminary results indicate that this type of schedule is safe and active in higher risk MDS and AML.

What are the options for patients that do not benefit or stop benefiting from azanucleosides?

Because the use of azanucleoside is now generalized in community practice, one problem we are facing is that of patients that lose response to either 5-azacitidine or decitabine. This is a major problem as survival has been documented to be very poor.[69] In the analysis of Jabbour *et al*. of 87 patients that had received decitabine, median survival was 4.3 months. Most of these patients were refractory to standard ara-C based therapies. The mechanisms of resistance to decitabine is not understood but could be pharmacological.[70] Why patients acquired secondary resistance is not understood either. In my opinion, there are ready available interventions for these patients and therefore, all patients should be considered for clinical trial. Studies with agents, such as clofarabine[71] and sapacitabine[72] are ongoing. A phase III study is evaluating the role of compound ON1910 in this context.

What is the role of allogeneic stem cell transplantation in MDS?

Until recently, alloSCT and non-alloSCT therapies (described above) have been viewed as two different and almost exclusive interventions. In the analysis of Cutler *et al*.,[73] it was proposed that patients with lower risk disease do not benefit in general from early transplantation. In contrast, patients with higher risk disease did derive a survival benefit if transplanted early. These results were obtained in a cohort of patients before access to other therapies, such as azanucleosides, was widely available. This data has generated multiple questions: 1) Is there a subset of patients with lower risk disease that could benefit from early transplantation? 2) What is the optimal therapy prior to alloSCT? 3) Do we have to consider the use of maintenance approaches post alloSCT? 4) Is there a subset of patients that may not benefit at all from alloSCT?

First, is the realization that only a small fraction of patients will eventually receive alloSCT. This is due partly due to the median age of patients, presence of other comorbidities, and economical and societal issues that limit access to alloSCT for MDS patients. For instance, US Medicare (that medically covers patients over 65 years of age) does not support the use alloSCT outside the setting a clinical trial. It is possible that in the near future, the use of true mini-transplant approaches, cord blood, and even haploidentical approaches could increase the number of potential candidates with MDS for alloSCT. In my opinion, this will have to be studied in MDS specific clinical trials.

Can we identify a subset of patients with lower risk disease that could benefit from early alloSCT? The question could be answered by identifying patients with lower risk and poor survival. The MDACC lower risk model indeed allows identification of such a subset of patients.[6] The main reason why alloSCT was not shown to improve survival in the analysis of Cutler *et al*. was that the inherent early mortality associated with alloSCT. Therefore, identifying a subset of patients with estimated poor survival could support the introduction of "early" transplantation in lower risk MDS. If the MDACC lower risk model was validated, a trial of early intervention would be an important study to conduct.

What is the optimal therapy prior to alloSCT? Results of upfront alloSCT in patients with excess blasts are questionable. Most clinicians would recommend some form of "debunking" type of therapy prior to alloSCT.

The two main options are some form of AML-like induction therapy or the use of azanucleosides. No study has really addressed this question. Therefore, this is my own current approach at the present time. In a young patient with diploid cytogenetics, and particularly if it is likely that an optimal (based on donor characteristics) alloSCT could be performed in the next few weeks, I would recommend AML-like therapy followed by alloSCT. But if the patient had abnormal cytogenetics or if I am unsure about the ready availability of donors or if the donor option is higher risk, I would proceed first with an azanucleoside analogue. The main advantage of this approach is the differential response rate in patients with abnormal cytogenetics and the lower risk of complications and mortality.[8] The main disadvantage is that it may take several months of therapy for any clear clinical benefit. One problem that I encounter when following this last approach is what to do with a patient that achieves a complete remission with the azanucleoside. Do you proceed with alloSCT at that time? Of course, patient preference is important here, so is the realization that the patient will eventually relapse without alloSCT. But patients with major complete cytogenetic remissions can survive for prolonged periods of time if maintained on the azanucleoside. This could also be an important research question.

Is there a role for maintenance therapy post alloSCT? One of the main problems that we encounter with alloSCT is not only the early mortality but also relapse disease. Recent data on clonal nature of stem cells in leukemia[74] indicate the possibility of multiple evolving clones developing during the course of the disease. Therefore, it is logical that patients that receive alloSCT could also benefit from interventions to control minimal residual disease. One approach is the use of azanucleosides post-transplant but also the eventual use of specific molecular targeted agents for patients with specific mutations (currently targets could be Flt-3 inhibitors, azanuecleosides for TET2 mut patients, and potentially JAK2 inhibitors and MEK inhibitors for patients with JAK2 and Ras mutations, respectively). At the present time, the best example of this type of approach is the use 5-azacitidine post alloSCT by DeLima *et al*.[75] In this trial, 45 patients with almost a universal expectation of relapse post alloSCT were treated on day 100 post alloSCT on a phase I trial of dose escalation of 5-azacitidine. These investigators demonstrate that this option is safe up to doses of 32 mg/m^2 daily x 7 days up to four cycles. Overall survival at 1 year was 77% and was better than expected in patients treated with 5-azacitidine. This is now being investigated in a randomized clinical trial ongoing at MDACC. Future specific therapies are needed in this area.

Finally, is there a subset of patients that we can anticipate are not going to benefit from alloSCT? Currently one approach is to offer alloSCT to patients with the worse anticipated prognosis, such as those with abnormal cytogenetics. A recent report from EBMTR indicated that survival of patients with monosomy 7 have a dismal prognosis with alloSCT. This data needs to be confirmed but already in 1997, the EBMTR reported that outcome of patients receiving either alloSCT or autologous transplantation was worse in the subset of patients with poor risk cytogenetics.[76] A study is needed comparing outcomes of patients with poor risk cytogenetics receiving either alloSCT or azanucleosides. This study is ongoing by the IBMTR.

Conclusions

Treatment decisions in MDS are evolving and increasing in complexity. An integrated approach evaluating the risk of the disease, molecular assessment, and evaluation of comorbidities are needed to select the most appropriate type of therapy.

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Issues in allogeneic hematopoietic stem cell transplantation in myelodysplastic syndromes

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A B S T R A C T

Allogeneic hematopoietic stem cell transplantation (HSCT) remains the only curative treatment option for myelodysplastic syndrome (MDS). Whilst there has been considerable interest regarding the use of new therapeutic agents for the treatment of MDS, the ability to achieve long-term disease eradication with demethylating or immunomodulatory drugs remains elusive.[1,2] However, HSCT may not be the most appropriate treatment for all patients with MDS, and the decision to proceed with transplantation should not be taken without due consideration of the probability of long-term benefit to the patient versus the inherent complications associated with the procedure. No prospective randomized controlled trials have directly compared non-transplant therapies with HSCT for MDS. Although the curative potential of allogeneic HSCT is recognized, certain controversies exist surrounding patient selection for such therapy, the optimal timing and the type of HSCT, and the use of chemotherapeutic agents to reduce the disease burden pre-transplant. This review will focus on the most recent data relating to these issues.

Patient selection for hematopoietic stem cell transplantation

Recipient age

MDS is predominantly a disease of the older person, with the median age at diagnosis around 70 years of age.[3] The majority of data relating to outcomes of HSCT in MDS patients is derived from retrospective studies, with a paucity of randomized controlled clinical trials performed in this area. Those patients considered suitable for HSCT, who eventually undergo the procedure and are reported in any clinical trials of HSCT, may not be representative of the cohort of MDS patients as a whole, since in many cases, patients older than 60 years are excluded from such trials.

The decision to proceed to HSCT for MDS patients is influenced by a number of factors, including disease stage (discussed below), donor availability, patient, and physician choice, but the over-riding factors within the patient are recipient age and the increasing frequency of comorbidities in older patients. Since the feasibility of HSCT for younger patients is established, the major interest in HSCT for MDS focuses on the older age groups. However, even here the boundaries between "younger" and "older" are blurred, with "older" often defined as 40 years onwards. The development of reduced intensity conditioning (RIC) regimens (Figure 1) for HSCT in MDS has enabled this procedure to be offered to older patients;[4-6] however, this is clearly a heterogeneous cohort and the decision to proceed to HSCT must be considered on a case-by-case basis, with the risks and benefits clearly outlined to the patient.

Previous (mainly registry) studies of HSCT in older patients with MDS have shown that advanced age is significantly correlated with a higher risk of non-relapse mortality (NRM).[7-9] The majority of such data comes from standard myeloablative conditioning (SMC) HSCT, with the "oldest" patients being up to around 60 years of age. The increasing use of RIC regimens and unrelated donors has extended the use of HSCT to older patients and as such, the effect of age on outcomes for HSCT in MDS has needed to be re-appraised.

Two recent reports have specifically addressed the influence of age on outcomes for HSCT in MDS (Table 1). A retrospective multicentre analysis by the European Group for Blood and Marrow Transplantation reported on the outcomes of 1,333 patients over 50 years of age transplanted for MDS between 1998 and 2006.[10] Patients were grouped into those aged 50–60 years and those over 60 years. The median age overall was 56 (and 63 in the over 60 age group). Sixty-seven percent of patients underwent a matched sibling allograft (33% unrelated donor source). Thirty-eight of patients underwent SMC, whereas 62% underwent RIC; of note, 60% of patients receiving SMC had more advanced disease at diagnosis and transplantation, compared with 49% of those receiving RIC HSCT. Four-year overall survival (OS) was 34% in those aged 50–60 years and 27% in those over 60 (p=0.23, HR=0.87). Estimated 4-year NRM was 36% in those aged 50–60 years versus 39% in the over 60s (p=0.39, HR 1.11). Patients receiving RIC HSCT (despite having less advanced stage disease at transplantation) and those

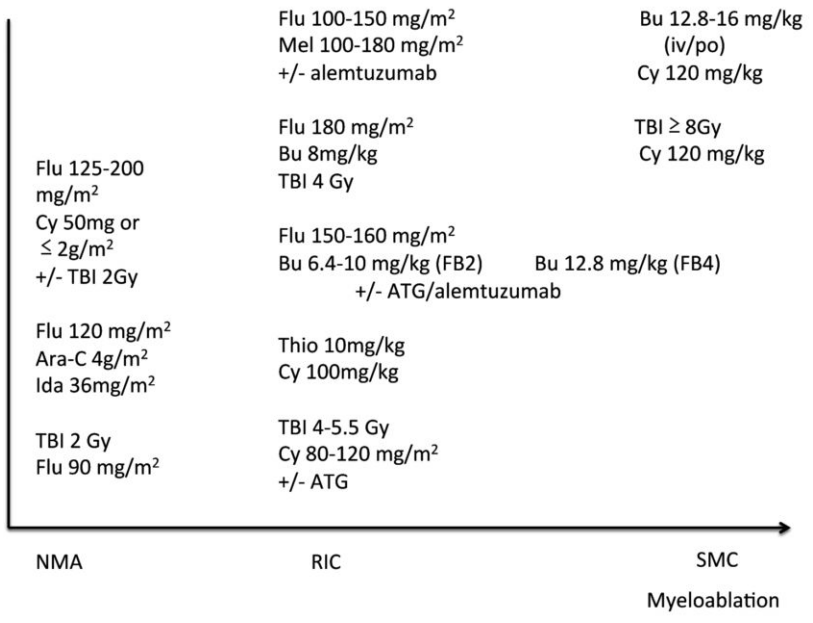

Figure 1. Diagram to illustrate intensities of commonly used regimens for MDS HSCT (ref.[12,41,59,60]). Flu = Fludarabine, Cy = Cyclophosphamide, TBI = Total Body Irradiation, Ara-C = Cytarabine, Ida = Idarubicin, Mel = Melphalan, Bu = Busulphan, ATG = antithymocyte globulin, Thio = Thiotepa, NMA = nonmyeloablative, RIC = Reduced Intensity Conditioning, SMC= Standard Myeloablative Conditioning.

Table 1. Major findings of three recent retrospective studies of RIC and SMC HSCT for MDS; see also.[58]

	Lim *et al*., EBMT 2010[10]	Clune *et al*., CIBMTR 2010[11]	Alessandrino *et al*., GITMO 2010[24]
N, disease type	1,333, MDS	1,080 total 535 MDS 545 AML (CR1)	357, MDS
Age range, y (median)	50–74 (56) 34% > 60y	40–79 (AML, 12%>65y) 40–78 (MDS, 10% >65y)	Transfusion dependent 21–72 (49) 18–65 (48)
Disease stage (FAB), N,%			
RA/RARS	313 (24%)	Not reported	
RAEB	471 (35%)		
RAEB-t	215 (16%)		
sAML	334 (25%)		
Disease stage (WHO), N,%			
RA/RARS/MDS with del 5q31	–		23 (7%)
RCMD/RS	–		61 (17%)
RAEB-1	–		53 (15%)
RAEB-2	–		107 (30%)
MDS-AML	–		113 (31%)
Conditioning, N, %			
SMC	500 (38%)	No SMC: all RIC/NMA	217 (61%)
RIC	833 (62%)		141 (39%)
Comments	4y OS 31%[a]	No effect of age on NRM, DFS, OS or relapse at multivariate analysis[b]	Transfusion dependency independently associated with inferior NRM and OS[c]

[a]No difference in OS for those aged 50–60y versus those aged >60y; use of RIC and advanced stage disease at transplant associated with inferior RFS.
[b]Adverse cytogenetics associated with inferior DFS and OS (AML) and inferior DFS (MDS).
[c]High pre-HSCT ferritin predictive of worse OS and NRM; adverse impact of transfusion dependency restricted to SMC HSCT.
RA=Refractory anemia, RARS=RA with ring sideroblasts, RCMD=Refractory cytopenia with multilineage dysplasia, RAEB=Refractory anemia with excess of blasts, RAEBt=RAEB in transformation, sAML=secondary AML. N=Number of patients.

patients over 60 years of age had higher rates of relapse at 4 years: relapse rate 41% versus 33% for RIC versus SMC (HR 1.39 and p<0.01) and 32% versus 41% for those aged 50–60 years versus older than 60 years (p=0.02, HR 1.32). At multivariate analysis, however, age did not significantly influence survival, relapse or NRM rates.

A CIBMTR study specifically evaluated the outcomes for 1,080 patients over the age of 40 undergoing RIC HSCT for MDS or AML in first complete remission (CR)

between 1995 and 2005.[11] Five hundred and thirty-five MDS patients (aged 40–78 years) and 545 AML patients (aged 40–79 years) from 148 centers underwent RIC or non-myeloablative (NMA) HSCT (although predominantly RIC). Patients over the age of 65 accounted for only 12% of the AML and 10% of the MDS cohort, underlining the low numbers of HSCT performed in this age range. The authors surmised that the low numbers are due to lack of data regarding outcomes for older patients undergoing HSCT and as a result, fewer referrals for this procedure within this age group. Significantly, there was no effect of recipient age on NRM, DFS, or OS in either the AML or MDS patient groups and 2-year survival was 30% across the ages, confirming the potentially curative role of HSCT for these diseases.

Such studies highlight the different conditioning regimens used worldwide (Figure 1) and indeed, the variation within regimens classified as SMC, RIC, or NMA. The distinctions between the categories are not clear-cut: under the umbrella of RIC conditioning, some regimens may be more or less myeloablative than others,[12] for example, those regimens using more than 10 mg/kg of busulphan versus less than10 mg/kg busulphan,[13] and this should be borne in mind when considering data collected from multiple, or even single, centers. Newer agents, such as treosulfan, are also being explored in RIC regimens, and one retrospective study showed an improved estimated 3-year RFS for patients undergoing HSCT conditioned with treosulfan (54%) versus 11% with total body irradiation-based conditioning.[14] The considerable variation between treatment regimens adds to the complexity of interpreting outcomes for "RIC" or "NMA" transplantation.

In summary, current data suggests that with the advent of RIC, age alone is not a barrier to HSCT in older patients and this procedure may be curative, with an acceptable NRM. With advancing age however, comes the attendant increased possibility of disease refractoriness to therapy and this remains an additional hurdle to overcome in the treatment of the older patient with MDS: the number of such patients who will enter a remission long enough to proceed to HSCT remains small. For those patients who can undergo HSCT, there is still an absence of data from randomized controlled clinical trials in this area to confirm the OS benefit and this should be a focus of future research.

Comorbidity

Since the majority of patients with MDS are in their 6th or 7th decade of life, it is to be expected that this patient group will be afflicted by comorbid conditions that may have an impact upon outcome following HSCT. Whilst clinical investigation of organ function provides objective data upon which to form opinions regarding a candidate patient's suitability to undergo HSCT, it is natural for there to be subjective biases which may influence this decision and lead to variations and selection bias between physicians. To standardize assessments of patients' suitability for HSCT, it is therefore useful to employ a scoring tool that not only identifies patients with greater relevant comorbidities, but also has sufficient prognostic impact to identify those patients for whom HSCT (irrespective of conditioning strength) has unacceptably high NRM. Universal adoption of such scores in analyses of outcomes of HSCT will also enable greater ease of comparison between studies.

Several comorbidity scores have been used to assess patients prior to transplantation previously,[15-17] but have limitations in that they may not have been developed specifically to evaluate patients undergoing HSCT for hematological malignancy or were developed in the era of SMC HSCT and therefore, may not be applicable to the predominantly older patients now undergoing RIC HSCT. A single scoring system may not be valid in the setting of transplantation for different malignant hematological diseases and this would need to be prospectively evaluated.

There has been considerable interest in the Haematopoietic Cell Transplantation Comorbidity Index (HCT-CI),[18] which has been demonstrated to provide prognostic information not only for patients with MDS undergoing transplantation, but also in those unsuitable for transplantation.[19] When specifically applied to patients with MDS or AML undergoing NMA or SMC HSCT, Sorror *et al*. (Table 2 and Figure 1) showed in a retrospective analysis that HCT-CI score was a strong predictor of worse outcome following HSCT at multivariate analysis (along with poor risk cytogenetics and high risk disease).[20] Additionally, patients could be stratified according to HCT-CI score and disease risk, with increasing NRM in patients with higher HCT-CI score and higher risk disease. Those patients with highest HCT-CI score and higher risk disease also suffered the worst OS (29% at 2 years) irrespective of conditioning regimen, with the reduced NRM of NMA HSCT being offset by an increased risk of relapse. Prospective use of HCT-CI and disease risk in clinical trials to analyze outcomes post RIC, NMA, and SMC HSCT are highly desirable.

There is limited data regarding the use of HCT-CI in MDS patients undergoing RIC HSCT. A retrospective analysis from our institution examined outcomes for 128 patients undergoing RIC HSCT (sibling and unrelated donors) with a uniform alemtuzumab-based conditioning regimen for high risk MDS and AML.[21] Patients with HCT-CI greater than or equal to 3 had the greatest NRM (42% at 3 years), thus defining a sub-group of patients for whom RIC HSCT may not be the optimal treatment option, especially with the emergence of novel therapeutic agents but this too requires evaluation prospectively.

Iron overload

An additional factor to be considered with regards to comorbidity in patients with MDS undergoing HSCT is the impact of iron overload secondary to repeated transfusion. An elevated serum ferritin (>1000 μg/L) has been associated with reduced OS and increased risk of infection (fungal and bacterial) following HSCT, largely in analyses of SMC HSCT patients.[22,23] Ferritin alone is considered an unsatisfactory marker of iron overload, given that as an acute phase reactant, it will be elevated in the presence of inflammation; for this reason, an evaluation of degree of transfusion dependency along with other biomarkers of body iron load add further impact to the suggestion of a detrimental impact of repeated transfusion on outcomes post HSCT. A recent retrospective analysis by the Gruppo Italiano Trapianto di Midollo

Table 2. 2-year NRM, Relapse, OS, and RFS incidences amongst patients receiving NMA HSCT compared with SMC HSCT as stratified into four risk groups based on HCT-CI scores and disease status, taken from Sorror *et al*. J Clin Oncol 2007, 25, 4246-54; with permission.

Risk Category	Type of Conditioning	NRM (%)	Relapse (%)	OS (%)	RFS (%)
Group I (HCT-CI scores 0-2 and low-risk diseases)	Myeloablative (n = 138)	11	14	78	75
	Nonmyeloablative (n = 28)	4	33	70	63
Group II (HCT-CI scores 0-2 and intermediate and high-risk diseases)	Myeloablative (n = 176)	24	34	51	43
	Nonmyeloablative (n = 34)	3	42	57	56
Group III (HCT-CI scores ≥ 3 and low-risk diseases)	Myeloablative (n = 52)	32	27	45	41
	Nonmyeloablative (n = 19)	27	37	41	36
Group IV (HCT-CI scores ≥ 3 and intermediate and high-risk diseases)	Myeloablative (n = 86)	46	34	24	20
	Nonmyeloablative (n = 44)	29	49	29	23

Abbreviations: NRM, nonrelapse mortality; OS, overall survival; RFS, relapse-free survival; HCT-CI, hematopoietic cell transplantation–specific comorbidity index.

Osseo (GITMO) of 357 patients undergoing SMC or RIC HSCT for primary MDS (Tables 1 and 2) demonstrated a significantly inferior OS and increased NRM for transfusion dependent patients and those with elevated serum ferritin undergoing SMC but not RIC HSCT;[24] only limited data exists to suggest a negative impact in the setting of RIC HSCT.[25]

Data is emerging regarding the relationship between serum ferritin and more specific parameters to assess parenchymal iron overload, for example, determination of liver and cardiac iron content by magnetic resonance imaging (MRI), measurement of serum hepcidin, and labile plasma iron[26] but results from larger, prospective datasets are required. Most importantly, randomized clinical trials to assess the potential for pre-HSCT iron chelation to improve outcomes with HSCT are needed.

Timing of allogeneic stem cell transplantation

The optimal time at which a patient should undergo HSCT during the natural course of the disease remains a point of discussion, and the lack of prospective data adds to the uncertainty in this area. Cutler *et al*.[27] developed a Markov-based decision model to assess the optimal timing for HSCT in MDS patients: either upfront transplantation at diagnosis, after a fixed time-delay from diagnosis but before progression to leukemia, or at the time of leukemic transformation. Eight hundred and sixty-eight patients were identified who had undergone SMC sibling HSCT fron 1990–1999 and were considered in the analysis. The authors concluded that upfront HSCT for patients with intermediate-2 and high risk MDS (classified according to the International Prognostic Scoring System[28]) maximized OS whereas for patients with low risk or intermediate-1 (Int-1) stage disease, HSCT should be deferred until disease progression to leukemia. However, this analysis did not include any patients over 60 years of age, and considered only patients undergoing SMC sibling HSCT. In addition, patients were stratified according to the IPSS, which does not reflect transfusion dependency, changes in prognosis during the natural course of the disease, or other features conferring an adverse prognosis, such as therapy-related MDS.

More recent studies have suggested that HSCT at an earlier time-point may also benefit patients with low risk/Int-1 MDS. A retrospective registry study by de Witte *et al*. analyzed outcomes for 374 patients with low risk MDS (refractory anemia with or without ring sideroblasts, RA and RARS) who underwent SMC or RIC sibling or unrelated donor HSCT. Notably, the median age of the patients was 39 years and there were sufficient data available to enable assignment of IPSS scores in only 53% of patients. Estimated 4-year OS and RFS were 52% and 48% respectively, with a relapse risk of 15% and NRM of 37% overall. NRM was 32% for those patients transplanted within 12 months of diagnosis but 42% beyond that time-point; this resulted in a significantly increased 4 year OS of 57% versus 47% for those transplanted at less than 12 months or beyond 12 months respectively.

The WHO classification-based scoring system (WPSS) may be advantageous over the IPSS with regards to stratifying patients into those most likely to benefit from early HSCT. It incorporates transfusion requirement into the score and enables a dynamic assessment of prognosis during the time-course of the disease, which has greater utility. The WHO classification of MDS has been shown to have prognostic utility in itself, through recognizing the impact of uni-lineage versus multi-lineage dysplasia on outcomes and by defining two categories of patients with excess of blasts (RAEB I&II), observed to have differing prognoses.[29] By basing the WPSS on the WHO classification, this beneficial predictive capability is retained.

The WPSS has been demonstrated to accurately predict OS and risk of transformation in MDS patients during the course of the disease by stratification of patients into five distinct risk groups.[30] It appears particularly useful with respect to patients with low risk MDS without excess of blasts, for whom the presence of transfusion dependency carries greater weight in terms of reflecting disease burden and comorbidity – for those with excess blasts, the effect of transfusion dependency on OS and transformation to acute leukemia is less, as would be expected.[30]

GITMO applied the WPSS in a retrospective registry-based study of patients with MDS undergoing HSCT, in which outcomes for 365 patients who underwent mainly SMC (33% had RIC) HSCT for MDS between 1990 and 2006 were analyzed.[31] More than 60% of the MDS patients undergoing HSCT had high/very high-risk disease according to the WPSS. Transplantation took place at a median of 9 months (range 1–189 months). For patients in the low and intermediate WPSS risk groups, 5-year OS was 80% and 63% respectively, with very low 5-year relapse rates (5% and 11%) and acceptable TRM (11% and 28%). Transfusion dependence was associated with reduced OS and increased TRM and had relevance (but no statistical significance) even in those patients with an excess of blasts. Notably, those

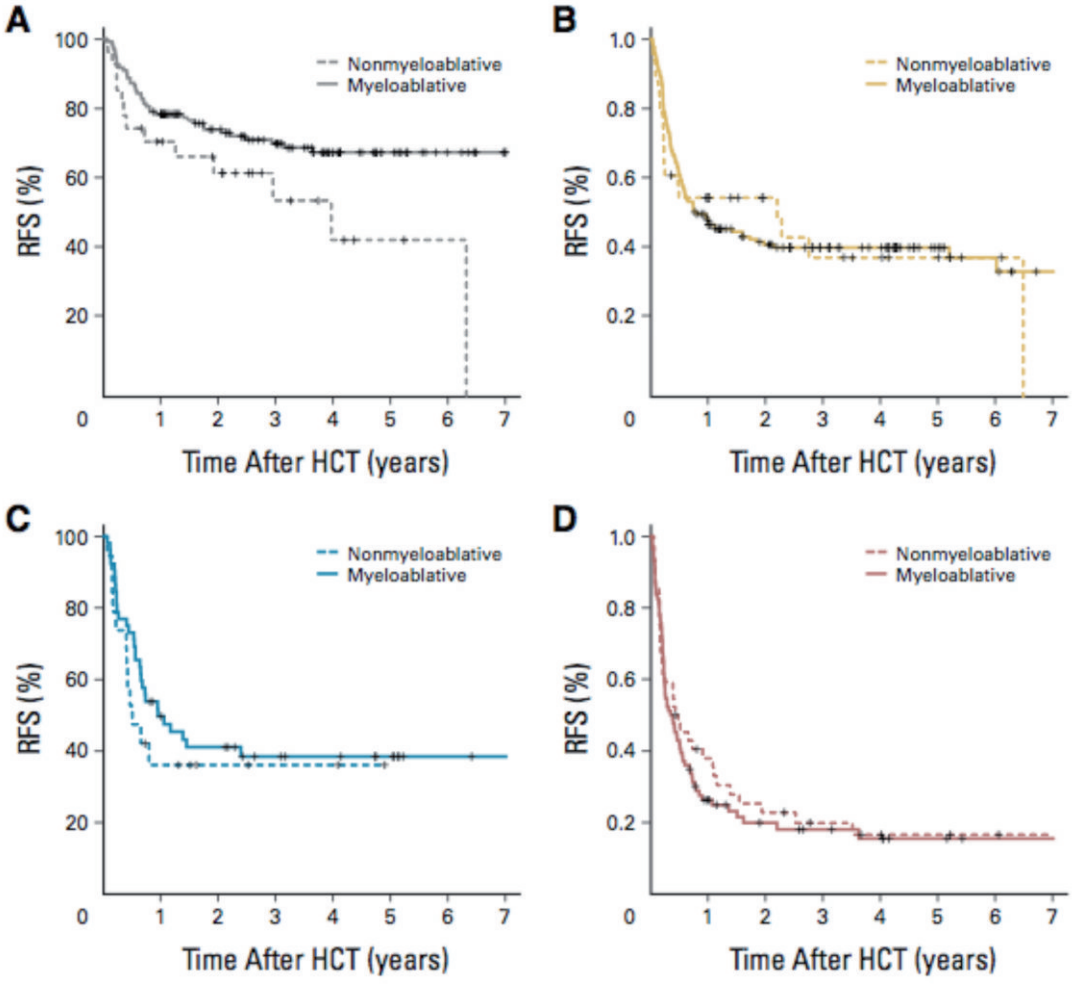

Figure 2. Kaplan-Meier curves of RFS, comparing NMA (—) vs SMC (___) patients among risk groups A(I), B(II), C(III), and D(IV) taken from Sorror *et al.* J Clin Oncol 2007, 25, 4246-54; with permission.

patients with an excess of blasts showed an OS at 5 years of only 25–28% despite HSCT.

Prospective evaluation of the contribution of dynamic changes, such as progression of cytopenia, acquisition of cytogenetic abnormalities, and transfusion dependence to outcomes for HSCT are required and will help to clarify the optimal timing for transplantation with respect to lower risk MDS. It is hoped that the identification of newer prognostic markers, using cytogenetic,[32-34] immunophenotypic,[34] and molecular techniques, such as high density single nucleotide polymorphism (SNP) analysis, gene-expression profiling,[35] and other molecular markers, such as EZH2, DNMT3A, and ASXL1[36] mutations will enable further improvement in terms of risk stratification, particularly for patients with low-risk disease.[37]

Requirement for cytoreductive therapy prior to hematopoietic stem cell transplantation

The persistent risk of relapse of MDS post HSCT remains the major factor undermining the potential for long-term cure provided by this treatment modality. Whilst RIC HSCT has extended the applicability of HSCT to older patients with MDS, this is offset by an apparent greater risk of relapse seen with RIC regimens. Prospective data is lacking, but several groups have reported a higher relapse rate following RIC HSCT.[10,38,39] In a retrospective comparison of 836 MDS patients undergoing RIC versus SMC matched sibling HSCT, the 3-year relapse rate was significantly increased in patients undergoing RIC (cumulative incidence 45% vs. 27% for patients undergoing SMC) but the low NRM in this group resulted in similar OS between RIC and SMC patients. Advanced phase disease and patients not in CR at transplantation also had a significantly increased risk of relapse. Bearing in mind that increasing RIC HSCT are likely to be performed for patients with MDS, it would seem reasonable to consider ways to prevent relapse, either by disease control prior to HSCT or by the adoption of measures post transplant to prevent relapse in those at greatest risk.

A recent EBMT study[10] described earlier (Tables 1 and 2), evaluating outcomes for 1,333 patients over 50 years

who underwent either RIC or SMC HSCT, showed no adverse effect of age on outcomes but at multi-variate analysis, both use of RIC and advanced disease stage (defined as >5% bone marrow blasts) at HSCT resulted in significantly increased relapse rates. Advanced disease stage at transplantation and use of an unrelated donor were significantly associated with increased NRM; most importantly, advanced disease stage at transplantation alone was the major independent variable associated with an inferior OS at 4 years. Other groups have previously demonstrated the adverse outcome associated with HSCT in the RIC setting, where patients are not in CR at transplantation.[13,40,41]

There are no prospective randomized controlled trials to determine the potential benefit of treatment with intensive induction chemotherapy prior to HSCT and data are derived mainly from small, single institution studies. Existing data are usually retrospective and gathered mainly from the setting of SMC HSCT and from small, single institution studies. Scott *et al*. found no benefit with respect to outcomes post SMC HSCT for patients with advanced MDS who did receive induction chemotherapy (IC) versus those who did not.[42] In their analysis, 18 of 33 patients who received IC pre-HSCT achieved complete remission and of these, 5 patients relapsed prior to transplantation. Ninety-two patients who did not undergo IC showed a relapse-free survival (RFS) of 26% at 3 years (13% for those who did undergo induction chemotherapy) and the difference was not significant, possibly due to the small sample size.

Oran *et al*. reported on 30 high-risk MDS patients, of whom 23 patients received IC followed by RIC HSCT, along with 82 AML patients.[43] In this small retrospective study, more than 50% of MDS patients had chemo-refractory disease. OS estimates at 2 years were 66% for patients in CR at HSCT, 40% in the presence of active disease but no circulating blasts, and 23% in patients with blasts detectable in the peripheral blood at the time of HSCT; 2 year cumulative rates of relapse in the same groups were 15%, 20%, and 46%, although follow-up was short (median 29.4 months). A high NRM was seen in those with active disease: 25–30% at 100 days and 35–65% at 2 years. At uni-variate analysis, the presence of circulating blasts was the sole factor significantly associated with disease progression – HR was 3.7 compared with those in CR (95% CI 1.4-9.8, p=0.01).

The lack of randomization between pre-HSCT IC treatment or no therapy renders such retrospective analyses difficult to interpret and biases are likely to operate. Certain patients will have been selected to undergo IC for particular reasons (for example, the perception of more aggressive disease) and as such, those patients who have chemo-sensitive disease may be "selected out" preferentially prior to HSCT. The response to IC needs to be sufficiently durable to persist until HSCT: such patients with advanced MDS are at high risk of relapse during the intervening period between remission induction and HSCT. Additionally, conventional IC carries the risk of death during treatment or causing toxicity, which may then prohibit the HSCT procedure.

For this reason, newer agents shown to be effective in treating MDS have been employed to de-bulk disease prior to HSCT without causing significant toxicity. 5-Azacytidine is one such agent used in this manner. A retrospective study of 54 patients with intermediate-high risk MDS or CMML who underwent sibling or unrelated donor HSCT included 30 patients who had received a median of 4 courses of 5-Azacytidine pre-HSCT and 24 who received no chemotherapy or induction chemotherapy. CR was only achieved in 4 of 30 patients who received 5-Azacytidine with PR in 10; 6 of these 30 patients progressed to AML and of these, 4 patients received standard induction therapy. At 2-years post transplant, the cumulative incidence of relapse was 31% in those who received 5-Azacytidine and 36% in those who did not. Thus, treatment with 5-Azacytidine was not demonstrated in this study to significantly affect remission rates, relapse rates or OS but has not as yet been evaluated prospectively. Decitabine has also shown feasibility as a pre-HSCT induction therapy but in a small study, with no clear evidence of improvement in outcomes following HSCT.[44] Many unanswered questions remain. For example, for those patients who do respond to hypomethylating agents prior to HSCT, when is the optimal timing for HSCT – at the time of best response or when no longer responding/disease progresses? Data from prospective trials is required.

Management of relapse

Despite the feasibility of HSCT even in older patients with MDS, the principal concern for transplant physicians and patients alike is the persistent potential for relapse. There is very little reported data to enable evidence-based decision-making regarding treatment of relapsed MDS post HSCT. Many of the larger retrospective multi-centre studies will report DFS and RFS but the details of therapy given to relapsing patients may be lacking. The two key issues of management of mixed chimerism and hematological relapse are discussed here.

Mixed donor/recipient chimerism

Donor and recipient chimerism may be closely followed to enable early identification of falling donor T cell chimerism and to guide withdrawal of immunosuppression along with the timing of administration of donor leucocyte infusion (DLI), although there is a paucity of data published in this area. Delayed attainment of donor chimerism is a recognized feature particularly of T-cell-depleted RIC transplants[45-47] and DLI can be given to improve low donor chimerism. We have previously reported, in a prospective study from our institution, on 10 patients (out of 75, all of whom underwent RIC HSCT for MDS, with a uniform fludarabine, busulphan, and alemtuzumab (FBC) containing regimen) and were treated with escalating doses of DLI for mixed chimerism after day 100. In 9 of 10 patients, full donor chimerism (FDC) was achieved.[46] This data was subsequently updated and identified 28 of 110 patients who had undergone FBC conditioned RIC HSCT for MDS and demonstrated falling donor chimerism. These patients received a median of two doses of DLI ($5x10^5$ CD3/kg and $1x10^6$ CD3/kg) and 17 of 28 subsequently attained FDC. Ten of 28 patients developed graft-versus-host disease (GvHD); 2 of those 10 patients succumbed to GvHD and 1 to relapsed disease despite

attainment of FDC post DLI. Interestingly, this study identified that patients with delayed attainment of FDC at day 100 showed superior DFS and OS compared with those with FDC by day 100 but similar RFS to those with delayed FDC. Deaths in those with early FDC were mainly attributable to GvHD or post-transplant complications, such as infection.[47]

In practice, with the limited objective data available to guide timing and dosage of pre-emptive DLI for falling donor chimerism, local policy will operate regarding institution of therapy. Additionally, the use of a variety of RIC regimens by different transplant centers means that treatment decisions should be guided by review of local outcomes; better reporting and if possible, prospective studies would add greatly to this field.

Hematological relapse

Data addressing management of patients relapsing with MDS HSCT is scant; however, in general, the long-term outlook for these patients is poor. Remissions, if attained, are frequently transient. The main options for therapy (following withdrawal of immunosuppression) in this setting comprise DLI, chemotherapy, second HSCT and supportive care only. Treatment with DLI alone achieves (often temporary) responses in a minority of patients with clear morphological relapse[48] and therefore, some form of induction chemotherapy is usually desirable in addition to DLI to improve outcomes but has not been the subject of prospective studies. In a report by Oran *et al*. of 63 patients relapsing with MDS or AML post RIC HSCT, therapy consisted of chemotherapy for aggressive relapse or DLI/second HSCT for more indolent relapses – response rates were in the region of 40–50% for chemotherapy or second HSCT but no patients responded to DLI alone.[49] Remission re-induction with chemotherapy may be consolidated by DLI or a second transplant – in the handful of patients who have received a second transplant in this setting, long-term remissions can be seen (2-year actuarial survival 50% reported in this study, where 18 patients received a second HSCT) but may be offset by increased TRM from a second procedure, for example, due to GvHD.[49]

Standard induction chemotherapy may be used for re-induction, but with the advent of newer therapies for MDS, could these agents be effectively employed following HSCT to treat and even prevent relapse? There is emerging data from a Phase I trial of low dose 5-Azacytidine post HSCT[50] supporting its tolerability and potential for treatment of relapsed/refractory MDS, and it has also been used in a small group of patients at risk of relapse as a maintenance therapy,[50,51] with a dose of 32 mg/m^2 for 5 days and four cycles shown to be both tolerable and safe.[51] However, larger scale, prospective, and randomized studies are required to evaluate the efficacy of these novel agents in such a setting in the future. Other treatments, which may come to the forefront of future therapy for relapsed myeloid disease post HSCT, include immunotherapeutic strategies, for example, peptide vaccination targeting leukemia-associated antigens, such as the Wilm's Tumor protein (WT1)[52-54] and whole cell leukemia vaccination with CD80 and IL-2 genetically modified leukemic blasts,[55-57] which is currently the subject of a Phase I clinical trial at our institution.

Conclusion

The landscape of therapy for MDS has undergone considerable change over the last several years with the advent of RIC HSCT and as a result of the increasing arsenal of therapeutic agents that may be employed. Improvements in prognostication increase confidence in decision-making in this area, and with discoveries in the fields of genetics and flow cytometry, this continues to evolve. However, along with these innovations come new challenges and questions to be answered. The lack of good quality data from prospective, randomized, controlled clinical trials results in conflicting data garnered from small, single institution studies. The focus of future studies in transplantation for MDS should be to strive towards the initiation of prospective trials to answer the most pertinent questions for the patient with MDS considering HSCT: what, when, and how?

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Molecular basis of myeloproliferative neoplasms

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A B S T R A C T

The molecular basis of myeloproliferative disorders (MPDs) involves constitutive or aberrant JAK-STAT signaling that leads to hypersensitivity or independence of myeloid progenitors from cytokines and malignant proliferation. Polycythemia Vera (PV) and Essential Thrombocythemia (ET) are characterized by excessive formation of mature red blood cells and platelets, respectively, while myelofibrosis appears as a scarring and fibrosis of the bone marrow, presumably as a consequence of abnormal myeloid progenitor proliferation. The unique acquired somatic mutation JAK2 V617F is present in more than 95% of PV patients, while exon 12 JAK2 mutations (around residue K539) are at the basis of the remaining 3–5% PV patients. In contrast to PV, only 40–50% of ET and PMF patients harbor JAK2 V617F and 8–10% are associated with activating mutations of the thrombopoietin receptor. Mutations in genes coding for down-modulators of the JAK-STAT pathway (LNK), the ras pathway (NF1), or cytokine/tyrosine kinase receptors (c-CBL) have been detected in a minority of MPN patients. Approximately 15% of MPN patients share mutations in epigenetic regulators with myelodysplasia and acute myeloid leukemia. Critical to progress in the MPN area will be the understanding of pathologic signaling in disease-initiating hematopoietic stem cells and of global chromatin effects of constitutive active STAT proteins.

Introduction

Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) have been grouped together with chronic myeloid leukemia (CML) under the entity of myeloproliferative disorders (MPDs) by William Dameshek[1] and more recently under the Myeloproliferative Neoplasm (MPN) entity.[2] PV, ET, and PMF are diseases initiated in the hematopoietic stem cell (HSC)[3,4] or in a myeloid progenitor that acquires HSC- and cancer-initiating stem cell features,[5] with a clinical picture given by a dramatic amplification of the myeloid compartment. MPNs can evolve to a very severe and treatment-resistant acute myeloid leukemia, and the precise cell type transformed at this blast stage is debated.

Identification of JAK2 V617F

The gene coding for Janus kinase 2, located on chromosome 9, became a candidate gene to be studied in detail in the pathogenesis of MPNs for several reasons. First, it had been shown that a JAK2 inhibitor was able to block Epo-independent colony formation from PV patients.[6] Second, in PV, genetic defects had been noted in chromosome 9.[7] Third, other tyrosine kinases, such as ABL and PDGFR, had been found to induce malignant proliferation in the form of fusion proteins. Fourth, JAK2 had been shown to exert a chaperone and stabilization effect for the maturation and traffic of the thrombopoietin receptor (MPL, TpoR), a process defective in MPN patients.[8]

A major advance came in 2005 with the discovery that the vast majority of MPN patients harbor a unique (V617F) acquired somatic mutation in the pseudokinase domain of JAK2.[9–12] The mutant was able to induce cytokine-independence of hematopoietic cell lines and to signal in the absence of cytokines. In such JAK2 V617F-expressing cell lines, one could demonstrate constitutive activation of STAT5, STAT3, MAP-kinase Erk1,2 phosphatydylinositol-3'-kinase, and Akt. Interestingly, overexpressing the wild type JAK2 was shown to prevent cell proliferation and signaling,[9] suggesting that JAK2 and JAK2 V617F compete for a limiting amount of cellular factor. Careful analysis demonstrated that at low levels of expression, co-expression of a dimeric cytokine receptor, such as the erythropoietin receptor (EpoR), TpoR, or G-CSFR, is required for transformation, while at higher levels of expression, endogenous JAK2-utilizing receptors can support transformation.[13] JAK2 V617F requires interaction with a cytokine receptor for oncogenic signaling, since mutation in the NH2-terminal FERM-like (band 4.1, ezrin, moesin, radixin) domain (Figure 1A), which binds to receptors, inhibits signaling.[14]

JAK2 V617F was discovered to be a preferred client of the Heat shock protein 90 (Hsp90) chaperone, and an inhibitor of Hsp90 was shown to destabilize JAK2 V617F.[15] Heat shock protein 90 (Hsp90) is an

ubiquitous chaperone that promotes ATP-dependent protein folding and has previously been shown to stabilize several oncogenic forms of tyrosine and serine threonine kinases.[16] An Hsp90 inhibitor, PU-H71, inhibited proliferation of JAK2 V617F cell lines and showed efficacy in a mouse model of MPN, with a reduction of the JAK2 V617F allele burden in mice. Complex formation between JAK2 V617F, Hsp90, and the inhibitor led to degradation of the JAK2 mutant.[15] The wild type JAK2 was not degraded in other tissues, suggesting that JAK2 V617F is a preferred client of Hsp90. Importantly, Hsp90 and JAK2 association can be demonstrated irrespective of the V617F mutation or of phosphorylation status, but degradation by the purine scaffold PU-H71 inhibitor appeared specific for mutant JAK2 and occurred via the proteasome pathway.[15] The precise E3 ligase required for JAK2 V617F ubiquitinylation remained unknown. Again, these data suggest that the conformation of JAK2 V617F might differ substantially from that of the activated wild type JAK2, but no structural data exist at the moment to substantiate this hypothesis.

Mechanisms of JAK2 V617F activation

The V617F mutation is located in the pseudokinase domain of JAK2 (Figure 1A), which was shown *in vitro* to prevent activation of the kinase domain. No crystal structure exists of the pseudokinase domain of any JAK or of an entire JAK protein.[17] The kinase domain of JAK2 was crystallized,[18] but how the pseudokinase domain triggers activation of the kinase domain remains speculative.[19] Since JAKs form functional units with cytokine receptors both in the cells and on the cell surface, a structure of the entire receptor-JAK complex will be required for understanding the mechanism of JAK2 V617F activation at atomic level. Several other residues, besides Phe, can activate at V617 (Trp, Leu, Ile, Met), pointing to large hydrophobic features triggering activa-

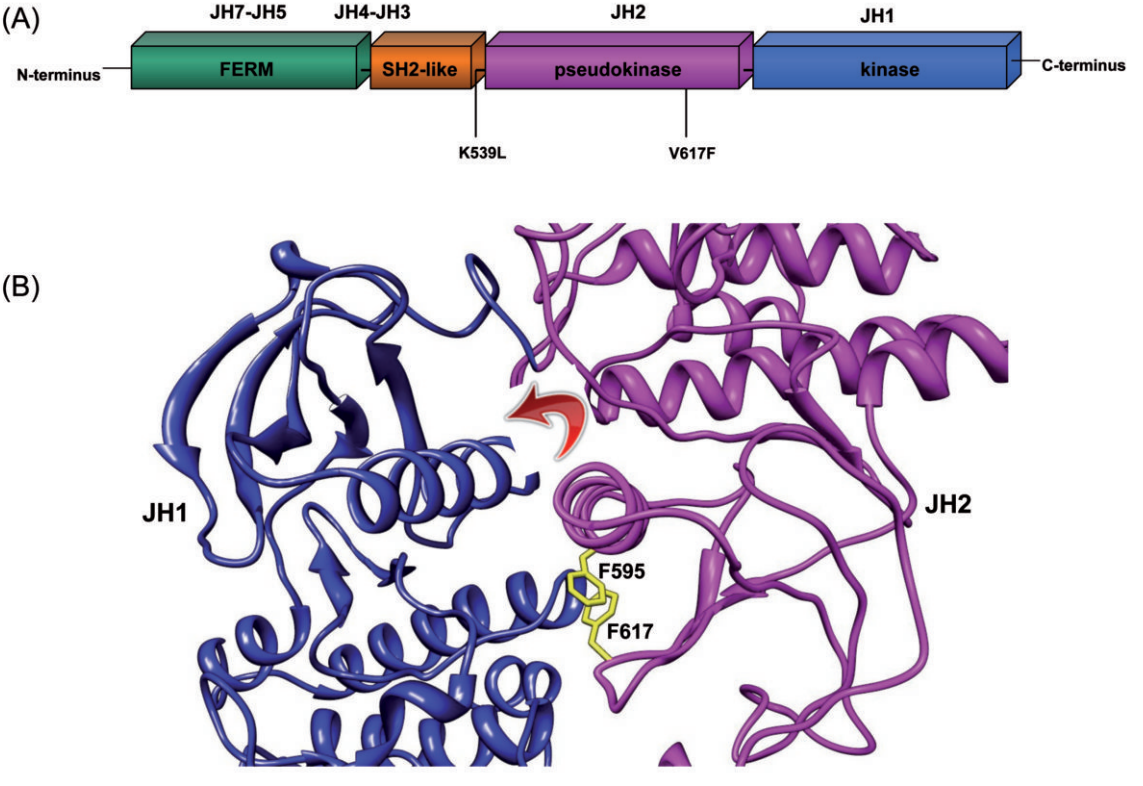

Figure 1. Structure of JAK2 and a model for JAK2 kinase domain activation by the pseudokinase V617F mutation.
(A) Janus kinase 2 contains several JAK homology (JH) domains: JH1, the kinase domain; JH2 the pseudokinase domain; after JH3-JH4 the SH2-like domain; and JH4-JH7, the FERM (band four point 1, ezrin, radixin, moesin)-like domain. The pseudokinase domain plays a major role in cytokine-dependent activation of the kinase domain, and was implicated in inhibiting the basal activity of the JH1 domain. The V617F mutation is activating the kinase activity of JH1, presumably by preventing the inhibition exerted by JH2 on JH1. Mutations in the linker between the SH2-like and the pseudokinase domain, usually around K539, also lead to activation of JAK2. The V617F mutation is detected in 98% of PV and approximately 50% of ET and PMF patients, while mutations around K539 (denoted exon 12 mutations) are detected in 2% of PV patients that do not harbor JAK2 V617F.
(B) The pseudokinase (JH2, pink) and kinase (JH1, dark blue) domains of JAK2 are modeled as adopting classical tyrosine kinase structures, interacting with each other.[19] Residue F595 of the helix C of JH2 is required for constitutive activation of JAK2 V617F and of other mutated JAKs proteins, but not for cytokine activation of wild type JAK2. F595 plays a pivotal role in transmitting the conformational change in JH2 to JH1 and eventually in activating the kinase activity of JH1. The region around V617F and F595 might be targeted by inhibitors that specifically decrease constitutive activation of JAK mutants.

tion.[20] While V617W would require three base pair changes, the V617L or V617I are weaker mutations that can be obtained by one base pair change; very rare MPN patients with V617I have been reported.[21]

The consequences of V617F mutation are ligand-independent activation of kinase activity,[22] and an unexpected decrease in K_M for substrates.[23] Modeling of the pseudokinase domain of JAK2 on structures of active kinases and functional assays point to a mechanism of activation where the V617F mutation requires a particular residue of the helix C of JH2, namely F595.[24] Only aromatic residues at F595 can support constitutive activation by JAK2 V617F, while mutants, such as F595A, lose constitutive activation without losing the ability to respond to cytokine-activated cytokine receptors.[24] The same residue was identified by computational approaches to maintain JAK2 inactive and promote activation in JAK2 V617F.[25] Interestingly, F595 mutations also decrease constitutive activation of JAK2 due to the exon 12 mutation K539L, or to the hinge R683G mutation, suggesting that F595 is pivotal for JAK2 activation (Figure 1B) in the absence of the large conformational change induced by cytokine receptor rotation provoked by cytokine binding.[24] Taken together, these data suggest that mutated JAKs can possibly be inhibited by targeting their pseudokinase domains. JAK1 and possibly TYK2 are predicted to behave similarly as JAK2 since the homologous V617F mutation activates both JAK1 and TYK2,[26] and since the homologous F595 residue in JAK1 is required for constitutive activation of JAK1 V658F.[24]

The current model of JAK2 V617F signaling posits that at the cell surface, complexes between JAK2 V617F and EpoR (erythroid cells), TpoR (megakaryocytes), and G-CSFR (granulocytic progenitors) signal autonomously, thus leading to a trilineage expansion (Figure 2). For the EpoR, inactive dimeric orientations[27] or inactive point mutants could support, like wild type EpoR, signaling by JAK2 V617F.[13,24] Thus, receptors must scaffold mutated JAK2, but the activation of the complex is triggered by the latter, and not by the receptor, as in the physiologic situation with cytokines. However, JAK2, and most likely also JAK2 V617F can interact with other JAK2-utilizing receptors, such as the beta chain of IL-3, the interferon gamma receptor 2 (IFNγR2), or gp130.[28]

Exon 12 JAK2 mutations

A minority of PV patients does not harbor JAK2 V617F, but carries mutations in exon 12 around residue K539.[29] Various deletions, insertions, and mutations are acquired in this region, which links the SH2-like and the JH2 domains of JAK2 (Figure 1A). Modeling suggests that these mutations induce activation of JAK2 kinase domain via a mechanism resembling that of V617F, given the space proximity between K539 and V617.[29] In contrast to JAK2 V617F, however, exon 12 JAK2 mutations have initially been detected only in Epo-independent erythroid colonies and not in granulocytes, and it

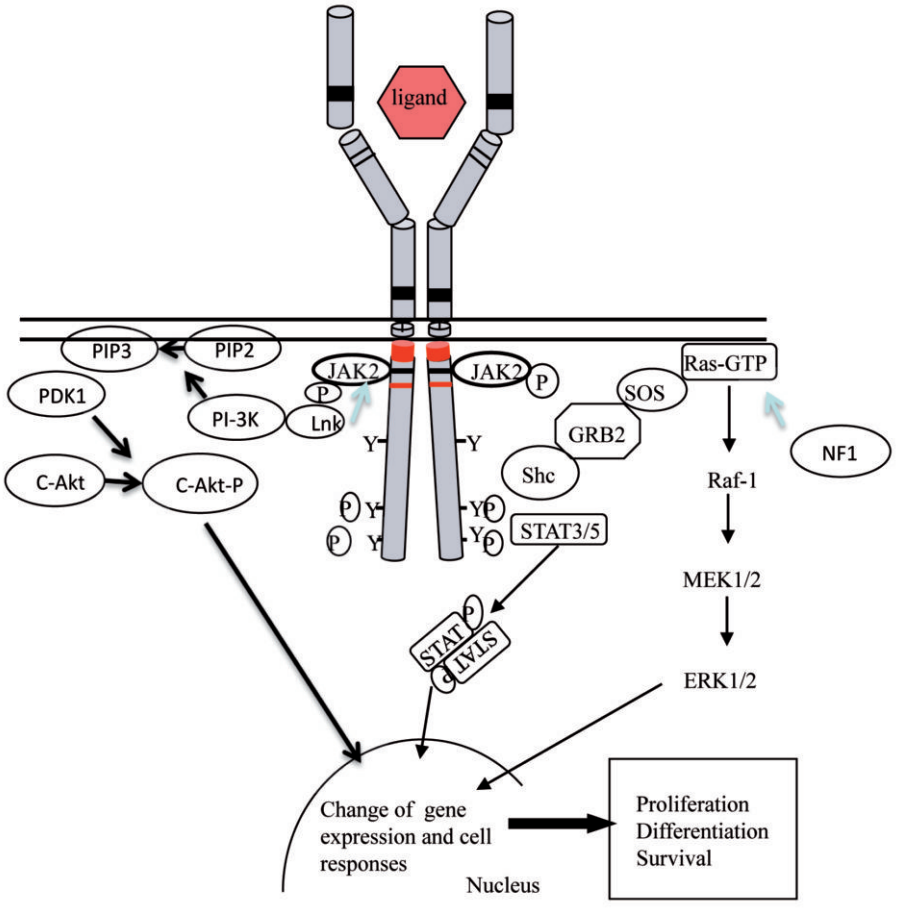

Figure 2. Homodimeric cytokine receptors are usually preformed dimers that change their orientation in response to ligand binding, adopting a conformation productive for JAK2 trans-phosphorylation and activation. In turn, activated JAK2 phosphorylates itself and the cytosolic tyrosine residues, which then attract SH2-(src homology 2) containing signaling proteins. Signal transducers and activators of transcription (STAT)-3 and 5 are thus attracted to the receptors and become themselves targets of JAK2 phosphorylation, which leads to their dimerization and translocation to the nucleus, where they regulate gene expression. The MAP-kinase pathway is activated via recruitment of the Shc adaptor and activation of ras signaling, which is counteracted by NF1 (Neurofibromatosis 1), a protein that stimulates the GTPase activity of ras. The phosphatydylinositol-3'-kinase (PI-3'-kinase) is also activated by cytokine receptor signaling. Negative regulators, such as LNK, and Suppressors of Cytokine Signaling (not shown) bind to JAKs and inhibit their signaling. Mutated forms of JAK2, such as JAK2 V617F, are presumed to activate signaling in complex with cytokine receptors in the absence of cytokine binding to the extracellular domain.

was thought that they are linked to a pure erythrocytosis phenotype. However, exon 12 JAK2 mutation patients develop thrombosis complications, secondary myelofibrosis, and leukemia similarly to those harboring JAK2 V617F.[30]

Thrombopoietin receptor mutations

Approximately 8–10% of ET and PMF patients that do not harbor JAK2 V617F carry mutations in the TpoR. Fascinatingly, most mutations seem to concern W515,[31,32] a tryptophan residue located in a cytosolic juxtamembrane amphipathic sequence (RWQFP (Figure 3)), that maintains the receptor inactive in the absence of Tpo.[33] Mutations of W515 to Leu, Lys, Ala, Arg have been reported, and in patients it was shown that TpoR W515 mutants occur at the HSC stage.[34] That many mutations at W515 activate the receptor indicate that it is the loss of Trp515 and not the acquisition of various residues that leads to activation. In bone marrow murine reconstitution experiments, TpoR W515L and TpoR W515A were shown to induce a rapid, fatal MPN with myelofibrosis, a phenotype that is much more severe than that induced by JAK2 V617F.[32,35] In fact, TpoR W515 mutants are probably the fastest myelofibrosis-inducing oncogenes known. Using mass spectrometry, it was shown that two cytosolic TpoR residues, Y592 and Y626, were found to be phosphorylated in cells transformed by TpoR W515A, and that these two residues play opposite roles in transformation.[35] The Y592F mutation leads to hyperactivation of JAK2 by TpoR W515A, while the Y626F mutation (or Y112F counting only intracellular residues), in contrast, abolished phenotype, without inhibiting JAK2 activation.[35] These results indicated that myelofibrosis induction requires pathways downstream of Y626, and indeed proteomics approaches showed that TpoR W515A induces excessive MAP-kinase Erk1,2 and STAT3 signaling.[35] A small molecule inhibitor of signaling by TpoR Y626 could be a new approach in the treatment of MPNs with mutated TpoR.

Familial and sporadic ET patients have been found to harbor with low frequency the transmembrane S505N activating mutation,[36–38] which leads to constitutive activation of TpoR. Another TpoR mutation, located in the extracellular juxtamembrane region at a position symmetrical to W515, namely T487A, was shown to induce a myeloproliferative phenotype *in vivo*, and has been detected in a non-Down syndrome childhood acute megakaryoblastic leukemia.[39] Overall, it is clear that the juxtamembrane and transmembrane regions of TpoR are true switch regions that can induce receptor activation (Figure 3).

At present, it is unknown why TpoR mutants induce a more severe and rapid phenotype when compared with JAK2 V617F. Signaling by TpoR W515A may differ from Tpo-activated TpoR, especially at the HSC level, where normal TpoR is responsible for maintaining quiescence, an effect opposite to its role in megakaryocyte proliferation and differentiation.[40–42] Thus, signaling or conformation by TpoR might not be similar in HSCs and megakaryocytes. Further experiments with knock-in mice will be interesting to understand the precise role of TpoR W515A and of megakaryocytes in the development of myelofibrosis.

Down-modulation of TpoR in MPN patient megakaryocytes and platelets

A feature of many MPN patients is down-modulation of TpoR cell surface and total levels in megakaryocytes and platelets, without down modulation of TpoR mRNA[8] (Figure 4). Examination of TpoR metabolism pointed to defective maturation, accumulation of glycosylation-immature (Endoglycosidase H-sensitive) TpoR,[43] characteristic of endoplasmic reticulum retention or

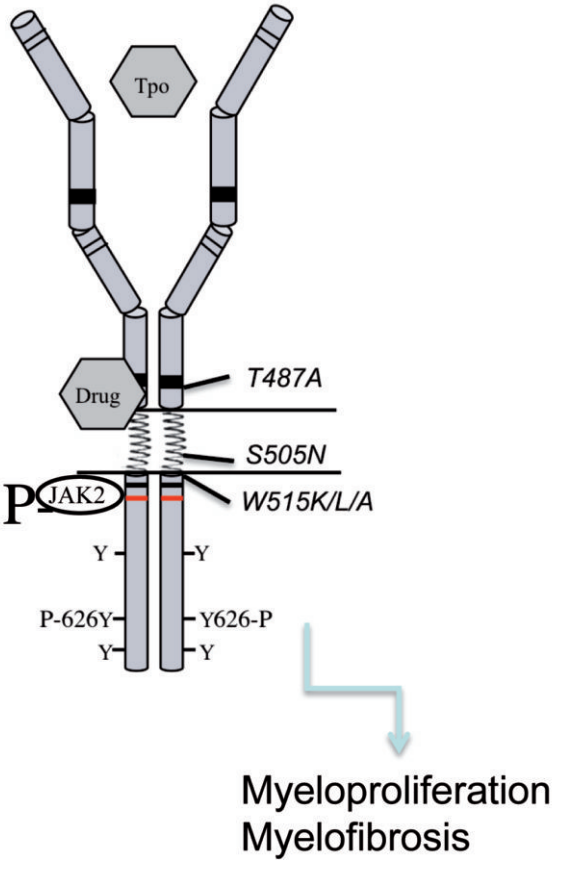

Figure 3. Several activating mutations in the thrombopoietin receptor (c-MPL, TpoR) have been identified in myeloproliferative disorders. Mutations cluster around the juxtamembrane and transmembrane domains. Most frequently W515 of the cytosolic juxtamembrane domain is mutated to Lys, Leu or Ala. W515 is part of a short amphipathic region that normally prevents self-activation of the receptor. Mutation of transmembrane S505 to Asn is also found in familial and sporadic MPNs. Mutation of a residue symmetrically located on the extracellular juxtamembrane region to W515, T487 to Ala also leads to constitutive activation of TpoR and induces a MPD in mouse reconstitution experiments. Furthermore, the human TpoR can be activated by small molecules that bind to the extracellular juxtamembrane region, further supporting the notion that this region can switch on receptor signaling. These mutations, especially W515K/L/A lead to constitutive activation of the receptor, activation of JAK2 and phosphorylation of Y626 of the cytosolic domain, which is required for induction of MPN and myelofibrosis *in vivo*.

reverse traffic from cis-Golgi to ER. JAK2 V617F allele burden inversely correlate with TpoR down-modulation, but this phenomenon can also be detected in patients that are negative for JAK2 V617F or positive for TpoR W515 mutations.[44,45] The reasons behind this anomaly are being explored. Given that in certain settings, TpoR signaling induces senescence and antiproliferative effects,[46] part of receptor down-regulation might be due to negative selection. On the other hand, JAK2 V617F appears to exert opposite effects on proteasomal degradation of TpoR from those of wild type JAK2 (Pecquet *et al.*, 2011 personal communication), which promotes receptor recycling[47] and surface localization.[48] It is yet to be determined whether receptor down-modulation is limited to late megakaryocytes and platelets, or is also manifest at the level of HSCs or CD34-positive cells.

Down-modulation of TpoR levels at the platelet and late megakaryocte levels, where Tpo is cleared from plasma, can by itself induce pronounced thrombocytosis.[49,50] This result has been obtained when c-Mpl (TpoR)-/- mice were rescued with transgenic constructs that utilized a proximal TpoR promoter that allowed TpoR expression at HSCs and early megakaryocyte levels, but was not sufficient to ensure physiologic levels of TpoR expression at late megakaryocyte and platelet levels. Because clearance of Tpo was defective, and early megakaryocyte progenitors could be stimulated by higher than normal Tpo levels, the phenotype induced was thrombocytosis.[49,50] Furthermore, the down-modulation of TpoR by JAK2 V617F might not only circumvent Tpo-induced senescence,[46] but may contribute to Tpo-induced proliferation through lower levels of cell surface TpoR. One prediction that can be made is that mutation in chaperones or traffic molecules that regulate TpoR expression might be detected in ET patients that do not harbor known mutations.

Another means of down-modulating TpoR levels is represented by induction of expression of a microRNA (miR-28) that targets for inhibition of translation the 3'-UTR (untranslated region) of TpoR (Figure 4).[45] miR-28 was reported to be overexpressed pathologically in platelets from 30% of MPN patients, especially those with high JAK2 V617F allele burden, or in ET patients negative for JAK2 V617F exhibiting high platelet numbers.[45] In addition to TpoR 3'-UTR, miR-28 also targets for translational inhibition several other mRNAs that code for proteins that regulate megakaryocyte differentiation.[45] As expected, expression of miR-28 in CD34-derived megakaryocytes led to inhibition of differentiation. Induction of miR-28 expression in cell lines depends on induction of expression of the host gene, Lipoma Preferred Partner (*LPP*).[45] Taken together, biochemical determinations on TpoR metabolism and

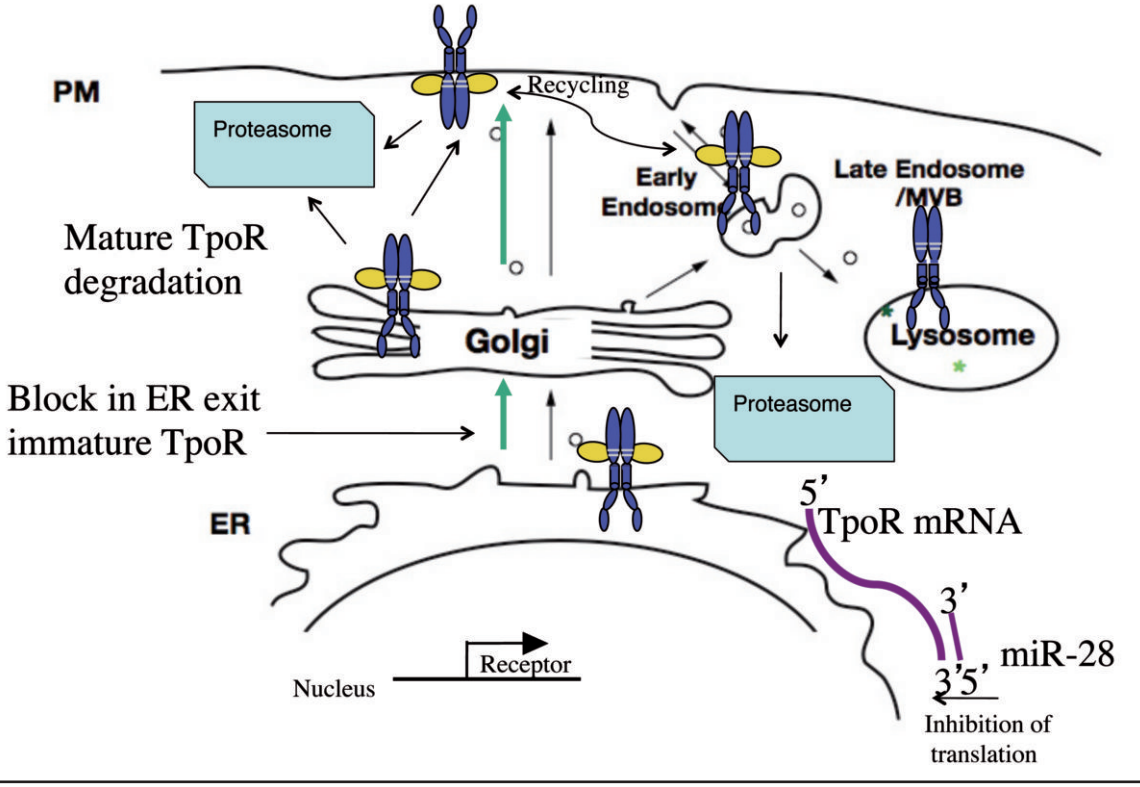

Figure 4. Several mechanisms promote thrombopoietin receptor down-modulation in MPN patients. Induction of expression of miR-28 by constitutive active STAT5 leads to inhibition of translation of MPL mRNA. Upon translation, TpoR (MPL) appears to remain in an immature glycosylated state (Endoglycosidase H sensitive) either in the ER, in the ER-to-Golgi intermediate compartment, or in the cis-Golgi. The receptor is coupled to JAK2 (yellow) intracellularly as a complex that traffics to the cell surface. The fate of this immature receptor is unknown, but one possibility is that it is degraded either by the proteasome or the lysosome. Cell surface TpoR (MPL) is known to recycle and this process is strongly stimulated by JAK2. Degradation of the receptor occurs via the proteasome and lysosome pathways. In MPN patients, both cell surface and total MPL levels are decreased. PM, plasma membrane; ER, endoplasmic reticulum, MVB, multivesicular bodies.

pathologic induction of miR-28 in MPNs suggest that several mechanisms are operating in patients to reduce levels of TpoR.

Negative signaling regulators: LNK, c-CBL, NF1, and SOCS proteins

Cytokine signaling involves tight dependency on cytokine binding to cytokine receptor extracellular domains in order to induce activation of JAKs and downstream signaling via STATs, MAP-kinase, PI-3'-kinase, and Akt. In the case of acute cytokine signaling, rapid induction of negative regulators, such as Suppressors of Cytokine Signaling (SOCS), Protein Inhibitors of STATs (PIAS), or activation of phosphatases and ubiquitin ligases concur to extinguish signaling. In the presence of mutated JAK2 or of mutated receptors, persistent signaling occurs, and it appears that these mechanisms are overwhelmed and cannot efficiently oppose continuous signaling.

A minority of MPN patients harbor LNK mutations,[51] and LNK appears to be play an important role in MPNs, as its level of expression in the absence of mutations correlates with JAK2 V617F allele burden in MPN patients.[52] Coded by a gene on chromosome 12q24.12, SH2-B (SH2B1) together with APS (SH2B2) and LNK (SH2B3) form a family of signaling adaptors that regulate signaling by several cytokine and growth factor receptors, the JAK-STAT pathway, myelopoiesis, and lymphopoiesis.[53,54] Particularly LNK has been shown to down-modulate KIT receptor tyrosine kinase signaling,[55] Tpo, and Epo signaling[56,57] and to decrease JAK2 V617F or TpoR W515L signaling.[58] LNK also restrains the phenotype of JAK2 V617F-induced MPD. All family members share a domain structure represented by an NH2-terminus dimerization domain, and proline rich motifs, a pleckstrin homology domain, and SH2 domain and a C-terminus domain containing a conserved tyrosine residue.[53] LNK binds to JAK2 V617F stronger than to wild type JAK2 both via the SH2 domain and a site in its NH2-terminus.[52] This would indicate that either the conformation of JAK2 V617F is different than that of wild type JAK2, or that constitutive signaling promotes LNK-JAK2 V617F complex formation. Without crystal structures of the full-length and mutated JAK2, it would be very difficult to understand the exact basis for this difference. Interestingly, in patients with MPNs, the levels of LNK expression correlated with the JAK2 V617F allele burden and LNK suppresses signaling by JAK2 V617F.[52] It is tempting to speculate that LNK exerts a negative pressure in signaling by constitutive active JAK-STAT, and that the extent of negative regulation and the mechanisms invoked to select against this pressure might be important for driving the precise phenotype of MPN in patients.

Two different LNK mutations were reported in JAK2 V617F-negative patients: one is a truncation and the other a missense mutation (E208Q) in the pleckstrin homology domain.[51] Other LNK mutations have been detected in JAK2 V617F-negative erythrocytosis[60] and leukemic transformation of MPN, with a higher frequency of 13%.[61]

CBL (Casitas B-cell lymphoma) proteins are ubiquitin ligases with major regulatory roles in receptor tyrosine kinase traffic and signaling termination.[62,63] c-CBL was also suggested to contribute to ubiquitinylation of TpoR cytosolic lysines.[64] c-CBL knock-out and double CBL and CBL-b deficient mice exhibit mild and severe MPN phenotypes, respectively.[65] c-CBL mutations have been reported in a low percentage of PMF (6%),[66] and in certain cases, c-CBL mutations are acquired during progression to leukemia.[66,67]

A high resolution 250K Single Nucleotide Polymorphism (SNP) array study on 151 MPN patients revealed microdeletions in the region encompassing the tumor suppressor NF1 gene (Neurofibromatosis 1) in two secondary myelofibrosis patients.[68] In one patient, the second allele was also mutated leading to bi-allelic loss of NF1.[68] NF1 is a well established negative regulator of ras signaling, and its inactivation leads to a progressive myeloproliferative disorder in mice.[69] Although not clear at this moment whether NF1 would play a specific role in MPNs, the presence of microdeletions in a substantial number of myelofibrosis patients[68] suggests that with highly sensitive genomics techniques, more alterations will be identified in the future.

SOCS proteins are negative regulators of JAK-STAT proteins due to their ability to bind to, inhibit, and target for ubiquitinylation JAK proteins. SOCS3 is recruited to certain cytokine receptors, such as EpoR and G-CSFR, via binding of their SH2 domains to phosphorylated receptor tyrosine residues, which triggers ubiquitinylation of receptor (for G-CSFR) lysine residues and lysosomal degradation, with an overall decrease in signaling.[70-72] Signaling by TpoR is down-modulated by SOCS1,[73] while SOCS3 could inhibit Tpo-induced megakaryocte colony growth, but not constitutive megakaryocyte growth in patient megakaryocytes.[74] Hypermethylation of promoter CpG islands and decreased expression of SOCS1 or SOCS3 have been reported in a minority of MPN patients.[75,76] Biochemically, it was shown that SOCS3 can inhibit JAK2 V617F signaling in an inducible 293 cell system.[77] In contrast, in transformed Ba/F3 EpoR JAK2 V617F or JAK2 K539L cells, where levels of expression of JAK2 mutants are higher, SOCS3 was unable to exert inhibitory functions, due to hyperphosphorylation of the SOCS3 SOCS box.[78,79] Whether the escape of cells from SOCS3 inhibition represents a qualitative difference or simply a quantitative issue due to persistent signaling, remains to be determined, but the fact is that the majority of MPN patients exhibit phenotype although SOCS3 is induced, and its phosphorylation can be a biomarker of overactive JAK2.[79] Furthermore, SOCS2 was shown to inhibit JAK2 V617F signaling, and its promoter is hypermethylated in some MPN.[80] Given that SOCS2 enhances degradation of SOCS3,[81] the picture might become more complex.

Stem cells in MPNs and the molecular basis of phenotype specificity

The key to understanding the pathogenesis of MPNs relies on the study of the mutated and non-mutated HSCs. The reasons for this are: i) HSC is the cell type where mutations like JAK2 V617F appear to be acquired;

ii) non-mutated and mutated HSCs coexist in the marrow of PV and ET patients,[82,83] but they are somehow inhibited, allowing clonal dominance of the mutated HSCs, possibly via the action of Tumor Necrosis Factor-α (TNF-α);[84,85] iii) in myelofibrosis, almost all HSCs belong to the mutated clone;[83] iv) xenotransplantation experiments with human JAK2 V617F-positive HSCs into immunocompromised mice and human bone marrow transplantation with JAK2 V617F-positive HSCs do not support a proliferative advantage for the mutated HSCs.[83,86] Similarly, in a knock-in JAK2 V617F model, apparently JAK2 V617F places HSCs at a disadvantage for blood reconstitution.[87] One yet unexplored scenario is that the MPN mutations can be acquired in different HSC subsets,[88] and that might influence disease latency and phenotype. For example, the recently described 'myeloid biased' subset[89] that increases with age and that is stimulated by TGF-β1 could be specifically targeted by mutations in myelofibrosis,[90] especially since TGF-β1 was already suggested to play a major role in myelofibrosis.[91]

Several hypotheses can be invoked to attempt to answer the central question of why one acquired mutation, JAK2 V617F, can induce three different diseases: i) gene dosage determines phenotype, as shown in transgenic mouse models[92] and in certain retroviral transduction models.[93] Although very attractive, the model of ET, PV, and PMF being induced by increasing JAK2 V617F kinase activity is not always seen in mouse models, where an erythrocytosis phenotype followed eventually by myelofibrosis is prevalent.[87,92–97] ii) individual SNP variations would favor a specific phenotype, for example, by favoring the interaction of JAK2 V617F with one of the three receptors: EpoR, TpoR, and G-CSFR.[98] iii) the TpoR might be specifically involved in inducing myelofibrosis since TpoR mutants induce rapid and severe MPNs with myelofibrosis in mice[32,35] and since strong megakaryocyte proliferation was reported to trigger myelofibrosis.[91,99–102] iv) depending on which type of HSC (myeloid-biased, lymphoid biased, or balanced)[90] acquires JAK2 V617F, a different phenotype would emerge. v) the recent discovery of mutations in genes, such as TET2[103–105] and several others, which give a clonal advantage to HSCs, makes it possible that acquisition of JAK2 V617F might give a different phenotype depending on pre-existent or subsequent mutations. Other factors must certainly exist that explain the phenotype specificity.

Recently, JAK2 V617F-positive individual patient Burst Forming Unit erythroid (BFU-E) colonies maintained in 0.01 U/ml Epo were compared between ET and PV patients with respect to *in vitro* gene expression profiles.[106] BFU-E colonies derived from ET patients expressed IFNγ- induced genes, due to constitutive activation of STAT1.[106] This was not the case for BFU-E colonies from PV patients, although the JAK2 V617F allele burdens were similar. The basis for this signaling difference via STAT1 remains to be identified. Expression of an activated form of STAT1 in normal CD34-positive progenitors induced an ET-like phenotype, whereas down-regulation of STAT1 activity in JAK2 V617F-heterozygous ET progenitors induced PV-like phenotype.[106] These studies demonstrated a key role for STAT1 signaling in preventing erythroid differentiation and favoring megakaryocyte differentiation.[106]

Signaling towards leukemic transformation

Exactly how chronic phase MPNs transform into acute myeloid leukemia (AML) remains unknown. While only 5–8% of ET and PV transform to AML, a larger proportion of PMF or of secondary MF (~18%) evolves to AML.[107,108] Genetic instability was reported in cells expressing JAK2 V617F,[109] and might contribute to leukemic transformation, although this remains rare in PV patients. The gene coding for the p53 protein, *TP53*, is not mutated in the chronic phase of MPNs, possibly because cytokine receptor signaling inactivates p53 functionally (Vainchenker *et al.*, 2011 personal communication). In contrast, p53 mutations are acquired in 20% of post-MPN AML patients,[67,110] suggesting that loss of p53 might play an important role in leukemic transformation. Heterozygous mutations of IDH1/2 (isocitrate dehydrogenase 1 and 2) are acquired to an extent of 21.6% in the blast crisis, while during the chronic phase IDH mutations are quite low.[111] The same is true for the deletion of the IKZF gene, coding for the Ikaros transcription factor,[112] and for mutations in the gene (RUNX1) that codes for the AML transcription factor, where mutations were found in 6/16 AML patients evolving from MPNs.[67] Since mutations or deletion of p53, IDH1/2, Ikaros, and AML1 are known to be associated with a substantial fraction of de novo AML,[113–116] it appears that these alterations could be common in AML and post-MPN AML, and might not be directly linked to the constitutive JAK-STAT signaling in MPNs, other than the latter predisposing for genetic instability and selection of a program and promotes blast transformation.

Chromatin effects of constitutive JAK-STAT signaling

Expression of JAK2 V617F or of mutated TpoR leads to constitutive STAT5 and STAT3 signaling, and also constitutive activation of MAP-kinase Erk1,2, PI-3'-kinase, and Akt. In *Drosophila melanogaster*, constitutive signaling induced by an overactivated JAK (JAK-D) leads to induction of common, but also to different genes from those normally targeted by *D. melanogaster* STAT (STAT92E).[117] Constitutive JAK signaling was found to disrupt heterochromatin gene silencing by derepressing genes not normally targeted by STATs.[117] On the other hand, non-phosphorylated STAT92E was shown to play a role in establishing the heterochromatin state, in association with heterochromatin protein 1 (HP1).[118] Whether this holds true for the mammalian system is not known, but several lines of evidence point towards a link between constitutive JAK-STAT signaling and global regulation of chromatin. First, JAK2 was shown to directly phosphorylate tyrosine 41 of histone H3, and by this to inhibit binding of the chromoshadow domain of HP1 alpha to histone H3.[119] These data uncovered a novel function for JAK2 in directly regulating heterochromatin,[119] although such regulation might also occur indirectly via activated

STATs. Second, levels of HP1 alpha bound to chromatin were found to be lower in embryonic stem (ES) cells engineered to express JAK2 V617F, and this effect was reversible upon addition of JAK2 inhibitors. Levels of phosphorylation of histone H3 tyrosine 41 were inversely correlated with HP1 alpha binding on chromatin.[120] Third, in mammalian hematopoietic cells, constitutive activation of STAT5, either by expression of JAK2 V617F, of TpoR W515 mutants, or of the active STAT5 1*6 mutant,[121] leads to induction of expression of the host gene for miR-28, Lipoma Preferred Partner (LPP), while acute STAT5 stimulation by cytokine did not.[45] This difference is due to the utilization of an alternative promoter containing a STAT5-binding site by the constitutively active STAT5 proteins.[45] Fourth, JAK2 V617F was shown to tyrosine phosphorylate and inhibit the catalytic activity of Protein Arginine Methyl Transferase 5 (PRMT5).[122] As a consequence, arginine methylation profiles of certain histones are altered in JAK2 V617F. A knock-down of PRMT5 leads in CD34-positive human cells to increased colony formation and erythroid differentiation, suggesting PRMT5 inactivation by JAK2 V617F might contribute to disease phenotype. It is, therefore, tempting to speculate that global chromatin changes are induced by constitutive JAK-STAT activation in MPNs and that these changes might play an important role in disease evolution.

Conclusion

The discovery of JAK2 V617F prompted attention towards the major feature of the majority of BCR-ABL negative MPNs, a constitutive active or aberrantly hyperactive JAK-STAT pathway. Since JAK2 is essential for formation of erythroid, megakaryocytic, and granulocytic myeloid cells, the trilineage expansion in these diseases could be well explained by the single JAK2 V617F event. More recently the picture became more complicated, with many other genetic events preceding or following the JAK2 V617F mutation, and with profound effects on chromatin that are only now beginning to be unraveled. While the understanding of the molecular bases of the phenotypes of diseases advanced rapidly, major unknowns remain with respect to signaling by JAK and receptor mutants in HSCs, epigenetic regulation induced by those mutants and mechanisms leading to leukemia transformation. Last but not least, reasons behind the acquisition of JAK2 V617F in HSCs remain completely unknown, as well as the molecular bases of MPN predisposition genes in families with MPNs.

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Diagnosis and treatment of polycythemia vera and essential thrombocythemia

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Introduction

Landmark discoveries occurred in the last few years have greatly advanced our knowledge of pathophysiology and contributed to a reassessment of the diagnostic criteria of polycythemia vera and essential thrombocythemia. On the other hand, the prognostic relevance of mutational abnormalities is still largely debated and very likely it is overall modest. Thus, the criteria employed for patients risk stratification are still exclusively based on clinical considerations. On the other hand, we are witnessing a remarkable number of ongoing clinical trials with drugs belonging to different classes that include interferon, inhibitors of histone deacethylases and JAK2 inhibitors, which hold the promise to improve the therapeutic approach to at least selected categories of patients

Polycythemia vera (PV) and essential thrombocythemia (ET) are stem-cell derived clonal myeloproliferative neoplasms (MPN) that have only recently witnessed significant advances in the understanding of their molecular basis.[1–3] A landmark discovery occurred in 2005, with the identification of a recurrent point mutation in exon 14 of *JAK2,*[4–7] the gene encoding the type I receptor-associated JAK2 tyrosine kinase. JAK2 is implicated in the intracellular transduction of signals originating from numerous cytokine receptors predominantly through the STAT pathway. However, the molecular complexity of MPN was surprisingly greater than anticipated, and the number of mutations being discovered has increased steadily in the last couple of years;[8,9] on the other hand, the hierarchy of molecular abnormalities and their original cell target remain largely to be defined.[8] Prompted by these seminal discoveries, experts of the 2008 WHO classification subcommittee revised the terminology underlying the "neoplastic" nature of the "myeloproliferative disorders", as they had been named after William Dameshek,[10] and at the same time, refined and improved the diagnostic criteria for the three "classic" MPN that include PV, ET, and primary myelofibrosis (PMF).[11,12] Finally, in an exceptionally short lapse of time thereafter, results of the first clinical trials employing small-molecule inhibitors that target JAK2 (and JAK1) became available, providing proof-of-concept of the effectiveness of a targeted-therapy.[13] In this review, I will briefly present the current approach to the diagnosis and treatment of PV and ET aiming at illustrating, whenever possible, how novel molecular information resulted in modification of our clinical approach.

The diagnosis of PV and ET

The detection of a *JAK2* V617F mutation in at least 95% of PV and 60% of ET patients has made diagnosis of these disorders easier and more accurate than in the "pre-JAK2" era, and is at the basis of the WHO 2008 revision of the diagnostic criteria (Table 1). Indeed, when the criteria for defining a raised hemoglobin level are satisfied, the presence of *JAK2* V617F mutation and subnormal serum erythropoietin levels support the diagnosis of PV with virtually absolute specificity, and distinguish it from conditions associated with reactive increase of hemoglobin. When erythropoietin levels are subnormal but the V617F mutation is absent, then searching for mutations in *JAK2* exon 12 is recommended;[14] the latter allow us to characterize molecularly a further approximate 2% of patients with V617F-negative PV. In a recent European collaborative study that recruited 106 *JAK2* exon 12-mutated PV patients, a total of 17 different mutations were identified.[15] *JAK2* exon 12 mutated patients had significantly higher hemoglobin level and lower platelet and leukocyte count at diagnosis compared with *JAK2*V617F-mutated PV subjects; two-thirds of the patients manifested isolated erythrocytosis only. The incidence of thrombosis, myelofibrosis, or leukemia, and the overall survival, were similar to JAK2V617F mutated subjects.[15] Mutations in *LNK*, a plasma membrane-bound adaptor protein that inhibits phosphorylation of wild-type and mutant JAK2, were originally described in one ET and PMF patient by Oh and colleagues;[16] however, functional defects of Lnk have been found in a large proportion of MPN patients also in the absence of mutations.[17] Finally, *LNK* mutations have been recently described also in two of eight *JAK2*-wild-type PV patients[18] who manifested isolated erythrocytosis without other clinical, laboratory, or bone

Table 1. The WHO criteria for diagnosis of PV and ET.

	Polycythemia vera	Essential Thrombocythemia
Major criteria	**1.** Hgb >18.5 g/dL (men) or >16.5 g/dL (women)	**1.** Platelet count ≥450 x 109/L
	or Hgb or Hct > 99th percentile of reference range for age, sex, or altitude of residence	**2.** Megakaryocyte proliferation with large and mature morphology. No or little granulocyte or erythroid proliferation
	or Hgb >17 g/dL (men) or >15 g/dL (women) if associated with a sustained increase of ≥ 2 g/dL from baseline that cannot be attributed to correction of iron deficiency	**3.** Not meeting WHO criteria for CML, PV, PMF, MDS, or other myeloid neoplasm
	or Elevated red cell mass >25% above mean normal predicted value	**4.** Demonstration of *JAK2*V617F or other clonal marker **or** no evidence of reactive thrombocytosis
	2. Presence of *JAK2*V617F or similar mutation	
Minor criteria	**1.** BM trilineage myeloproliferation	
	2. Subnormal serum Epo level	
	3. EEC growth	
Diagnostic combinations	Both major criteria + 1 minor criterion	All four criteria must be met
	or First major criterion + 2 minor criteria	

Epo, erythropoietin; EEC, endogenous erythroid colonies; LDH, lactate dehydrogenase.

marrow morphologic features of classic PV.

The diagnostic approach to patients presenting with thrombocytosis as the main hematologic abnormality is indeed more cumbersome; as a matter of fact, the reliability of some histologic criteria included in the WHO classification employed for differential diagnosis has been questioned.[19] Disorders that mimic ET include reactive thrombocytosis, PMF, "masked" PV, chronic myelogenous leukemia (CML), and refractory anemia with ring sideroblasts and marked thrombocytosis (RARS-T), as well as other unusual chronic myeloid neoplasms.[20] The *JAK2* V617F mutation is harbored by approximately 60% of the patients who are finally diagnosed as ET, while an additional 5–8% present mutations in *MPL* exon 10 at codon 515.[21–24] When these clonal markers are present, they reliably exclude the possibility of reactive thrombocytosis, while negativity for *BCR-ABL1* rules out CML; in RARS-T, *JAK2* V617F mutation is present in more than 50–60% of cases[25] and is invariably associated with signs of dyserythropoiesis.[26] Thus, at the present time, at least 30–40% of ET patients remain molecularly not characterized, and according to the WHO criteria, the diagnosis most relies on bone marrow hystology; the degree of trilineage proliferation, megakaryocyte morphology, and topography, and the extent of fibrosis are employed as key variables in distinguishing ET from prodromal stages of PV and PMF.[27]

A particularly debated issue concerns the distinction between so-called "true ET" and "prefibrotic myelofibrosis".[19,28–30] Overt fibrosis is absent in bone marrow biopsy of prefibrotic myelofibrosis, possibly leading to a spurious diagnosis of ET.[31] In order to contribute to solving these issues, in a recent international study, 1,104 bone marrow biopsies from patients diagnosed as ET in seven experienced centers were collected and centrally re-reviewed by one author of the WHO classification, with the aim to ensure strict adherence to the WHO histologic criteria for diagnosis.[32] It was found that the overall survival and the rate of transformation to leukemia and to overt myelofibrosis were significantly worse in early/prefibrotic myelofibrosis compared with ET patients, while thrombotic complication rates were similar . However, whether "early/prefibrotic myelofibrosis" and "true ET" are two different entities or rather they reflect distinct evolution stages of a single disease remain to be clarified. The negative impact of reticulin accumulation at diagnosis has been demonstrated in a large series of ET patients from the PT-1 trial.[33] Elevated reticulin levels predicted higher rates of arterial thrombosis, major hemorrhagy, and myelofibrotic transformation independently of known risk factors. Elevated reticulin levels at presentation predicted higher rates of arterial thrombosis (hazard ratio [HR], 1.8; 95% CI, 1.1 to 2.9; P = .01), major hemorrhage (HR, 2.0; 95% CI, 1.0 to 3.9; P = .05), and myelofibrotic transformation (HR, 5.5; 95% CI, 1.7 to 18.4; P = .0007) independently of known risk factors.

The International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) has developed criteria for diagnosing myelofibrotic evolution of PV and ET.[34]

Therapeutic approach to ET and PV

Clinical needs of patients with PV and ET

PV and ET are relatively indolent disorders which, according to most studies,[35–37] result in a modest reduction of survival, usually after the first decade from diagnosis. However, in a recent retrospective study from the Swedish Cancer Registry that included 4,389 and

2,559 patients with a diagnosis of PV and ET, respectively, a significant overall excess mortality compared with reference population was found.[38] The relative survival ratio (RSR) at 10 years was 0.64 (95% CI, 0.62–0.67) for PV and 0.68 (0.64–0.71) for ET. There was a trend towards improved survival in the last decade, mostly ascribable to a reduction in the number of deaths due to cardiac disorders and thromboembolic events; thus, the commonest causes of death were represented by hematological malignancies and solid tumors. Evolution to post-polycythemic or post-thrombocythemic myelofibrosis (PPV-/PET-MF) and transformation to acute myeloid leukemia (AML) contributed heavily to reduced survival.

Much attention has been paid in last years to the patients' quality of life (QoL) that is burdened by a spectrum of complications, including thrombosis, hemorrhages, constitutional symptoms, fatigue, pruritus, microvascular manifestations, and increased risk of miscarriage. Also the side effects of treatment, such as phlebotomy-induced iron deficiency or the mucous and skin toxicities due to hydroxyurea, can contribute to an overall reduced QoL. In an Internet-based symptom survey of 1,179 patients with MPN, of whom 405 were PV and 304 were ET, more than 70% of the patients reported symptoms ascribable to the underlying disease: fatigue in 72–85%, night sweats in 40–49%, and bone pain in 40–43%. Pruritus was prevalent in PV (65% vs. 39% in ET).[39] To improve and standardize the measurement of QoL-related issues, Mesa *et al*. recently developed an internationally validated Myeloproliferative Neoplasm Symptom Assessment Form (MPN-SAF) that complements and extends the SAF previously developed specifically for Myelofibrosis (MSAF).[40,41] The MPN-SAF contains 27 items that address symptoms related to spleen enlargement, myeloproliferation, vascular events, manifestations due to abnormally increased pro-inflammatory cytokine levels, fatigue, and psychiatric aspects. It is expected that this instrument will be an essential part of any novel clinical trial and will permit us to assess reproducibly the response of disease-associated symptoms to novel (and conventional) therapies.

These patients' "clinical needs" represent the goals of therapy in PV and ET that, according to the management recommendations recently developed by the European Leukemia Net (ELN), are: to avoid first occurrence and/or recurrence of thrombotic and/or hemorrhagic complications; to minimize the risk of evolution to PPV-/PET-MF or transformation to AML; to control systemic symptoms; and to optimally manage potentially risky situations, such as surgery or pregnancy.[42]

Indications for treatment based on risk stratification

Established risk factors for reduced survival in PV and ET include advanced age and history of cardiovascular events. Leukocytosis and anemia have also been reported to impact on survival negatively, but they have not been validated prospectively yet.[36,43,44] Considering that therapy is not curative, the rationale behind current risk stratification in PV and ET is based on an estimate of the risk of thrombotic complications. Age older than 60 years and a history of thrombosis are the criteria used to classify patients into a "high-risk" (when either of these is present) or "low-risk" (when both are absent) category. In a cohort of 1,638 PV patients who were screened as part of the European Collaboration on Low-Dose Aspirin (ECLAP) trial, the rate of events was 2.5 per 100-persons/year among low-risk subjects compared with 10.9 per 100-persons/year among those who were older than 65 and had a prior thrombosis.[45] The role of additional, generic, risk factors for thrombosis (diabetes, obesity, hypertension) is not clearly defined, and whether subjects presenting such abnormalities configure an "intermediate-risk" category is not currently supported by evidence. Smoking is associated with an increased risk of arterial thrombosis in PV,[46,47] and should be strongly discouraged in PV and ET patients; avoidance of hormonal therapy in women is recommended. On the other hand, "extreme" thrombocytosis (where "extreme" means >1,000x10^9/L platelets for some and >1,500x10^9/L for others) is considered a risk factor for hemorrhages, possibly due to an acquired von-Willebrand like disorder,[48,49] but it is not associated with an increased rate of thrombosis. Paradoxically, in a retrospective study in ET, a platelet count greater than 1,000x10^9/L was found to exert a protective effect on thrombosis,[50] thus supporting a number of previous evidences that thrombosis is not directly associated with high platelet count (reviewed in [51]). A correlation between thrombosis and leukocytosis,[47,52–57] *JAK2* mutated genotype, or the *JAK2*V617F allelic burden[58–60] is suggested in some studies but denied in others; therefore, leukocytosis and *JAK2* mutational status are not included in current risk stratification and should not guide therapy. However, due to their possible relevance for the pathogenesis of thrombosis and as "surrogate" end-points for therapy, it would be very important that their significance is addressed in prospective controlled studies.

Conventional treatment of ET and PV

The cornerstones of the management of patients with ET and PV are the control of (i) erythrocytosis, through phlebotomies, alone or in association with cytoreductive drugs; (ii) thrombocytosis, through the use of platelet-lowering agents; and (iii) abnormal platelet function by antiplatelet therapy with aspirin. Indications vary according to the patient's risk category (Table 2). It should be pointed out that these indications represent shared opinions among experts but evidence based on clinical trials has been produced only for the use of low-dose aspirin in patients with PV[61] and for hydroxyurea in high-risk patients with ET.[62,63]

Low-risk patients with PV are managed only with therapeutic phlebotomies while in high-risk patients with PV and ET, cytoreduction is recommended. In PV and ET patients, low-dose aspirin (81 to 100 mg daily) should be prescribed independent of the risk category; however, in the youngest, asymptomatic patients with ET who do not have additional generic cardiovascular risk factors, a watch-and-wait attitude is justified as well. Conversely, due to the risk of bleeding, aspirin must be used with caution in the presence of extreme thrombocytosis or other relevant contraindications (allergy, asthma, previous severe gastric bleeding, laboratory evidence of acquired von Willebrand disease in subjects with a history of hemorrhagic manifestations). Whether

Table 2. Risk-oriented conventional treatment in PV and ET.

Risk category	Risk factors	PV	ET
Low	Age <60 yr and no thrombosis history	· Phlebotomy · Low-dose aspirin	· Low-dose aspirin
High	Age >60 yr and/or thrombosis history	· Cytoreduction ± Phlebotomy · Low-dose aspirin	· Cytoreduction · Low-dose aspirin

anti-aggregants other than aspirin offer the same protection against thrombosis remains to be evaluated in clinical trials. Cytoreduction is sometimes employed in low-risk patients for a number of reasons that include poor tolerance to, or too high frequency of, phlebotomies (in case of PV); symptomatic splenomegaly; evidence of progressive myeloproliferation manifested by leukocytosis or extreme thrombocytosis; presence of severe constitutional symptoms; and/or pruritus. Generic cardiovascular risk factors are usually not a reason for treating otherwise low-risk patients, and decision in this regard should be individualized; however, those risk factors should be corrected with diet, physical exercise, anti-diabetics, anti-hypertensive drugs, lipid lowering agents, as more appropriate.

It is usually recommended that the hematocrit target is set at less than or equal to 45% and less than or equal to 42% in men and women with PV, respectively.[64] However, these targets derived mainly from a small trial[65] and have not been substantiated rigorously. As a matter of fact, a large retrospective study in Europe found no difference in the rate of thrombosis or death in patients who had their hematocrit maintained under 45% or above 45% (up to 55%) and received concomitantly low-dose aspirin.[66] A large prospective trial (CYTO-PV) that compares two different hematocrit target levels (≤45% and 45 to 50%) is undergoing in Italy. Similarly, there is no based evidence to support the commonly chosen target level of $400x10^9$/L platelets in high-risk patients with ET.[67]

Drugs currently employed as first-line therapy are represented by hydroxyurea and interferon-α in countries where it is available to such a purpose, although it is not approved by FDA or EMA. Hydroxyurea is usually started at 500–1000 mg/die, and the dose is titrated based on the target level of hematocrit and/or platelet count. The drug is usually well-tolerated, but gastrointestinal intolerance, mucous, or cutaneous ulcers, skin toxicity, fever, or in rare instances, pulmonitis can limit its use in individual patients. Conventional formulations of INF-α have been successfully employed to control hematocrit in 50–94% of PV patients[68] or platelet count in greater than 70–80% of ET patients (reviewed in[69]). However, INF-α has severe side effects leading to discontinuation in more than 30–40% of the patients, including fatigue, flu-like syndrome, worsening or developing of autoimmune diseases, psychiatric manifestations, and myelosuppression. More recently, pegylated forms of INF-α have been developed to increase drug half-life and to reduce the frequency of administrations. In two recent studies, pegylated INF-α2a, usually at 90 μg/week, was employed in PV (40 patients)[70] or in PV and ET (40 and 39 subjects, respectively).[71] Greater than 80% of the patients obtained hematologic remission accompanied by a continuous decrease of *JAK2* V617F allele burden; up to 10% of the subjects achieved the complete disappearance of measurable *JAK2*V617F allele. On the other hand, about 20% of the patients had to discontinue the treatment due to side effects. As a whole, results of these two studies confirm the hematologic efficacy of INF-α in PV and ET and the potential for achieving the eradication of *JAK2*V617F mutated cells (which could not correspond to eradication of the disease, according to a recent report that demonstrated persistence of TET2 mutated cells in some patients who became JAK2V617F-negative after interferon).[72] A controlled randomized study of pegylated INF-α2a versus hydroxyurea in high-risk patients with PV and ET has been launched recently by the MPD-RC group, and will hopefully provide definite conclusions about the superiority of interferon to hydroxyurea as first-line therapy for PV or ET. A second study of the MPD-RC is a single arm salvage therapy with pegylated INF-α2a in high-risk PV or ET patients who are resistant or intolerant to hydroxyurea or have had splanchnic thrombosis.

If hydroxyurea is ineffective, poorly tolerated, or causes significant toxicity, the drugs commonly employed as second-line therapy are represented by INF-α, particularly in younger patients, busulfan, pipobroman, or anagrelide in case of ET. Radiophosphorus is rarely employed in older PV patients, and in studies of the Polycythemia Vera Study Group (PVSG) in the 1980s, this treatment was associated with an increased rate of leukemia transformation.[73] Busulphan is preferred in older patients; the drug needs careful titration because of its potent myelosuppressive effects. Anagrelide is approved in Europe as second line for hydroxyurea resistant or intolerant patients with ET. A large randomized trial, the PT-1, that compared hydroxyurea with anagrelide on the top of low-dose aspirin, in high-risk ET patients concluded for an overall superiority of hydroxyurea due to a significant less risk of arterial thrombosis, major hemorrhages, and fibrotic transformation, although anagrelide proved superior against venous thrombosis.[63] In a recent randomized study, anagrelide was not inferior to hydroxyurea as a single drug in the treatment of newly-diagnosed high-risk ET patients;[74] however, results of this study should be interpreted with caution because of the low statistical power due a "non-inferiority" trial design. Side effects of anagrelide include headache, flushing, cardiopalm, and arrhythmias,[75] and led to discontinuation in a greater proportion of the patients than hydroxyurea in the above mentioned trials. Due to the anti-platelet activity of anagrelide, the concomi-

tant use of aspirin should be carefully evaluated.

One still largely debated issue about the use of hydroxyurea concerns its leukemic potential; however, this is not substantiated by any of the clinical trials available to date, although it should be acknowledged that none of these was specifically designed to this endpoint. Conversely, the combined use of multiple chemotherapeutic agents has been associated with a higher rate of leukemic transformation than expected.[76] Leukemia is part of the natural history of these disorders, as supported by the observation that the rate of evolution to leukemia among PV or ET patients who were un-treated or had received hydroxyurea only was 7.4% and 3.3%, respectively.[36,43] In another study that included three French prospective trials with hydroxyurea and pipobroman, the rate of leukemia transformation was 12 to 15%, with an excess for pipobroman. Most cases of transformation occurred after 15 years, and there was no evidence of a plateau.[77]

A standardized definition for clinical resistance and intolerance to hydroxyurea in ET and PV was developed by a group of experts of the ELN using consensus methodologies (Table 3).[78,79] These criteria can be conveniently used for decision-making when assessing the opportunity to move patients to second-line therapies and/or for identifying those suitable for enrollment in clinical trials with novel drugs. Furthermore, criteria for monitoring the response to treatment in PV and ET have been developed by the ELN group (Table 4). These criteria involve the measurement of levels of response and their ranking according to three sets of categories: clinical-hematological, molecular, and histological response.[80] Presently, only clinical-hematological criteria should be employed for monitoring the response to conventional cytoreductive therapy, since no drug, with the exception of interferon, has produced yet relevant effects on mutated allele burden. In fact, *JAK2* V617F allele levels fluctuate over time even in the absence of any treatment, and claims of significant decline of *JAK2*V617F allele burden in patients treated with hydroxyurea have not been universally reproduced.[81–85] Thus, sequential monitoring of molecular response can be recommended only in the settings of clinical trials and not in routine management; conversely, there is no indication to perform serial bone marrow evaluations if not clinically indicated.

Novel drugs

A part for interferon, two novel categories of drugs, the "JAK2" inhibitors and the histone deacethylase inhibitors, have recently been evaluated in PV and ET and preliminary reports have been presented.

INCB018424 is a JAK1 and JAK2 inhibitor that has been first used in a clinical trial including 153 patients with primary or PPV/PET-MF.[86] The drug was overall well tolerated, with few and low-grade toxicities; most common side effects were due to on-target activity and included reversible thrombocytopenia (that represents the dose limiting toxicity) and anemia, that could be managed with dose titration. As many as 44% of the evaluable patients presented a greater than or equal to 50% reduction of spleen enlargement, and more than 80% experienced significant improvement of constitutional symptoms, including pruritus, night sweats, early satiety, abdominal discomfort, and fatigue. These effects are probably due to a normalization of increased proinflammatory cytokines mediated by the anti-JAK1 activity.[13,86,87] The drug has also been employed in a phase 2 trial in patients with PV and ET.[88] This study included 39 subjects with ET and 34 with PV who were intolerant/refractory to hydroxyurea (ELN criteria). The overall response rate was 97% (50% complete and 47% partial) in PV and 90% (26% complete and 74% partial) in ET. In PV, 97% of the patients achieved control of hematocrit to less than 45% in the absence of phlebotomies, and 68% experienced a complete resolution of enlarged spleen; more than 70% of patients with leukocytosis or thrombocytosis at baseline normalized their blood count. Among ET patients, 49% achieved a normal platelet count while 79% reached a platelet count less than $600x10^9$/L or a decrease greater than 50% at last follow-up visit. In 13 of 14 patients

Table 3. European LeukemiaNet criteria of resistance or intolerance to hydroxyurea in patients with PV and ET.

PV:

1. Need for phlebotomy to keep hematocrit <45% after 3 months of at least 2 g/day of HU, *OR*
2. Uncontrolled myeloproliferation, i.e., platelet count >$400x10^9$/L *AND* white blood cell count >$10x10^9$/L after 3 months of at least 2 g/day of HU, *OR*
3. Failure to reduce massive splenomegaly by more than 50% as measured by palpation, *OR* failure completely to relieve symptoms related to splenomegaly, after 3 months of at least 2 g/day of HU, *OR*
4. Absolute neutrophil count <$1.0x10^9$/L *OR* platelet count <$100x10^9$/L or hemoglobin <100 g/L at the lowest dose of HU required to achieve a complete or partial clinico-hematological response, *OR*
5. Presence of leg ulcers or other unacceptable HU-related non-hematological toxicities, such as mucocutaneous manifestations, gastrointestinal symptoms, pneumonitis or fever at any dose of HU

ET:

1. Platelets >600,000/µL after 3 months of at least 2g/day of hydroxyurea (2.5 g/day in patients with a body weight >80 kg)
2. Platelets >400,000/µL and WBC less than 2,500/µL at any dose of hydroxyurea
3. Platelets >400,000/µL and Hb less than 10 g/dL at any dose of hydroxyurea
4. Presence of leg ulcers or other unacceptable muco-cutaneous manifestations at any dose of hydroxyurea
5. Hydroxyurea-related fever

Table 4. Criteria for definition of clinico-hematologic, molecular and histologic response in patients with PV and ET according to the European LeukemiaNet.

	Polycythemia Vera	Essential Thrombocythemia
	Clinico-hematologic response	
Complete Response	Ht lower than 45% without phlebotomy, AND Platelet count ≤400 x 10^9/L, AND WBC count ≤10 x 10^9/L, AND Normal spleen size on imaging, AND No disease related symptoms	Platelet count ≤400 x 10^9/L, AND No disease-related symptoms,* AND Normal spleen size on imaging, AND White blood cell count ≤10 x 10^9/L
Partial Response	In patients who do not fulfil the criteria for complete response: Ht lower than 45% without phlebotomy, OR Response in 3 or more of the other criteria	In patients who do not fulfill the criteria for complete response: Platelet count ≤600 x 10^9/L OR decrease >50% from baseline
No Response	Any response that does not satisfy partial response	Any response that does not satisfy partial response
	Molecular response	
Complete response	Reduction of any specific molecular abnormality to undetectable levels	
Partial Response (Applies only to patients with a baseline value of mutant allele burden greater than 10%)	A reduction equal to or greater than 50% from baseline value in patients with less than 50% mutant allele burden at baseline, ***OR*** A reduction equal to or greater than 25% from baseline value in patients with more than 50% mutant allele burden at baseline	
No Response	Any response that does not satisfy partial response	
	Histologic response	
Bone marrow histological remission	Presence of age adjusted normocellularity and no reticulin fibrosis	

with platelet count greater than 1,000x10^9/L, a greater than 50% reduction was observed. Similar to the myelofibrosis trial, INCB018424 was well tolerated after a medium follow-up of 21 months. A phase 3 trial in PV, the RESPONSE trial, started to recruit patients in the first quarted of 2011. This is a randomized trial where 300 patients with PV who are refractory or intolerant to hydroxyurea will be randomized to receive INCB018424 or best available therapy for 32 weeks, with the possibility of cross-over in case of failure to reach the endpoints at that time.

Another inhibitor of JAK2 kinase activity, lestaurtinib (CEP-701), was found to inhibit *in vitro* the expansion of $CD34^+$ cell-derived erythroid cells from MPN patients preferentially when compared with controls.[89] This agent was tested in a cohort of 39 *JAK2* V617F-positive subjects, 27 and 12 of whom had a diagnosis of "high-risk" PV and ET, respectively.[90] The primary endpoint of the trial was a reduction in *JAK2* V617F neutrophil allele burden; secondary endpoints included reduction in phlebotomy rate, improvement in hemoglobin, white cell and platelet counts, reduction in hydroxyurea dose, and improvement of spleen size. At last update, it was found that among the patients who concluded the scheduled 18 weeks of treatment more than 80% had a reduction of spleen size and amelioration of pruritus; reduction of phlebotomy rate was seen in some patients, but occurred after 6 months of therapy and was not associated with concomitant improvement of white cell or platelet count. Conversely, platelet and white cell counts increased in many patients while on the drug. It was unexpected that amongst the serious adverse events that occurred there were six arterial and venous events in five patients,[90] a complication that has not been reported yet in other trials with JAK1/JAK2 inhibitors in myelofibrosis or in "high-risk" PV and ET. It remains to ascertain whether these events are related specifically to the drug or simply reflect its ineffectiveness in preventing trombosis.

The orally available HDAC inhibitor ITF2357 (Givinostat) has been administered to 12 PV and 1 ET patients in a phase 2 study; also included were 16 subjects with myelofibrosis.[91] The rationale underlying this study was that Givinostat was shown able to induce a specific down modulation of the phosphorylated JAK2 V617F protein and inhibition of its downstream signaling while it minimally affected the wild type JAK2 in cells lacking the *JAK2* V617F mutation.[92] The drug was usually well tolerated, although most patients experienced grade 2 gastrointestinal toxicity. Among the 13 PV/ET patients, 1 complete, 6 partial, and 4 no responses were documented, while 2 patients went off-study prematurely. Spleen enlargement improved in 75% of PV patients, and the majority experienced improvement of constitutional symptoms and pruritus. There was evidence of a trend towards reduction of the V617F allele burden, although the short treatment period (median of 20 weeks) precluded any firm conclu-

sions at this regard. Based on these encouraging results, a clinical study envisioning the concomitant administration of low dose Givinostat and hydroxyurea has completed enrollment as of January 2011.

It is of note that both INCB18424 and Givinostat had remarkable effects on the symptomatic control of pruritus; indeed, intractable pruritus, typically aquagenic, is complained by most PV patients, and sometimes represents a disabling condition. It is very poorly responsive to conventional treatments, including antihistamines, the serotonin uptake inhibitor paroxetine, photochemotherapy with UVA light, and psolaren. Remissions after interferon therapy are more common. The pathogenetic mechanisms are still largely unknown, but abnormal activation of *JAK2* V617F mutated basophils and mast cells has been recently considered causative.[93,94]

Conclusions

The improved understanding of the molecular pathogenesis of ET and PV that followed the seminal discovery of *JAK2* V617F mutation in 2005 has rapidly translated in an improved diagnostic approach, as it is outlined in the WHO 2008 revised classification of MPN. While this is certainly true for PV, owing to the almost universal presence of a mutation in *JAK2*, diagnostic uncertainties still remain in approximately 40% of cases of ET negative for *JAK2* V617F or *MPL* mutations. Also, are ET and PV two unique diseases or just one, "continuum" disease? Furthermore, the relationships between so called "true" ET and "pre-fibrotic" myelofibrosis are still largely a matter of debate that goes beyond simple classification, if the latter condition holds a worse prognosis as recent data suggest. Therefore, notwithstanding the simplification and increased robustness of current diagnostic criteria, we are not yet in the position to enlist MPN using only molecular criteria nor to use molecular signature as a valid criterion for disease entity sub-classification, patient risk stratification, therapeutic decision, or monitoring the response to therapy. Furthermore, we can reasonably expect that in the next years, a number of prospective controlled clinical trials will provide information as to whether any of the novel agents, alone or in combination, is able to improve the achievement of major therapeutic goals, that is, prevention of thrombosis, amelioration of the quality of life, and better leukemia-free and overall survival. Because of these reasons, the field of MPN will certainly continue to receive much attention and interest as it never had in the past.

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Individualized care plans for myelofibrosis

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A B S T R A C T

Myelofibrosis (MF) is a potentially fatal myeloproliferative neoplasm (MPN) with a very heterogeneous group of patients with widely variable prognosis. Clinical management options range from low-risk/low-reward observation to high-risk/high-reward allogeneic stem cell transplantation. Medical therapy ranges from off label utilization of palliative agents for MF associated anemia or splenomegaly; however, none of these latter agents impact the natural history of the illness. The discovery of the JAK2-V617F, and subsequent additional MPN associated mutations has led to development of a spectrum of selective inhibitors of JAK2. Clinical trials with these latter agents have led to meaningfully reductions in MF-associated splenomegaly and constitutional symptoms. Several additional therapies that do not directly target JAK2 (e.g., immunomodulatory drugs, histone deacetylase inhibitors) may ameliorate MF-associated anemia and morbidity-inducing symptoms. Balancing the potential benefits of these new agents against the risks and benefits of allogeneic stem cell transplantation requires an accurate estimation of the prognosis for an individual patient and development of individualized treatment plans. Evolving information regarding the efficacy of new medicines (alone or in consolidation) will continue to modify MF treatment strategies and plans.

Introduction

Myelofibrosis (MF) in 2011 remains the myeloproliferative neoplasm (MPN) that induces the most morbidity and is associated with the poorest life expectancy.[1] Although pathogenetic origins may vary, clinically MF is an amalgam of individuals with apparently *de novo* myelofibrosis (so-called primary myelofibrosis, or PMF), as well those who evolved from a clear antecedent MPN, either polycythemia vera or essential thrombocythemia (Post ET/PV MF).[2] Regardless of subtype of MF, patients suffer from a spectrum of complications arising from the malignant clone, including ineffective hematopoiesis with resulting cytopenias, splenomegaly, bothersome constitutional symptoms, risk of vascular events including thrombosis and hemorrhage, and risk of blastic transformation.[3]

A major clinical challenge for the management of MF patients is that it is a very heterogeneous illness in terms of symptomatic burden, morbidity, and expected mortality. Additionally, the clinical course can be dynamic with certain benchmarks developing, which can be worrisome in a previously stable patient. An accurate assessment of prognosis is essential in choosing the appropriate therapy for an individual patient, given that some patients may live more than 15 years,[4] while others, such as those who progress to acute leukemia, have a life expectancy as short as 2 months.[5]

Historically, peripheral blood findings at the time of diagnosis, such as anemia and changes in leukocyte count, have been the most prognostically significant variables in MF, and were incorporated into the 1996 Lille criteria (Dupriez score)[6] (Table 1). In 2009, the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) retrospectively analyzed over 1000 cases of PMF at diagnosis and developed a four-tier International Prognostic Scoring System (IPSS) incorporating five factors with independent negative prognostic impact: age older than 65, anemia, constitutional symptoms, leukocytosis, and circulating blood blasts (Table 1).[3] Of these five factors, anemia was the variable with the greatest negative prognostic significance.[3] Further efforts to apply the IPSS factors at any point in time along the course of the illness resulted in a dynamic IPSS (DIPSS),[7] which demonstrated that the acquisition of these factors at any point along the course of the illness is detrimental, particularly anemia (Table 1). Finally, within each DIPSS category (low, intermediate-1, intermediate-2, and high risk), three additional factors (erythrocyte transfusion dependence, karyotype, and the presence of thrombocytopenia) further refines dynamic prognosis in the DIPSS-Plus system.[8] Finally, young patients (<50 years of age) can live in excess of 15 years if they lack thrombocytopenia and have no more than one adverse IPSS risk factor.[4]

Current prognostication systems have several limitations. All current systems are derived from retrospective experience and solely from PMF patients (excluding post-ET and post-PV MF), and all systems incorporate clinico-pathological variables that offer limited insight into mechanisms of disease progression, as these mechanisms are incom-

Table 1. Prognostic scoring systems for primary myelofibrosis (PMF).

		Lille 1996[6] (At Diagnosis of PMF)	IPSS 2009[3] (At Diagnosis of PMF)	DIPSS*[7] (Valid throughout – PMF)	DIPSS Plus@[8]
Factors	Anemia (Hb <10g/dL)	X	X	X	X
	Leukocytes	X (<4 or >30 x 10(9)/L)	X (>25 x 10(9)/L)	X	X
	Blasts in Peripheral Blood		X	X	X
	Constitutional Symptoms		X	X	X
	Age >65 years		X	Age Adjusted for individuals <65	X
	Unfavorable Karyotype#				X
	Platelet Count <100 x 10(9)/L				X
	Erythrocyte Transfusion Dependent				X
Categories	Low	***0 Factors*** Median Survival 93 Months	***O Factors*** Median Survival 135 Months	**WBC count greater than 25 x10(9/)L** *Hazard Ratio = 1.7*	***O points*** Median Survival 185 Months
	Intermediate 1	***1 Factor*** Median Survival 26 Months	***1 Factor*** Median Survival 95 Months	**Hemoglobin level lower than 10 g/dL** *Hazard Ratio = 3.7*	***1 point*** Median Survival 78 Months
	Intermediate 2		***2 Factors*** Median Survival 48 Months	**Peripheral blood blasts 1% or higher** *Hazard Ratio = 2.59*	***2–3 points*** Median Survival 35 Months
	High	***2 Factors*** Median Survival 13 Months	***3 or More Factors*** Median Survival 27 Months	**Constitutional symptoms** *Hazard Ratio = 3.04*	***4-6 points*** Median Survival 16 Months

**Hazard ratio for the development of the risk factor during the course of MF in patients < 65 years of age.*

 # Unfavorable Karyotype: sole or two abnormalities with any of the following (+8, -7/7q, i(17q), -5/5q-, 12p-, inv(3), 11q23) or complex (≥ 3 abnormalities).

@ DIPSS Plus Points (DIPSS Low risk – 0 points; Int 1 – 1 point, Int 2 – 2 points, High – 3 points), (transfusion dependence, adverse karyotype (see above), or thrombocytopenia – all 1 point each).

pletely understood. Despite these limitations, current prognostic systems (Table 1) are very useful in determining true low risk disease (median range of survival 93–185 months) and true "high-risk" disease (median range of survival 13–27 months). Intermediate risk remaining the most varied group (median range of survival 35–95 months). Given these heterogenous categories of outcomes, it is important we approach these patients with individualized treatment plans.

MF Scenario 1: the asymptomatic low risk patient

The low risk patient with MF has a survival which can range from 8 years to even decades (Table 1). When an individual is asymptomatic, the optimal management remains expectant observation. Rationale for this approach is based on the reality that no medical therapy has altered the natural history for patients with MF, no medical therapy has been shown to delay or avoid progression, and allogeneic stem cell transplantation has a median survival less than the expected survival of low risk MF patients (Table 2). Observation comes with two responsibilities for the treating clinician. The first is that observation can be stress inducing for the anxious patient, and we should be appropriately aware and explain well the rationale for observation. The second is we must be mindful of evidence of disease progression, which signals a need for a change in our therapeutic approach, particu-

Table 2. Recent major series of allogeneic stem cell transplant (ASCT) trials for myelofibrosis.

Author	No. of Patients	Conditioning	Median Age (Range)	Non-Relapse Mortality at 1 Year	Acute GVHD (Grade 2–4)	Chronic GVHD (Grades 2–4)	Overall Survival
Rondelli[38]	21	RIC	54 (27–68)	10%	33%	45%	85% (at 2,5 years)
Kerbauy [39]	104	Ablative	49 (18–70)	34 % (at 5 years)	64%	85%	61% (at 7 years)
Kröger [40]	103	RIC	55 (32–68)	16%	27%	43%	67% (at 5 years)
Bacigalupo[41]	46	RIC	51 (24–67)	24%	37%	31%	45% (at 5 years)
Snyder* [42]	23	RIC	58 (39–69)	33% (Group 1) 0% (Group 2)	60% (Group 1) 10% (Group 2)	61%	55% (2 years)(Grp 1) 93% (2 years)(Grp 2)
Ballen[27]	289	Ablative	47 (18–73)	18% (100 days–HLA Matched) 35% (100 days–unrelated)	40–43%	40%	37–40%

RIC: Reduced intensity conditioning.
**Group 1:GVHD prophylaxis Cyclosporine A plus mycophenolate moffetil +/- methotrexate.*
Group 2: GVHD prophylaxis tacrolimus/ sirolimus +/- methotrexate.

larly the acquisition of features identified in the DIPSS prognostic system (Table 1) (development of anemia, leukocytosis, constitutional symptoms, or circulating blasts) but not asymptomatic splenomegaly.

MF Scenario 2: the "symptomatic" low risk patient

Given that low risk individuals with myelofibrosis may have survival that can exceed 8 years (Table 1) and can be associated with survival that can be from 1–2 decades or in rare circumstances, even greater, the focus of therapy for these patients would flow from their symptomatic burden.[9]

Constitutional symptoms, which are prognostically detrimental through IPSS (Table 1), are narrowly defined (weight loss >10%, significant night sweats, documented fevers). However, symptomatic burden in MF patients can be significant, with a prevalence of symptoms, which can be severe from the illness not being accounted for in current prognostic systems. In the prospective validation trial of the Myeloproliferative Neoplasm Symptom Assessment Form (MPN-SAF), we observed prevalence of fatigue, decreased quality of life, pruritus, vascular symptoms all to be present in over 50% of patients.[10]

In the future, if we have therapies that clearly alter the natural history, that is, prolonged survival over a lifetime, it creates complete remissions at minimum of toxicity expense, then these recommendations obviously will change. In 2011, given we have no such therapy, our choice of options flow both by reasonably minimal toxicity and that symptomatic burden can be an important target, given their prevalence in the illness. The choice of therapy really would be directed toward the symptom, which is predominantly difficult.

Splenomegaly

The reduction of splenomegaly in the low risk symptomatic patient should be done so only if the spleen is causing mechanical pain, abdominal distention, early satiety, or having difficulties with splenic infarctions. In this scenario, the JAK2 inhibitors that cross the spectrum of these agents should be considered for this group of patients (Figure 1, Table 3). Currently in the setting of a clinical trial and soon, these agents will be approved in Europe and in the United States for clinical use. The ability of these agents to improve both splenomegaly and symptomatology is central in their clinical benefit (Table 3). Next, hydroxyurea has long been the standard bearer of therapy for splenomegaly in myofibrosis activity in 40% patients[11] achieving a clinical improvement by IWG-MRT or EUNMET response criteria. It is an inexpensive medication; in the ideal world, I would consider it second line if an individual had failed JAK2 inhibition, but given the limiters and availability, it could be frontline depending upon the clinical scenario. Finally, other myelosuppressive therapies, particularly interferon alpha 2a[12] or pegylated interferon alpha 2a[13] is a reasonable consideration if clinically available for the patient covered by insurance and the splenomegaly is not extreme. The likelihood of extreme splenomegaly, that is, greater than 20 cm below the left costal margin, is probably unlikely without developing a higher IPSS risk score.

Pruritus

Pruritus is its own challenging complication and difficulty. Antihistamines can be helpful, perhaps by their effect on mast cells[14] but frequently are inadequate for meaningful relief. Again, JAK2 inhibition is shown to be highly efficacious in this group of patients up to this point in time. Again, therapy strictly on the basis of pruritus should only occur if this is truly problematic. Interferon alpha 2a[13] is another agent, as either front or second line,

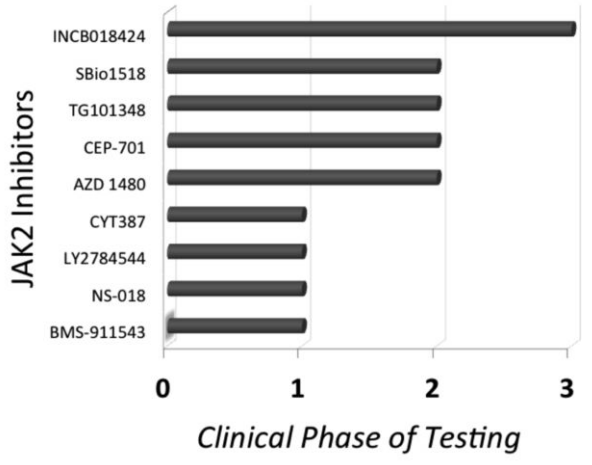

Figure 1. Current inhibitors of JAK2 and their current phase of clinical testing for patients with primary and post essential thrombocythemia – post polycythemia vera myelofibrosis.

which would be a reasonable consideration. The use of selective serotonin reuptake inhibitors has been reported to be beneficial and is a reasonable consideration,[15] but probably after having failed one of the other therapies, and finally, therapeutic UV light has been used with symptomatic benefit. Toxicities could potentially include the development of cutaneous malignancies and need to be done by a certified dermatologist. Patients need to have aggressive monitoring of their skin for complications. Initiating therapeutic UV light with concurrent hydroxyurea would be problematic as it would likely accelerate the risk of cutaneous malignancies.

Vascular/proliferative symptoms

Symptoms that are microvascular in nature, problems with concentration, headaches, numbness, or tingling can exist on a spectrum with early myeloproliferative disorder with essential thrombocythemia polycythemia vera.[10] Here, primary agents for controlling myeloproliferation in patients: hydroxyurea,[16] interferon alpha 2a,[13] or anagrelide[16] are all reasonable considerations, with hydroxyurea probably our primary agent of efficacy given its data in polycythemia vera and essential thrombocythemia.

Other Symptoms

JAK2 inhibitors are probably the most efficacious across the spectrum of the remaining symptoms (Table 3), which could be significantly problematic, including very severe fatigue and other debilitating symptoms not strictly proliferation related and would not be related to anemia or other symptoms, which would move patients to a higher risk category.

JAK2 inhibitors – Status report

2005 heralded a watershed moment for MF with the discovery of constitutively activating mutations in the JAK2 tyrosine kinase, most commonly JAK2-V617F.[17] Subsequently, investigators observed several other clonally restricted MPN-associated mutations, such as MPL-W515L/K[18] and allelically heterogeneous mutations of TET2.[19] A stream of additional mutations (typically with low prevalence and uncertain pathogenetic implications) associated with MPNs continue to be described, including *LNK, CBL, ASXL1, IDH, IKZF1, and EZH2.* The discovery of these mutations prompted rapid development of selective JAK2 inhibitors, with

Drug	Target	Phase	Disease	Efficacy	Toxicity	Ref
INCB18424	JAK2 JAK1	III	MF PV/ET	Splenomegaly Symptoms	Anemia Thrombocytopenia	20,43
TG101348 (SAR302503)	JAK2 FLT3	II	MF	Splenomegaly Symptoms	Anemia Thrombocytopenia Gastrointestinal	23,44
SB1518	JAK2 FLT3	II	MF	Splenomegaly Symptoms	Gastrointestinal	24
CEP701	JAK2 FLT3	II	MF PV/ET	Splenomegaly Symptoms	Gastrointestinal Anemia Thrombocytopenia	45,46
CYT-387	JAK1 JAK2	I	MF	NPR	NPR	47
LY2784544	JAK2	I	MF ET/PV	NPR	NPR	48,49
AZD1480	JAK2 JAK3	I/II	MF	NPR	NPR	
NS-018						50,51
Non JAK2 Inhibitors						
LBH-589	HDAC	II	MF	Splenomegaly Anemia	Anemia Thrombocytopenia Gastrointestinal	52
RAD-001	mTOR	II	MF	Splenomegaly Symptoms	Minimal	53
Pomalidomide	IMID	III	MF	Anemia	Minimal	33,54
Pegylated Interferon Alpha-2a	Biological	III	PV/ET	Erythrocytosis Thrombocytosis Symptoms	Myelosuppression Depression	13,55-57

NPR: No results in public domain on these ongoing trials.

Table 3. Current status of clinical trial testing of JAK2 inhibitors in Myelofibrosis.

the first of these agents entering clinical phase I testing a little over 2 years from the discovery of JAK2-V617F (Figure 1).

The agent that is farthest along in development is **INCB018424** (JAK1/2 inhibitor), which was tested in 153 patients with PMF and post-PV/ET myelofibrosis in a Phase I/II study.[20] Substantial clinical benefit and improvement in symptoms were observed, such as a reduction in pruritus, and patients gained weight and increased their walking distance. In addition, serial administration of the MF-SAF throughout this trial demonstrated a significant improvement in MF associated symptoms.[21] The greatest improvements in MF-SAF scores were reported by patients experiencing abdominal discomfort, night sweats, pruritus, and fever. Responses were equivalent regardless of subtype of MF (i.e., PMF vs. post-ET or post-PV MF) or JAK2 mutation status, and responses appear durable (median duration of response >1 year). Reduction in splenomegaly (>50% decrease in size according to criteria of the IWG-MRT) was seen in 52% of patients at the 15 mg BID dose and across all dose levels for 44%.

TG101348 is a selective JAK2 inhibitor s active against FLT3 and RET. [22] In a phase I study in 59 high- or intermediate-risk primary or post-PV/ET and PMF patients, 47% achieved a spleen response (IWG-MRT) at 12 months, with a mean response duration of 315 days.[23] Leukocytosis and thrombocytosis normalized in 57% and 90%, respectively. In patients with symptoms at baseline, early satiety resolved in 56%, night sweats in 89%, cough in 67%, pruritus resolved in 50%, and fatigue improved in 63% of patients. JAK2V617F allele burden was significantly reduced.[23]

SB1518 is a selective JAK2 inhibitor active at low nanomolar concentrations against both wild-type and mutant (V671F) JAK2, as well as FLT3.[24] In a Phase I/II study of 33 with previously treated PMF and splenomegaly at enrollment, activity was demonstrated with 17 of 30 patients (57%) achieving a 25% or greater reduction in spleen volume by MRI. Side effects were of GI nature (diarrhea, nausea, vomiting, abdominal pain) and fatigue with little myelosuppression.[24]

CYT387 inhibits Jak1/2 and TYK2, as well as other kinases (CDK2/cyclin A, MAPK8, PRKCN, PRKD1, ROCK2, and TBK1).[25] The overall response rate (ORR) by IWG-MRT in the Phase I/II study was 62%. Anemia response was 50% overall and higher in transfusion dependent patients with 57% and 69% at 150 mg and 300 mg, respectively. Interestingly responses were seen in 5 of 9 patients previously treated with other JAK2 inhibitors and 5 of 12 patients treated with pomalidomide. Splenomegaly was reduced in 47%, and constitutional disease symptoms were controlled in more than 80% of patients, including night sweats (88%), bone pain (80%), pruritus (92%), and fever (100%). Hyperlipasemia, transaminitis, headache, and a first-dose effect (lightheadedness, hypotension) were DLTs or side effects. Greater than or equal to grade 3 thrombocytopenia, anemia, and neutropenia were seen in 27%, 7%, and 5% of patients, respectively. These results and especially anemia results are early, need confirmation, and a full manuscript is awaited.

MF Scenario 3: the intermediate I risk patient

Patients who are intermediate risk are probably the most heterogeneous group amongst those with myelofibrosis (Table 1). Individuals in this group may have survivals that could range anywhere from as low as 3 years to as much as 8 or 9 years. Individuals by current prognostic scores fall into either Intermediate 1 or Intermediate 2 group (Table 1). Given that individuals who are intermediate 2 have survivals that range from 35–48 months, their management is best, though amongst similar strategies as those used for high risk patients (see next section). When deciding individualized therapy in the heterogeneous intermediate I risk group, one needs to try to delineate where amongst this group an individual may lie with perhaps the current DIPSS-Plus criteria being the most helpful to sub-stratify an individual's prognosis. Initially, an individual has to be mindful of a patient's goals, wishes, age, comorbidities, and philosophy. Therapeutic options really fall into two categories, one, those which would be disease altering and potentially have higher risks, but higher reward in terms of survival (allogeneic stem cell transplant – Table 2) or therapies that may be disease altering; but we do not have definitive proof of these agents at this point in time, including JAK2 inhibition, interferon alpha 2a, or hypomethylation therapy, such as azacitidine[26] (Table 4). The next group of options would revolve around medicines that are helpful amongst palliative goals and finally clinical trials, which as an umbrella term incorporates medicines of a targeted and non-targeted nature, including JAK2 inhibitors, which remain on clinical trials and other agents, which are efficacious against myelofibrosis.

Goal of disease altering therapy

Now amongst these patients, if the goal is disease course alteration, where an individual lies in that spectrum of prognosis from 3 years to 8 years, this becomes very important. Allogeneic stem cell transplant could either be considered frontline therapy in this group for individuals in the higher end of the intermediate risk category who are appropriate stem cell candidates based on their comorbidities and a suitable donor (Table 2). Likewise, for individuals at the longer end of the spectrum, transplant may remain a more reasonable backup option if other lines of therapy fail or risks increase. Outside of stem cell transplantation, JAK2 inhibition is probably our most interesting group of agents in this group of patients (Table 3). It may be a disease-altering therapy, but there is no definitive proof at this time, and randomized clinical trials are expected with anticipation.

Allogeneic stem cell transplant (ASCT) offers the possibility of cure to patients with MF. However, the risk of graft versus host disease (GVHD), transplant related mortality (TRM), and post-transplant relapse of the MF itself make it essential to use this form of therapy only in the most appropriate candidates. So who are the optimal candidates for ASCT in MF? This is a highly controversial question. We have no randomized prospective data from which to draw conclusions. As with all decisions in medicine, the choice and timing of ASCT in MF is based on weighing of the risk-to-benefit ratio, as well

Table 4. A risk-based approach to develop individualized management plans for myelofibrosis.

Risk Status	Therapeutic Options	Sequence of Use in MF
Low Risk - Asymptomatic	Observation	Observation only appropriate option
Low Risk - Symptomatic	Hydroxyurea Interferon alpha – 2a JAK2 inhibition Selective Serotonin Reuptake Inhibitors Therapeutic UV light	Splenomegaly 1. JAK2 inhibition on Study 2. Hydroxyurea off study 3. Other myelosuppressives a. Interferon alpha – 2a Pruritus 1. JAK2 inhibition on study 2. Interferon alpha – 2a 3. Selective Serotonin Reuptake Inhibition 4. Therapeutic UV light Vascular/ proliferative symptoms 1. Hydroxyurea 2. Interferon alpha -2a Other Symptoms (fatigue, etc) 1. JAK 2 inhibition
Intermediate Risk	Disease Altering Allogeneic Stem Cell Transplant ? JAK2 inhibition ? Interferon alpha – 2a ? hypomethylation therapy Palliative Goals Hydroxyurea Immunomodulators (IMIDS) Erythropoietin Androgen Therapy Splenectomy Splenic Radiotherapy **CLINICAL TRIALS**	Goal is disease alteration 1. JAK2 inhibition **2. CLINICAL TRIALS** 3. Possible second line therapy a. Interferon alpha-2a or hypomethylation 4. Allogeneic stem cell transplant (sequence needs refinement of where in intermediate risk they stand) Goal is Palliation Splenomegaly (see low risk section for initial options) **1. CLINICAL TRIALS** 2. Splenectomy or splenic radiotherapy if other options exhausted Anemia 1. IMIDS 2. Erythopoietin or Androgens **3. CLINICAL TRIALS** Symptomatic Burden (see low risk section)
High Risk	Disease Altering Allogeneic Stem Cell Transplant ? JAK2 inhibition ? Interferon alpha – 2a ? hypomethylation therapy Palliative Goals Hydroxyurea Immunomodulators (IMIDS) Erythropoietin Androgen Therapy Splenectomy Splenic Radiotherapy **CLINICAL TRIALS**	Goal is disease alteration 1. Allogeneic stem cell transplant if candidate 2. JAK2 inhibition **3. CLINICAL TRIALS** 4. Hypomethylation therapy Goal is Palliation Splenomegaly (see low risk section for initial options) **1. CLINICAL TRIALS** 2. Splenectomy or splenic radiotherapy if other options exhausted Anemia 1. IMIDS 2. Erythopoietin or Androgens **3. CLINICAL TRIALS** Symptomatic Burden (see low risk section)
Blast Phase of Myelofibrosis	Allogeneic stem cell transplant Induction chemotherapy Low intensity chemotherapy (i.e. low dose cytarabine) Hypomethylation therapy **CLINICAL TRIALS**	Curative path (only if transplant candidate) 1. Induction chemotherapy (either standard or experimental induction "strength" chemotherapy). 2. Salvage induction therapy for primary refractory blast phase (very low likelihood of success) 3. Allogeneic Stem Cell Transplant Non curative path **1. CLINICAL TRIALS** or supportive care alone (+- hydroxyurea for control of leukocytosis) 2. Hypomethylation therapy (can be first line if no valid trial available and therapy is appropriate)

as a thorough understanding by the patient of the complexity and potential adverse effects of the therapy they might choose to undertake.

Analysis of recent large series of ASCT in MF is sobering (Table 2), with TRM ranging from 10–30% (depending on age, donor compatibility, and conditioning regimen) and rates of acute GVHD (grades II–IV) ranging from 10–60%. Rates of chronic GVHD are even higher, with certain series reporting 85% of patients with grades II–IV chronic GVHD. Overall survival ranges from 30–60%; the largest retrospective IBMTR series suggests about one-third of patients with MF who are transplanted might expect long term disease-free survival.[27]

Given the risks and unanswered questions in 2011, which patients with MF should be transplanted? The group of patients where the risk-benefit ratio appears most favorable is those progressing towards blastic transformation. Given the median survival of patients after transformation is less than 3 months,[5] ASCT, if it is at all an option, should occur before transformation occurs.

Clinical trials with newer agents that still may have a greater impact on the natural history of these diseases are viewed with great interest, but their impact on natural history is of course, by definition, uncertain. Finally, therapies may help to delay the natural progression of the illness, but this again remains uncertain and their use depends on the scenario. In patients with earlier myelofibrosis, there is increasing data suggesting interferon alpha 2a may be a consideration (Table 3). Individuals with higher risk disease, that is, specifically an increase in blasts hypomethylationtherapy, may be a reasonable option.[26,28]

Goal of palliation

If palliation is the goal for the MF patient, then we must determine what is it that we are trying to palliate. For splenomegaly, I would refer you to the low risk section as the initial option that we would consider. For individuals who have failed other options, one might consider a clinical trial next if available and appropriate, and finally, in select scenarios of overwhelming mechanical symptoms, we will consider splenectomy[29] or splenic radiotherapy.[30] With limiting toxicities of both of these latter therapies being first for splenectomy, short and long-term morbidity and mortality, thrombosis, and hemorrhage in the perioperative setting, and long-term complications of leukocytosis and thrombocytosis, splenic radiotherapy leading to profound cytopenias and short duration of response.

Anemia based palliation revolves around the use of immunomodulatory drugs, thalidomide,[31] lenalidomide,[32] and now pomalidomide[33] with individuals who have a deletion 5q[34] in their cytogenetic profile being the clearest candidates for lenalidomide therapy. The selective use of erythropoietin for individuals with an inadequate erythropoietin response is a reasonable option for intermediate risk myelofibrosis patients. Androgen based therapy, simply agents, such as danazol,[35] are reasonable considerations. Clinical trials, specifically trying to improve anemia, remain of interest but clearly have an experimental endpoint.

And finally, for generally symptomatic burden, I would refer you to the low risk section, as it would

depend on which symptom was being palliated, but again this would be a consideration for JAK2 inhibition.

MF Scenario 4: the intermediate II or high risk patient

The high risk patient is an extension of the intermediate risk patient, but the timeline is more compressed, with high risk patients having survivals that really are in the 2–3 year range, but could be significantly less (Table 1). In these individuals, the role of allogeneic stem cell transplant is as front line therapy. If a patient is a candidate for stem cell transplant, then the goal should be to have the transplant performed in the most expeditious manner possible (Table 4). If transplant is not a consideration, then JAK2 inhibition is a reasonable thought, may have an impact on the natural history of the illness, and may also help to palliate the disease. Clinical trials of other agents would be reasonable backup options for this group of patients. Finally, if disease alteration is a goal, hypomethylation therapy[36] may play a role in this group of patients. The data in the literature remains relatively thin, with a goal of avoiding disease progression as a target and all agents must be considered experimental with such an endpoint.

Goal of palliation

For the high risk patient who has a palliative goal, it is very clear and important to be clear with the patient of their short-term expected survival and have appropriate expectations. Here, for patients with splenomegaly, we again use the options discussed in the low risk section, but clinical trials, as an initial option if they have failed other options, is very reasonable. Additionally, splenectomy and splenic radiotherapy are short-term palliative options. Palliation of anemia, symptomatic burden, and other difficulties would mirror those palliative efforts performed in the intermediate risk patient.

MF Scenario 5: acute myeloid leukemia from antecedent MF

Blast phase of myelofibrosis represents an extremely dangerous and life-threatening progression for individuals with this illness.[5] Here, the likelihood of long-term success is difficult. Patients truly exist in one of two categories: if a curative path exists, specifically, the only potentially curative option for the patient in blast phase of myelofibrosis would be stem cell transplantation, but it has been well demonstrated that undergoing an allogeneic stem cell transplant in the phase overt blast phase illness, that is, greater than 20% blasts in the bone marrow, will be met with failure if some short-term remission from the blast phase is not achieved. Therefore, the path for these individuals involves induction chemotherapy, either standard, that is, cytarabine administered for 7 days continuous infusion with standard or high dose daunorubicin or some equivalent induction therapy. For these groups of patients, it would be reasonable to consider high risk acute myeloid leukemia induction clinical trials if they have a reasonable expectation of being able to achieve a remission. If remission is achieved, it probably will not be long lived.[5] It is important to try to go to stem cell transplant as soon as humanly possible; in the ideal world, to go from induction to the transplant itself (Table 2). If the timing of transplant and obtaining a donor delays the transplant, then an appropriate consolidation regimen administered during this interval would be reasonable. Salvage induction therapy for those who are primary refractory to blast phase for either their day 14 bone marrow or after the induction phase can be considered, but the likelihood of success is low, there is very limited data in the literature, and the likelihood of success in the patient who has needed multiple induction regiments for blast phase of myelofibrosis is quite low.

The final group is those individuals who have a non-curative path for the blast phase of myelofibrosis. In this group of individuals, it is appropriate to either consider clinical trials of low and intermediate intensity, but not of induction intensity, that is, most appropriate outpatient therapy if possible or supportive care alone. Clearly the use of hydroxyurea for the control of patients with significant leukocytosis in high circulating blast percentages is reasonable. It is very important for patients in this group to realize that they have a fatal illness with a short life expectancy and that they understand well that the therapy being given is for palliation and modest disease control, but that the illness will be taking their life and they must plan accordingly. Finally, hypomethylation therapy has been shown in high risk MDS patients and those with early AML; that is, those without extremes of leukocytosis, significant organ dysfunction, is that azacitidine 75 mg/m^2 for 7 days can be a reasonable option.[36] Amongst patients who have transformed from an MPN (including MF) to AML, preliminary data shows the potential for response (39% with a return to a chronic MPN phase in those who had transformed to either AML or myelodysplastic syndrome).[37] These latter preliminary data are interesting and suggest further prospective trials alone or in combination therapy approaches are warranted given the limited therapeutic that currently exist for these patients.

Conclusion

The understanding and therapy of MF has advanced significantly over the past 5 years, with several approaches and agents making a meaningful difference for MF patients even today. Several trials seeking FDA approval for agents in MF are ongoing – a situation without precedent in this disease. Current management of our MF patients requires treating clinicians to assess prognosis accurately, consider risks and benefits of each line of therapy, and engage in a careful shared decision-making process with the patient. Future advancements in therapy will likely result from combination therapeutic approaches, targeted therapies based on new pathogenetic insights, or both.

In conclusion, myelofibrosis represents a very heterogeneous illness, in which one must be mindful not only of the presenting features of the illness but be astute to observing the clinical course of each patient, watch how their disease unfolds, continue to weigh the strengths and merits of each therapeutic option, as they evolve, and come up with an individualized tailored plan with

the flexibility to be adjusted as the disease changes. It is important to note that one can rapidly progress at times from one phase of risk to another, and have the options for higher risk intervention be available; that is, have appropriate stem cell transplant donors identified so that delay does not occur for higher risk therapies when they need to be employed.

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Angiogenesis and antiangiogenesis in patients with multiple myeloma

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A B S T R A C T

Angiogenesis is a constant hallmark of multiple myeloma (MM) progression and has prognostic potential. The pathophysiology of MM-induced angiogenesis involves both direct production of angiogenic cytokines by plasma cells and their induction within the bone marrow microenvironment in stromal cells. Cytokines promote plasma cell growth, survival and migration, and angiogenesis. It has been demonstrated that bone marrow macrophages and mast cells are closely involved in vasculogenic mimicry, thus contributing together with circulating endothelial cells and endothelial precursor cells (EPCs) to the MM neovascularization.

An improved understanding of the importance of angiogenesis-related signaling in MM has allowed for the rational use of antiangiogenic therapies in this tumor.

First evidence of an increased angiogenesis in bone marrow of multiple myeloma patients

In 1994, Vacca and colleagues demonstrated for the first time that bone marrow microvascular density was significantly increased in multiple myeloma (MM) compared with monoclonal gammopathy of undetermined significance (MGUS), and in active versus nonactive MM. The authors first hypothesized that progression from MGUS parallels an increase in bone marrow microvascular density.[1] As progression from *in situ* to invasive and metastatic solid tumors is accompanied and enhanced by the switch from the prevascular to the vascular phase, these findings suggest that active MM represent the 'vascular phase' of plasma cell tumors, and nonactive MM and MGUS their 'prevascular phase'. Subsequent studies by others confirmed the observation of increased angiogenesis in active MM compared to MGUS or healthy individuals.[2-5]

Cytokines involved in the angiogenic switch

The mechanisms of induction of the vascular phase are the subject of current investigation.[6] Several studies show overexpression and secretion of the vascular endothelial growth factor (VEGF) by the clonal plasma cells. VEGF stimulates proliferation and chemotaxis in both endothelial cells via VEGF receptor-2 (VEGFR-2) and bone marrow stromal cells (BMSCs) via VEGFR-1. VEGFRs are rapidly phosphorylated by the interaction with VEGF, and signal via extracellular signal-related kinase-2 (ERK-2).[7] Plasma cell-derived VEGF also stimulates interleukin-6 (IL-6) and VEGF secretion in BMSCs, whereas BMSCs-derived IL-6 promotes proliferation, survival, and VEGF production in plasma cells.[8]

Fibroblast growth factor-2 (FGF-2) is also secreted by MM cells.[9] Levels of FGF-2 are significantly higher in the plasma cell lysates of patients with active MM compared with nonactive MM and MGUS patients. Inhibition of FGF-2 by an anti-FGF-2 antibody suppressed the angiogenic potential of plasma cells from patients with active MM.[9] Moreover, FGF-2 triggers paracrine myeloma-BMSCs interactions in an IL-6/FGF-2 paracrine loop.[10] Syndecan-1 (CD138), a low affinity receptor of FGF-2, is also expressed by MM cells.[11]

Hepatocyte growth factor (HGF) and its receptors c-Met and angiopoietin-1 (Ang-1) have been described as additional angiogenic factors in MM.[12-14] HGF has been identified in human MM cell lines12 and in freshly isolated MM cells.[13] Serum levels of this factor are high in approximately 40% of patients at diagnosis and decline to normal levels with response to induction therapy. There is no decline if the therapy is ineffective, and the levels become high again upon release.[15] Giuliani *et al*. found that Ang-1 expression is up-regulated in MM cell lines or in patients' plasma cells, and that the Ang-1 expression in the patients' samples correlated with bone marrow microvascular density.[14] Furthermore, they demonstrated that the Ang-1 receptor Tie-2 is up-regulated in the bone marrow endothelial cells in the presence of MM cells, and that an anti-Tie2 antibody blocked the *in vitro* angiogenic activity of MM cells.

Insulin like growth factor-1 (IGF-1) plays a role in the angiogenic process by stimulating MM cells to secrete VEGF.[16] Plasma IL-8 is increased in MM.[17] Osteopontin (OPN) was

found to contribute to angiogenesis in MM,[18] and OPN expression correlated with bone marrow microvascular density.[19]

Matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) secretion is increased in patients with active versus nonactive MM or MGUS.[9] A number of MM cell lines and freshly isolated bone marrow plasma cells produce MMP-9, although correlation with disease activity has not been assessed.[20] MMP secretion of MM cells is triggered by BMSCs or endothelial cells.[21]

We have demonstrated that platelet-derived growth factor (PDGF)-receptor beta (PDGFRβ) and pp60-Src as constitutively activated tyrosine kinases (TKs) are expressed in plasma cells of MM patients.[22] The PDGF-BB/PDGFRβ axis promoted MM tumor growth by activating ERK-1/2 and AKT, and this activity was selectively induced by VEGF.

Airoldi *et al*. demonstrated that IL-12 receptor B2 (IL-12Rβ2) was expressed in primary MM cells but down-regulated compared with normal plasma cells.[23] IL-12 reduced the pro-angiogenic activity of primary MM cells *in vitro* and decreased significantly the tumorigenicity of NCI-H929 cell line in SCID/NOD mice by inhibiting cell proliferation and angiogenesis.

Hose *et al*. have reported that normal bone marrow plasma cells express several angiogenic genes, including *VEGF-A, IGF-1, and adrenomedullin (ADM)*, and their culture supernatants significantly induce *in vitro* angiogenesis.[24] MM plasma cells show a pattern of aberrant expression of several angiogenic factors, and culture supernatants of primary MM cells and human cell lines induce *in vitro* angiogenesis. However, none of these factors was expressed in any of the MM patients' samples, and no correlation with microvascular density could be found.

The role of the bone marrow microenvironment

The MM microenvironment is formed by clonal plasma cells, extracellular matrix proteins, and BMSCs, which are intimately involved in all biological stages of intramedullary growth.[25] Reciprocal positive and negative interactions between plasma cells and BMSCs are mediated by an array of cytokines, receptors, and adhesion molecules. Interactions between these components give proliferation, migration, and survival of plasma cells, as well as their drug resistance.[26–28] Plasma cells secrete several cytokines, such as tumor necrosis factor alpha (TNF-α),[29] transforming growth factor beta (TGF-β), VEGF, FGF-2,[9] HGF,[12,13] Ang-1,[14] and MMPs,[9] into their microenvironment. Moreover, binding of plasma cells to BMSCs triggers transcription and secretion of cytokines by the latter, such as IL-6,[8] IGF-1,[30] VEGF,[31] and CXCL12/stromal cell derived factor-1α (SDF-1α)[32] that mediate cell growth (IL-6, IGF-1, VEGF), survival (IL-6, IGF-1), drug resistance (IL-6, IGF-1, VEGF), migration (IGF-1, VEGF, MMPs, SDF-1α), and angiogenesis (VEGF).

The role of bone marrow endothelial cells

Vacca *et al*. demonstrated that the endothelial cells of MM bone marrow (MMECs) are characterized by an enhanced expression of specific angiogenic factors/receptors, such as VEGF/VEGFR-2, FGF-2/FGF-2R-2, and Ang-2/Tie-2, and by an increased *in vitro* and *in vivo* angiogenic activity.[33] These endothelial cells express more mRNA and secrete larger amounts of the CXC-chemokines CXCL8/IL-8, CXCL11/interferon-inducible T-cell alpha chemoattractant (I-TAC), CXCL12/SDF-1α, and CCL2/monocyte chemotactic protein-1 (MCP-1) than human umbilical vein endothelial cells (HUVECs). Since paired plasma cells express cognate receptors to a variable extent, paracrine loops between MMECs and plasma cells involving CXC-chemokines and their receptors may be operative in patients and mediate plasma cell proliferation and homing.[32]

We have demonstrated that pp60c-Src is a key signaling effector of the VEGF loop required for MMECs' survival, migration and angiogenesis, and assessed the antiangiogenic activity of dasatinib, a novel orally bioactive PDGFRβ/Src TK-inhibitor that significantly delayed MM angiogenesis *in vivo*.[22]

We have carried out a comparative gene expression profiling of MMECs versus MGUS endothelial cells (MGECs) with an Affymetrix assay.[34] Twenty-two genes were found differentially expressed (14 down-regulated and 8 up-regulated) in MMECs versus MGECs. Deregulated genes were mostly involved in extracellular matrix formation and bone remodeling, cell adhesion, chemotaxis, angiogenesis, resistance to apoptosis, and cell-cycle regulation. Validation was focused on *DIRAS3*, *SERPINF1*, *SRPX*, *BNIP3*, *IER3*, and *SEPW1* genes, which were not previously found to be functionally correlated to the overangiogenic phenotype of MMECs. Small interfering RNA for the up-regulated genes *BNIP3*, *IER3*, and *SEPW1* affected critical endothelial cell functions mediating this overangiogenic phenotype, for example, proliferation, apoptosis, adhesion, and capillary tube formation.

The role of circulating endothelial cells and endothelial precursor cells

Zhang *et al*. demonstrated that circulating endothelial cells (CECs) and endothelial precursor cells (EPCs) in peripheral blood were six-fold higher in MM patients than in controls and correlated with serum M protein and β2-microglobulin.[35] Circulating EPCs displayed late colony formation/outgrowth and capillary-like network formation, which were inhibited by thalidomide treatment. Co-expression of VEGFR-2 and CD133 characterized EPCs and VEGFR-2 mRNA elevations correlated with the M protein levels.

We have demonstrated that in patients with active MM, plasma cells and stromal cells in the microenvironment recruit hematopoietic stem precursor cells (HSPCs) and induce their differentiation into mature MMECs.[36] In fact, when incubated with VEGF, FGF-2, and IGF, HSPCs differentiate into endothelial-like cells expressing typical endothelial markers, such as FVIII-RA, VEGFR-2, and VE-cadherin, and form capillary-like networks *in vitro*.[36] Bone marrow MM but not MGUS biopsies revealed HPSCs inside the neovessel wall, suggesting that in the former, HSPCs contribute to the

neovessel building together with MMECs. Therefore, besides angiogenesis, HSPC-linked vasculogenesis contributes to neovascularization in MM patients.

The involvement of MM macrophages and mast cells in vascular mimicry

When bone marrow macrophages from MM patients are exposed to VEGF and FGF-2, they transdifferentiate into cells overlapping functionally and phenotypically paired MMECs, and generate capillary-like networks mimicking those of MMECs.[37] In patients with active MM, FACS analysis of freshly isolated bone marrow mononuclear cells revealed higher percentages of CD14/CD68 double-positive cells than in nonactive MM and MGUS patients. In active MM patients, bone marrow biopsies displayed macrophages with both endothelial cell-like (i.e., CD68/FVIII-RA double positive) and apparently typical (i.e., CD68 positive/FVIII-RA negative) features located in the microvessel wall and collaborating with MMECs to line the vessel lumen. Figures of this type were rare in nonactive MM and absent in MGUS patients. Thus, macrophage involvement in the vasculogenic pathway proceeds in step with MM activity, as well as with progression of plasma cell tumors.[37]

Chen *et al*. demonstrated that monocytes induce vascular endothelial cell gene expression and develop tube-like structures when cultured with bone marrow from MM patients who express pleiotrophin; this effect was blocked with anti-pleiotrophin antibodies.[38] Moreover, when co-injected with human MM cells into SCID mice, green fluorescent protein-marked human monocytes were found to be incorporated into tumor blood vessels and expressed human vascular endothelial cell proteins and genes that were blocked by anti-pleiotrophin antibodies. These results suggest that vasculogenesis in human MM may develop from tumoral production of pleiotrophin, which induces the transdifferentiation of monocytes into vascular endothelial cells.

Bone marrow angiogenesis and mast cell density counts are highly correlated in patients with nonactive and active MM and in those with MGUS, and both parameters increased simultaneously in active MM.[39] Vessels from MM biopsies are lined by mast cells whose cytoplasms was filled with numerous and irregularly shaped electron dense granules.[40] These ultrastructural findings have been confirmed by confocal laser microscopy using double anti-tryptase (a mast cell marker) and anti-FVIII-RA (an endothelial cell marker) antibodies. Vessels from MM biopsies displayed regions stained by FVIII-RA alternating with regions stained by both tryptase and FVIII-RA. In the MGUS biopsies, the vessels were uniformly stained by the anti-FVIII-RA antibody only, while tryptase-positive mast cells were only recognizable perivascularly.[40] Overall, these data suggest that in MM, mast cells contribute to neovascularisation.

Antiangiogenesis in MM (Table 1)

Thalidomide and lenalinomide

Both drugs have emerged as highly active agents for the treatment of MM. The antiangiogenic properties of thalidomide supported its use in MM.[41] Furthermore, in addition to its antiangiogenic activity, thalidomide enhances T-cell- and NK-cell-mediated immunological responses, induces caspase-8-mediated apoptosis, and downregulates IL-6 production within the microenvironment.[42,43] Singhal *et al*. firstly used thalidomide on a compassionate basis to treat 84 patients with relapsed and refractory MM in 1997. They found it remarkably effective, with a 32% response rate.[44]

We analyzed the antiangiogenic properties of thalidomide45 and demonstrated that therapeutic doses of thalidomide markedly downregulate dose-dependently key angiogenic genes in MMECs, but upregulate them or are ineffective in endothelial cells of patients with nonactive MM or MGUS. Secretion of VEGF, FGF-2, and HGF also diminished dose-dependently in conditioned media of active MMECs, whereas it did not change in the other conditioned media.[45] The FDA led several clinical studies in 2006 for the drug approval in the treatment of newly-diagnosed MM, in combination with dexamethasone. Rajkumar and co-workers reported that 470 patients with untreated symptomatic MM were randomized to thalidomide/dexamethasone versus placebo plus dexamethasone.[46] The overall response rate was significantly higher and the time to progression significantly longer with thalidomide/dexamethasone.[46]

Lenalidomide is a 4-amino-glutarimide analogue of thalidomide with antiangiogenic proprieties.[47] It inhibits the interactions between cadherin 5, beta-catenin, CD31, and adherens junctions, which are critical events for angiogenesis to develop. Furthermore, lenalidomide inhibits VEGF-induced PI3K-Akt pathway signaling and hypoxia inducible factor-1 alpha (HIF-1α) expression,[48] exerts an anti-TNF-α activity, modulates the immune response by T cells and NK cells, induces apoptosis of tumor cells, and decreases the binding of MM cells to bone marrow stromal cells.[42,43,49,50] In 2006, lenalidomide received FDA approval for the treatment of MM patients who had at least one prior therapy. A retrospective analysis of clinical trials on previously treated relapsed/refractory MM demonstrated an improved response rate and increased median for patients treated with lenalidomide and dexamethasone, compared with those treated with dexamethasone alone.[51–53] Lenalidomide sensitizes MM cells to bortezomib.[54] In a phase 2 study, lenalidomide/bortezomib/dexamethasone produced 84% responses in

Table 1. Antiangiogenic properties of drugs used in clinical trials in multiple myeloma.

Drug	Antiangiogenic properties
Thalidomide	Inhibition of VEGF, FGF-2, HGF
Lenalidomide	Inhibition of VEGF, VEGFR-2, NF-kB
Bortezomib	Inhibition of VEGF, VEGFR-2, Ang-1, Ang-2, NF-kB, ERK-1/-2
Tyrosine kinase inhibitors	Inhibition of VEGFR-1, -2, -3, PDGFR-β, c-kit
Zoledronic acid	Inhibition of VEGF, VEGFR-2

relapsed/refractory patients, including complete response or near complete response in 21%. This regimen produced responses in 98–100% of newly-diagnosed MM patients.[55]

Recently, we have demonstrated that lenalidomide, at clinically achievable concentration, is antiangiogenic *in vivo* and inhibits MMECs migration.[47] Lenalinomide halts the MMECs' overangiogenic potential by down-regulating key angiogenic genes and VEGF/VEGFR-2-mediated downstream signaling pathways involved in cell motility, and NF-kB. A comparative proteomic analysis reveals that lenalidomide-treated MMECs modulate the expression levels of angiogenesis-related genes controlling cell motility and invasiveness, cell shape, and cytoskeletal dynamic remodeling, as well as energy metabolism pathways and protein clearance.[47]

Bortezomib

It is a proteasome inhibitor, which induces endothelial cell apoptosis,[56] inhibits VEGF, IL-6, Ang-1 and Ang-2, and IGF-1 secretion in BMSCs and endothelial cells from MM patients,[57,58] HIF-1α activity,[59] downregulates caveolin-1 tyrosine phosphorylation, which is required for VEGF-mediated MM cell migration, and also blocks the caveolin-1 phosphorylation induced by VEGF in endothelial cells, thereby inhibiting ERK-dependent cell proliferation.[60]

Roccaro *et al*. demonstrated that bortezomib inhibits the proliferation of MMECs in a dose- and time-dependent manner.[57] Moreover, in endothelial cell functional assays, including chemotaxis, adhesion to fibronectin, capillary formation on Matrigel, and chorioallantoic membrane (CAM) assay, bortezomib demonstrated a dose-dependent inhibition of angiogenesis. Bortezomib has been previously approved for MM patients who failed at least one prior therapy,[61] and for initial treatment in a pivotal, multicenter, open-label trial, in which 682 previously untreated patients, who were ineligible for high-dose therapy plus stem-cell transplantation, were randomized to receive melphalan and prednisone combination alone (control group) or with bortezomib.[62] The time to progression among patients receiving bortezomib plus melphalan/prednisone was 24 months, as compared with 16.6 months among those receiving melphalan/prednisone alone (control group). The overall survival and response rates were also better in the bortezomib group.[62]

The use of bortezomib in pre-transplant induction therapy revealed a higher response rate, compared with other induction regimens.[63] Recently, we demonstrated that bortezomib and zoledronic acid display distinct and synergistic activities on bone marrow macrophages of MM patients.[64] Drugs inhibited macrophage proliferation, adhesion, migration, and expression of angiogenic cytokines and capillarogenesis on Matrigel. Moreover, VEGFR-2 and ERK-1/2 phosphoactivation, as well as NF-kB were also inhibited. Preclinical studies with new proteasome inhibitors are underway.[65,66]

Tyrosine kinase inhibitors in the treatment of MM

Receptor tyrosine kinases (RTKs) are transmembrane proteins containing an extracellular lectin binding domain and an intracellular catalytic domain. Many of the processes involved in tumor growth, progression, and metastasis are mediated by signaling molecules acting downstream from activated RTKs. Tyrosine kinase inhibitors (TKIs) are small molecules able to pass the plasma membrane.[67] The tyrosine kinase VEGFRs are crucial mediators in angiogenesis. Stimulation of VEGFRs and other RTKs causes massive activation of signaling pathways in endothelial cells. TKIs inhibit not only VEGFRs but also other receptors in the super-family of RTKs, including PDGFR. Inhibitors of VEGF signaling not only interfere with angiogenesis but also cause regression of some tumor vessels,[68] giving changes in all components of the vessel wall, consisting in loss of endothelial cell fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts.[69]

In 2005, the FDA granted regular marketing approval for sorafenib, a small oral inhibitor for the treatment of patients with advanced renal cell carcinoma.[70] It is a small oral multi-TKI of VEGFR, PDGFR, *c-kit*, and Flt-3 kinase activity.[71] TKIs can be taken orally, if necessary in a salt form of the inhibitor. For example, sunitinib is taken as sunitinib malate, while sorafenib as tosylate sorafenib. Lin *et al*. showed that vatalanib (PTK787/ZK222584), an orally administered broad-spectrum TKI of VEGFR-1,-2,-3, PDGFR-β, *c-kit*, inhibited proliferation and migration of MM cells.[72] Pandiella *et al*. showed that imatinib mesylate (STI571) blocked cell-cycle progression in MM and potentiated the effects of conventional anti-MM agents *in vitro*.[73] However, in a phase II trial in patients with refractory/relapsed disease, no response was obtained.[74]

Zangari *et al*. and Kovacs *et al*. evaluated the activity of SU5416, a small TKI of VEGFR-1,-2,-3, and vandetanib (ZD6474) in patients with refractory MM, and observed a decrease in VEGF serum levels in patients with stable disease, but not with objective response.[75,76] Podar *et al*. demonstrated that pazopanib (GW786034B) and GW654652, two broad-spectrum TKIs of VEGFR-1,-2,-3, PDGFR, *c-kit*, inhibited *in vivo* MM cell proliferation, migration, and survival, VEGF-induced up-regulation of adhesion molecules on both endothelial and tumor cells, and exerted an antiangiogenic activity *in vivo*.[77] However, a phase II trial in 21 MM patients treated with pazopanib gave no appreciable response.[78]

Ramakrishnan *et al*. showed that sorafenib exerted a significant anti-MM activity and synergized with common anti-MM drugs.[79] Coluccia *et al*. have shown constitutive activation of PDGFR-β/*Src*, two dasatinib targets, in plasma cells and MMECs.[22] Moreover, dasatinib significantly delayed MM tumor growth and angiogenesis *in vivo*, showing a synergistic cytotoxicity with other anti-MM drugs, that is, melphalan, prednisone, bortezomib, and thalidomide.

In about 10–20% of MM patients, a translocation [t(4;14)] involving FGF receptor-3 (FGFR-3) is associated with poor prognosis.[80–82] Small molecules with selective TKI activity (SU5402, SU10991, PD173074, PKC412) have been validated in preclinical models of MM.[83–85]

Zoledronic acid ZOL

This is a bisphosphonate used for MM bone disease and hypercalcemia. ZOL has a direct cytotoxic activity on tumor cells and suppresses angiogenesis.[86,87] We have demonstrated that therapeutic doses of ZOL markedly inhibit *in vitro* proliferation, chemotaxis, and angiogenesis of MMECs, and *in vivo* angiogenesis in the CAM.[88] These effects are partly sustained by gene and protein inhibition of VEGF and VEGFR-2 in an autocrine loop.

Mevastatin, a specific inhibitor of the mevalonate pathway, which prevents prenylation of several proteins leading to cellular apoptosis, antiangiogenesis, and activation of γ/δ T-cells, reverts the ZOL antiangiogenic effect, indicating that the drug halts this pathway.

Interleukins

Cocco *et al*. have demonstrated that IL-27 directly inhibits MM cell growth both *in vitro* and *in vivo* primarily through the inhibition of angiogenesis.[89] Moreover, IL-27 inhibits osteoclasts differentiation and activity, and induces osteoblast proliferation and damps *in vivo* tumorigenicity of human MM cell line through inhibition of angiogenesis. These results open new perspectives for MM therapy because IL-27 may block MM progression and metastatic bone resorption.

Concluding remarks

Angiogenesis is intimately involved in the pathobiology of MM in both preclinical and clinical models. An improved understanding of the importance of angiogenesis-related signaling in MM has allowed for the rational use of antiangiogenic therapies in patients with this malignancy.

The median survival for patients with MM has almost doubled since the introduction of thalidomide, lenalidomide, and bortezomib. These agents have been incorporated into conventional cytotoxic and transplantation regimens and used as a treatment for newly diagnosed MM. Nevertheless, most patients still relapse after an initial response to treatment and multidrug resistance often emerges over time.[54]

Considering the complex angiogenesis regulatory network that involves multiple angiogenic factors produced by various cell types, any antiangiogenesis therapy aimed at a single angiogenic factor is not likely to be highly effective. Areas of future investigation include identifying factors other than VEGF critical in the angiogenic cascade, elucidating mechanisms of therapeutic resistance, and developing markers for identifying patients most likely to benefit from antiangiogenic treatment.

Novel agents, as well as various emerging compounds targeting cell surface receptors, inhibiting signaling pathways or unfolded protein response, interfering with the cell cycle, as well as epigenetic agents are currently under investigation.

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Role of genetics in myeloma risk stratification

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A B S T R A C T

Risk stratification, mostly done via genetic testing is of paramount importance in the prognostication of myeloma for counseling treatment planning and prognostication. This paper discusses the implications of these novel genetic factors and current therapy for the disease.

Introduction

It is now widely accepted that multiple myeloma (MM) is a heterogeneous disease with the major subtypes being defined by the underlying genetic aberrations of the clonal plasma cells (PC).[1,2] These genetic aberrations have been used as the foundation to classify the disease, establish prognostic categories, and to a limited degree, serve as predictive indicators.[1–3] Many aspects of the disease are directly influenced by the presence of specific genetic changes. These include clinical features, "natural history of the disease," aggressiveness markers and other.

Obtaining this genetic information is now considered routine clinical care for MM, and should be required for all ongoing and future clinical trials.[4,5] In general, five to eight subgroups of MM have been identified using the various methodologies, although many of these categories largely overlap.[6–8] Subdivision into many small subgroups carries the risk of creating fragmented analyses that lack statistical power to be reliable[9] In response to this challenge, a dichotomy classification has been proposed that segregates patients into high and standard risk categories (+/- and intermediate risk category) (www.msmart.org).[9,10] While no specific therapy exists that is primarily directed at high-risk MM, three important corollaries have emerged: i) clinical trials aimed at high-risk MM are being developed; ii) identification of risk categories has allowed standard-risk patients to be treated with more chronic disease controlling strategies; iii) and identification of high risk features is critical for proper counseling of patients with these variants of the disease (avoidance of "MM is a chronic disease" hue to the patient counseling discussion).

Background and techniques

The field of MM cytogenetics has invigorated since the first observations were made in the mid 1980s and 1990s regarding a potential role for cytogenetic changes to discriminate patients with more aggressive disease.[11–13] The field quickly evolved to the application of molecular cytogenetics (i.e., FISH)[3,14,15] and more recently high throughput genomic platforms (i.e., gene expression profiling (GEP)[16–18] and array based comparative genomic hybridization (aCGH).[19,20] Deployment of more modern genomic tools has been hampered by practical limitations regarding reimbursement and regulatory considerations. However, a recent commercialization of GEP provides a test case in which modern genomics is introduced into routine clinical care for the disease.[21]

Until such advances are widespread, FISH remains the standard for disease prognostication.[2,3,14,15] It is imperative, however, that FISH be done in such a manner that the scoring is restricted to PC only.[22] Many commercial laboratories continue to perform FISH in unsorted, or otherwise unselected, nuclei making results interpretation unreliable. Samples submitted to cytogenetic laboratories often represent the last pull of those collected for clinical purposes. It is, therefore, not uncommon to observe a much lower fraction of PC in those aspirates, mostly as a consequence of hemodilution. Clinicians must be wary and look for the specific nomenclature provided in the cytogenetic response. A standard "*nuc ish*" (for nuclear interphase FISH) should be considered evidence of unreliable testing. PC must be either sorted (for instance using anti-CD138 magnetic beads)[3] or the FISH analysis must be coupled with immunofluorescence detection of the clonal PC.[22] PC identification can readily be done using cytoplasmic light chain staining (e.g., cIg-FISH).[22]

Diagnosis and disease classification

All cancers can be classified from various perspectives that describe features and attributes of the clonal cells (and sometimes the host as well).[23,24]. In MM, the disease

diagnosis does not require genetic studies *per se*, as the morphology is obvious and consistent enough that no specific genetic marker is required for diagnosis. A rare exception to this occurs in cases where MM must be differentiated from other late B-cell malignancies, such as Waldenström macroglobulinemia (WM).[25,26] For instance, the rare version of MM that produces IgM monoclonal proteins (IgM MM) can be differentiated from WM, since the former has a high prevalence of t(11;14)(q13;q32)[26] and the latter is more likely to have 6q deletions and never has t(11;14)(q13;q32).[27]

Despite recent treatment advances, namely derivates of thalidomide and proteasome inhibitors[28,29] being touted as overcoming some of the negative prognostic implications for patients with high risk genetic features (e.g., t(4;14)(p16;q32)), these patients still tend to have more aggressive clinical courses with overall worsened prognosis from the time of diagnosis.[15,30,31]

Biological classification

A biological classification of the disease is primarily driven by the orderly acquisition of genetic changes that drive clonal proliferation.[2,7,8,32] Because the classification identifies discrete subgroups of the disease, these classes may be associated with dissimilar outcome, but not necessarily. This biological classification is primarily driven by genetic features that can be observed since the premalignant phases of the disease are associated with specific genetic progression events (from benign stages to malignant).[33,34] In MM, this is best exemplified by chromosome 14 translocations as one group and trisomies leading to hyperdiploidy as another.[35–37] Accordingly, at the very top hierarchical level, MM can be classified into hyperdiploid and non-hyperdiploid.[36] This classification is one that stands the test of time and is usually revealed by testing performed via various technologies.[2]

Genetic prognostic classification

While many systems exist that can estimate prognosis for MM patients, recent efforts are all focused on the identification of genetic markers that can discriminate clinical outcomes.[3,15,38] These genetic prognostic markers may be the same that also form biology subclasses (e.g., t(4;14)(p16;q32)), but can also include genetic progression events, such as chromosome 17 deletions and abnormalities of chromosome 1 (deletions of 1p and 1q amplifications). Genetic prognostic classifiers are accordingly exclusively focused on those features of the clonal cells that dictate outcome. In contrast, clinical classifications identify host features that may portend a higher likelihood of death only because of host specific features and not truly describing a more aggressive nature of the clonal process.[23,24]

Genetics have been used by several large studies to predict the outcome of MM patients treated with conventional[14,39] and high dose chemotherapy followed by stem cell transplant (SCT).[3,15,16] The majority of these studies were done when treatments were primarily based on alkylator therapy. Now multiple studies are underway to understand better the prognostic implications of classic genetic classifiers in cohorts of patients treated with proteasome inhibitor and imuunomodulatory drugs.

While there is still a paucity of data regarding the value of prognostic factors for novel therapeutics, it appears that bortezomib can neutralize some of the negative effects of some of the high-risk markers,[40–42] and emerging data exists regarding thalidomide and lenalidomide.[43–45] Despite the initial excitement all of the studies addressing the role of proteasome, inhibitors in patients with high-risk disease have been relatively underpowered to address the question conclusively, and most have been done in the setting of relapsed and refractory MM, with only few addressing the question in the upfront setting.[30,41,43] It is also important to note that the value of prognostic factors needs to be validated according to the specific stage of the disease; prognostic factors validated in the upfront treatment of the disease may not have similar effects in previously treated patients.

Predictive capacity

In some instances, genomic markers may be used to predict responsiveness to specific therapy (e.g., herceptin in her2neu positive breast cancer). As of yet, there are no validated predictive markers for MM. This information, if available in an expeditious, economical, and practical manner could be of major significance in choosing the various therapeutic options for the disease. Since most patients currently still receive all agents at one point or another of their disease, it is likely this information would only determine sequencing of treatment. The only exception to this would be if we had such predictive power to know with great certainty that a proposed therapy would be of no value in patient care, and then attempts at it would be futile. One unvalidated example of a predictive marker is determination of constitutive upregulation of the NF-kB pathway via mutations.[46] Another possibility is that a pathway or a cell surface marker is specific enough so that therapy would only be dictated in cases with such phenol/genotype.

Specific genetic categories

Deletions of 17p

Among all MM, genetic factors deletions of chromosome 17 remain the single most important prognostic factor.[3,14–16,47–50] These abnormalities were identified originally in patients treated with conventional forms of chemotherapy and SCT but have persisted as prognostic in patients treated with lenalidomide and bortezomib.[43–45,51] A recent study of a large group of patients receiving induction treatment with bortezomib and dexamethasone followed by SCT showed that novel therapy has had a minimal impact among patients with 17p13 deletions.[45] One large study continues to show the importance of -17 as a marker of negative outcomes.[30] While the exact gene involved in the negative prognosis associated with these deletions has not been fully elucidated, all evidence points to *p53* deficiency as a culprit in the more aggressive clinical course.[47]

t(4;14)(p16;q32)

This translocation was originally described as associated with an inferior outcome in a series of patients treated with alkylators, both at standard doses and with

SCT.[14,16,52–54] This translocation is associated with unfavorable outcomes and more aggressive clinical features at the time of diagnosis.[14,16,52–54] In the study of Shaughnessy and colleagues, patients with this translocation appear to be more likely to have a high risk GEP signature.[16] While recent data initially showed that the negative prognostic implication of the translocation was eliminated with the use of bortezomib,[40,41] subsequent studies have failed to show this. In fact, the aforementioned large French series shows that while bortezomib has improved outcome in t(4;14)(p16;q32) patients as compared with older therapies, the genetic marker is still prognostic in a large group of patients treated with this drug.[45] Another large Spanish study has shown similar results and confirms that high-risk genetic features still portend an unfavorable outlook for patients.[30] It can now be summarized that while novel therapeutics have improved the outlook of most patients, these gains are distributed unequally, being minimal for -17 patients, moderate for t(4;14)(p16;q32), and greatest for standard risk disease.[30]

t(14;16)(q32;q23) and other MAF abnormalities

We originally identified abnormalities of C-MAF, associated with the t(14;16)(q32;q23) as a negative prognostic feature in MM among patients treated with conventional doses of alkylator therapy.[14] Subsequent studies showed similar effects when patients were treated with high dose chemotherapy. In a GEP study, those with t(14;16)(q32;q23) were more likely to exhibit features of disease aggressiveness.[16] Another study looking at MAF-b translocations also identified this factor as associated with an inferior outcome amongst MM patients.[55] Routine testing for MAF abnormalities has not been universally embraced given the less frequent nature of this aberration. A recent report by the IFM has questioned the prognostic significance of t(14;16)(q32;q23) given that a large set of patients tested showed a neutral effect on prognosis.[56] This remains puzzling, as all other previous reports have shown important effects on prognosis. In this French study, those with t(14;16)(q32;q23) had a high propensity to display many circulating cells, a hallmark of aggressive MM.[56] While we think it is quite possible this may be due to type II error, further studies are needed to clarify or dispel the importance of this translocation as a prognostic marker.

Chromosome 1

Multiple studies have shown that chromosome 1 abnormalities are associated with an inferior outcome in MM.[57–61] The interpretation of these studies is confounded since these aberrations, chromosome 1p deletions and 1q amplifications, are highly correlated, and discerning individual contribution is nearly impossible.[37] Chromosome 1 aberrations have not yet made their way into standard clinical practice and continue to be investigated.

Genomic testing beyond FISH

GEP

The power of GEP as a prognostic tool[17,18] has been best exemplified by the work of investigators at the University of Arkansas.[8,16] Using a large patient dataset, they have been able not only to provide a classification of the disease (albeit very similar to all others proposed),[8] but also more importantly, identify 15% of patients with a very poor prognosis.[16] The data have been validated in other scenarios but need further validation in the context of novel therapies, although it is likely to be useful in these setting as well.

Practical limitations exist for widespread applicability of this test, but they are being overcome with standardized methods for cell processing. The test must be done in purified PC that needs to be shipped to reference laboratories. The results are interpreted as "signatures" indicative of high-risk genetic features. A new commercial venture has begun offering the test as a reference laboratory.[21] GEP also needs to be validated further as the test of choice in patients treated with various combinations of modern agents. There is no doubt from the scientific perspective that these strategies represent the best method for prognostic estimation of MM. A balance between the power of the predictive ability of genomics and practical implications is needed to integrate this fully into clinical practice.

aCGH

A series of studies has looked at the role of aCGH as a prognostication tool for MM.[19,20] The advantages of this platform are that the results are more stable and therefore more reliable. The other advantage is that the test can be easily converted to other standard clinical testing, such as FISH. Nevertheless aCGH has not gained mainstream acceptance as a routine test for MM given some of the same practical limitations as GEP (such as the need for purified plasma cells, regulatory efforts, etc.). One study showed that patients could be readily classified into three major prognostic subgroups based on a combination of genomic loss/gain (amp5q and deletions of 12p) and 2-microglobulin.[19,20] Another smaller study also looked at the use of aCGH as a classifier for the disease and with possible prognostic implications.[19,20]

Sequencing

There is minimal information regarding sequencing strategies as it relates to clinical outcomes and prognosis in MM. Some studies have shown that classic oncogenes and tumor suppressor genes can be altered in MM, but these have not reached the point of clinical utility.[47,62,63] Recent efforts using next generation sequencing will soon be published but have not been yet implemented with a clinical slant (Chapman *et al*, In press, Nature 2011).

Practical aspects of genetic testing

As aforementioned, the accurate genetic classification of patients can have profound consequences on clinical decisions, including counseling and treatment selection.[10] Furthermore, identification of risk categories should be paramount in developing specific clinical trials that address a major unmet medical need; the management of high-risk MM.[2] Results obtained from clinical genetic testing should be actionable and provide information with utilitarian value to clinicians.

Genetics prognostic tools should be reliable in predicting outcome of patients and also have desirable features to be developed as standard clinical laboratory tests (i.e., good reproducibility and accuracy). Ideally, they should be widely available and easy to interpret. Prognostic estimation with FISH-based strategies fulfills many of these requisites, albeit with GEP having a greater ability to discern outcome. If current genomic platforms can be employed in determining the outcome in a community setting for MM patients, they are likely to replace FISH as the diagnostic tool of choice (Table 1).

Recommended genetic clinical testing in myeloma

It is now accepted that all MM patients should have disease risk stratification done by employing one or more genetic tools.[2,10,15,30] In the past, this was mostly done using standard karyotype analysis, given the powerful implications of on outcome for patients with chromosome abnormalities. The limitations of this test made the diagnostic community move forward towards more modern techniques, such as FISH and now GEP. The GEP strategy promises to deliver the highest impact in selecting patients with high risk myeloma but still practical limitations have prevented widespread use.[16]

GEP recommendations

GEP is limited in that it is only available at reference laboratories,[21] needs to be done using purified PC, and is still fraught with complexities regarding reimbursement, coverage, and so on. It assumes a sufficient quantity of plasma cells is available for RNA extraction and of sufficient quality to perform the test. Unfortunately, not all patients have enough cells to be able to have GEP done, perhaps with smaller RNA requirements, the fraction of patients with successful results will increase. The purification process must be validated *post-hoc* for assurances that the product is composed predominantly of clonal PC. The cells and products should be maintained under optimal conditions to avoid degradation. The test is very appealing as it provides the most desirable genetic information in one simple step and has been shown to have the highest predictive value for any genetic classifier of MM. It has the capabilities of detecting those with the more aggressive disease, and can serve as comfort for those without this high-risk signature.[16] There is no doubt that GEP strategies will and should become standard of care in the future.

FISH testing recommendations

The recommended clinical testing will undoubtedly change over time if one uses FISH as a primary diagnostic tool, as new probes and markers will be discovered and validated (and some eliminated).[2] Some of the testing could be done only once as the information provided will not change, and some can be repeated since there may be changes over time.

FISH testing must be done always using a concurrent marker that identifies the PC or only with nuclei of previously sorted PC. Otherwise, the results should be considered unreliable. FISH is limited in that it addresses genomics only in a "close ended" question format and falls short of proving a comprehensive perspective on the genomic complexity of the disease. Its categorical allocation of patients into subsets, therefore, is intrinsically limited and overall, can only provide general guid-

Table 1. Comparison between FISH and GEP.

	FISH	GEP
Ability to discern highest risk categories	++	+++/++++
Clinical test	Yes	Now being deployed
Information obtained	Limited	Global genomics
Expense	Similar	Similar*
Can do overnight testing?	Yes	Yes
Need for reference laboratories	Yes	Yes
Number of cells needed for analysis	100	>100,000
Requires cell enrichment/purification?	No for clg	Yes
Suitable for cases of minimal plasmacytosis?	Yes	No
Number of cases with informative results	Most (>90%)	Low (<50%)
Covered by insurance	Yes	Sometimes

** Cost may be less for GEP depending on the number of probes used for FISH.*
*** Needs metadata generation and interpretation.*

Table 2. Risk stratified testing in myeloma.

Test	Markers	Frequency
High risk translocations	T(4;14) and t(14;16)	Needs to be tested only once to establish category
17p13 deletions	FISH -17p13	Can be tested at multiple times as it is acquired with progression
GEP	High risk GEP UAMS High risk IFM signature	Centrosome index Can be done at baseline and may be repeated. Data also emerging on predictive ability

ance. It is quite likely that a future that involves GEP would make FISH potentially dispensable (the only exception now being determination of 17p deletions not adequately covered by GEP).

Frequency of FISH testing recommended

An attempt has been made to summarize current recommendations and thoughts regarding the frequency of testing needed for myeloma (Table 2). While undoubtedly this will change over time, this can be used as a general guide on what to test and when.

Conclusion

Until the time that genomic tools (i.e., GEP, mRNA sequencing, genome sequencing) become standard clinical tools, at minimum, FISH must be done to establish prognostic criteria for MM patients. FISH identifies 25% of patients with high-risk disease and is fundamentally not that dissimilar to GEP, although clearly of less power. For now, the application of FISH techniques are likely to remain as the main way of determining the prognostic outcome of MM based on genomics.

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Frontline treatment of multiple myeloma

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A B S T R A C T

This review will focus on the current frontline treatment of multiple myeloma. High-dose therapy and autologous stem cell transplantation in younger patients, and new treatment modalities including the use of novel agents in elderly patients will be discussed.

Introduction

The decision to treat a patient is based on the criteria set for the diagnosis of symptomatic multiple myeloma (MM), which includes the CRAB (hypocalcaemia, renal dysfunction, anemia, and bone lesions) criteria. Patients with clearly defined monoclonal gammapathy of unknown significance (MGUS) or smoldering multiple myeloma (SMM) do not need initiation of therapy irrespective of any associated risk factors except specifically targeted protocols.[1]

Treatment of patients less than 66 years of age

Autologous stem cell transplantation ASCT versus conventional chemotherapy CC

A key therapeutic advance was the introduction almost 25 years ago of high-dose melphalan therapy supported by autologous hematopoietic stem-cell transplantation.[2] The Intergroupe Francophone du Myelome was the first to conduct a randomized trial showing the superiority of this approach compared with CC in terms of response rate, event-free survival (EFS), and overall survival (OS).[3] These results were confirmed 7 years later by the British Medical Research Council.[4] As a consequence of these two studies, ASCT became the standard of care for frontline therapy, at least in younger patients (up to 65 years of age) with normal renal function. An important finding from these studies was a significant increase in the rate of CR (negative immunofixation) or very good partial remission (reduction of the M-component by more than 90%) with ASCT, which was significantly correlated with longer progression-free survival (PFS) and OS. Overall, the use of ASCT did improve the median OS from 3 years to 50 to 55 months. The results of randomized trials comparing conventional chemotherapy to high-dose therapy and ASCT are summarized (Table 1).

The clinical results of HDT were obtained with two types of conditioning regimens, using either high-dose melphalan alone or regimen additionally containing total-body irradiation (TBI). The IFM group compared melphalan 200 mg/m² (Mel200) and melphalan 140 mg/m² plus 8 Gy TBI and showed that Mel200 was at least as effective and better tolerated than the regimen containing TBI.[10] Moreover, while EFS was identical in both groups, OS was significantly longer with Mel200 due to a longer OS after first relapse. As a result, Mel200 became the preferred preparative regimen, and up to now, no randomized trial has shown the superiority of any other conditioning regimen compared with Mel200.

Tandem transplantation

Strategies based on further intensification were explored with the aim of improving on the results obtained with single ASCT. The IFM was the first to conduct a randomized trial comparing single and double ASCT in 599 patients up to 60 years of age.[11] On an intent-to-treat basis, the 7-year EFS and OS were significantly improved in the double ASCT arm. The benefit for EFS, but not for OS was confirmed by two other randomized studies (Table 2).[12,13] All three published studies showed that achievement of CR (or at least a VGPR) had a favorable impact on EFS and/or OS. However, many investigators considered the benefit of this approach to be marginal, and were concerned by cost and morbidity. Therefore, defining which patients could derive benefit from this aggressive approach was important. The only parameter that predicted the patients who did or did not benefit from double ASCT was response to the first ASCT. Patients with less than 90% reduction of their M-component after one ASCT had a longer OS in the double ASCT arm, whereas patients achieving CR or VGPR after the first ASCT had the same OS with or without the

Table 1. Conventional chemotherapy vs. high-dose therapy: results of randomized studies.

Group/Trial (reference)	Nb of patients	Age	Median follow-up	CR rate (%)		Median EFS (mo)		Median OS (mo)	
				CC	HDT	CC	HDT	CC	HDT
IFM 90[3]	200	<65	7 years	5	22	18	28	44	57
MAG91[5]	190	55-65	56 mo	5	19	19	24	50	55
Pethema[6]	164	<65	44 mo	11	30	33	42	66	61
Italian MMSG[7]	195	<70	39 mo	6	25	15.6	28	42	58 +
MRC7[4]	407	<65	42 mo	8	44	19	31	42	54
MAG95[8]	190	55–65	10 years	20	48	19	25	48	48
US S9321[9]	516	≤70	76 mo	15	17	14% at 7 years	17% at 7 years	38% at 7 years	38% at 7 years

second ASCT step. Therefore, at the end of 1990s, many investigator considered single ASCT with Mel200 as the preparative regimen as the standard of care, and that a second ASCT could be proposed in patients achieving less than VGPR after the first HDT.

Novel agents to improve response rates during induction

Until recently, VAD was the induction regimen most widely used prior to ASCT and considered the standard of care. The primary objective of incorporating novel agents in this setting is to increase the CR rate not only prior to, but also after ASCT. A further objective of incorporating novel agents during induction is to reduce the proportion of patients requiring a second ASCT because of a suboptimal response (less than VGPR) to the first ASCT step.

Thalidomide was the first novel agent to be compared with VAD, in combination with dexamethasone alone (TD),[14] or with adriamycin plus dexamethasone (TAD).[15,16] Overall the benefit of TD or TAD compared with VAD remained modest. Other thalidomide combinations have also been evaluated. The CTD regimen (Cyclophosphamide, Thalidomide, Dexamethasone) is currently being investigated in a large randomized study in the UK, and preliminary results show high CR rates both before and after ASCT.[17]

The second novel agent to become available, bortezomib was investigated in combination with dexamethasone in a large trial (IFM2005-01) and prospectively compared with VAD.[18] Post-induction CR/nCR, greater than or equal to VGPR, and overall response rates were significantly higher with bortezomib-dexamethasone versus VAD. These superior response rates in the bortezomib-dexamethasone induction arms of the trial translated into better response rates after HDT. This improvement also impacted the outcome of the disease and the median PFS was 36 versus 30 months with bortezomib-dexamethasone versus VAD. Survival was not superior in the bortezomib-dexamethasone arms of the study, due to effective salvage regimens at the time of relapse. The level of response achieved with bortezomib-dexamethasone post-induction in the IFM2005-01 trial is now considered as the goal of current therapies, and as a result, bortezomib-dexamethasone has become the backbone of induction therapy prior to ASCT to which other more complex regimens should be compared.

The addition of a third agent to bortezomib-dexamethasone (thalidomide -VTD,[19] doxorubicin - DVD or PAD,[20] lenalidomide - RVD,[21] or cyclophosphamide - CVD22) has been tested in several small phase 2 studies and the outcome appears even better, with response rates around 90% and CR rates up to 24%. In all of these studies, the frequent, rapid, and deep responses consistently translated into improved outcomes. Three prospective studies have already shown that VTD is superior to TD or bortezomib-dexamethasone.[23–25] The Italian group prospectively compared TD versus VTD in 474 patients with newly diagnosed MM prior to tandem ASCT and found that VTD resulted in higher CR and greater than or equal to VGPR rates as compared with TD, which translated into better PFS after high-dose therapy.[23] The Spanish group also compared TD versus VTD versus a more complex chemotherapy regimen, including bortezomib, prior to ASCT in 390 patients and confirmed that VTD was able to achieve the best pre- and post-ASCT CR rates.[24] In the IFM2007-02 trial, four cycles of the "standard" bortezomib-dexamethasone induction regimen were prospectively compared with four cycles of VTD with lower doses of bortezomib (1 mg/m^2 instead of 1.3 mg/m^2) and thalidomide (100 mg/day instead of 200 mg/day, as in the Italian and Spanish trials) to reduce the neuropathy rate.[25] VTD was

Table 2. Single versus double autologous stem-cell transplantation: results of published randomized trials.

	Number of patients	EFS	OS
IFM 94[11]	399	7 years: 10% versus 20% (p<0.03)	7 years: 21% versus 42% (p<0.01)
Bologna 96[12]	321	Median 23 months versus 35 (p<0.001)	7 years: 46% versus 43% (p=0.90)
Hovon 24[13]	304	Median 22 months versus 21 6 years: 15% versus 7% (p=0.013)	Median 50 months versus 55 (p=0.51)

again found to result in superior CR plus VGPR rates both before and after ASCT. The reduction in the doses of both bortezomib and thalidomide was associated with a reduction in the incidence of neurotoxicity, with grade 3–4 peripheral neuropathy (PN) occurring in only 3% of the patients in the VTD arm. The IFM2007-02 study therefore confirmed the superiority of a 3-drug combination over a 2-drug combination as induction prior to ASCT. The Hovon group recently reported the final results of a phase 3 randomized prospective trial comparing VAD versus PAD as induction prior to HDT. This study confirmed the superiority of the bortezomib-based triplet induction over VAD, and OS was also superior in the bortezomib arm of the trial.[26]

No data are available to draw conclusions regarding the superiority of one combination, VTD, RVD, VCD, PAD, and so on, over the other. Although response rates are clearly improved with novel agent cocktails, the demonstration of a significant OS advantage will often be difficult given the large number of patients and the long duration of follow-up required, as well as the availability of effective salvage therapies. Thus, based on response rates, depth of response, and PFS as surrogate markers for outcome, 3-drug combinations, as tested in the phase 2 and 3 studies described above, are, in 2010, the standard of care prior to ASCT (Table 3).[27]

Table 3. Response rates to novel-agent-containing induction therapy, and clinical outcomes after HDT-ASCT in phase III trials.

Study by induction regimen	No.	Postinduction (%)		Post-Transplant (%)		Long-term Outcomes (%)
		ORR	CR/nCR/VGPR	ORR	CR/nCR/VGPR	
MAG [14]						
TD	100	NR	25 ≥ VGPR	NR	44 ≥ VGPR	NR
VAD	104	NR	7 ≥ VGPR	NR	42 ≥ VGPR	NR
HOVON-50[15-16]						
TAD	268	71	3 CR 37 ≥ VGPR	88	31 CR 66 ≥ VGPR	Median EFS: 34 months Median PFS: 34 months Median OS: 73 months
VAD	268	57	2 CR 18 ≥ VGPR	79	23 CR 54 ≥ VGPR	Median EFS: 22 months Median PFS: 25 months Median OS: 60 months
IFM 2005-01[18]						
Vel/Dex	240	79	6 CR 15 CR/ nCR 38 ≥ VGPR	84	16 CR 35 CR/ nCR 54 ≥ VGPR	Median PFS: 36 months 3-year OS: 81
VAD	242	63	1 CR 6 CR/ nCR 15 ≥ VGPR	79	9 CR 18 CR/ nCR 37 ≥ VGPR	Median PFS: 30 months 3-year OS: 77
GIMEMA MMY-3006[23]						
VTD	236	94	19 CR 62 ≥ VGPR	NR	55 CR 87 ≥ VGPR	2-year PFS: 85 2-year OS: 96
TD	238	79	5 CR 31 ≥ VGPR	NR	38 CR 69 ≥ VGPR	2-year PFS: 75 2-year OS: 91
PETHEMA/GEM05MEN0S65[24]						
TD	104	62	14 CR 28 ≥ VGPR	92	37 CR 64 ≥ VGPR	NR
VTD	102	82	31 CR 59 ≥ VGPR	97	52 CR 78 ≥ VGPR	NR
VBMCP/VBAD/Vel	100	71	23 CR 37 ≥ VGPR	96	49 CR 65 ≥ VGPR	NR
IFM 2007-02[25]						
Vel/Dex	99	81	12 CR 22 CR/ nCR 35 ≥ VGPR	84	33 CR 54 CR/ nCR 59 ≥ VGPR	NR
VTD	100	90	13 CR 32 CR/ nCR 51 ≥ VGPR	90	30 CR 61 CR/ nCR 73 ≥ VGPR	NR
HOVON-65/GMMG-HD4[26]						
VAD	373	55	5 CR/ nCR 15 ≥ VGPR	77	15 CR/ nCR 36 ≥ VGPR	Median PFS: 27 months
PAD	371	78	11 CR/ nCR 42 ≥ VGPR	88	30 CR/ nCR 61 ≥ VGPR	Median PFS: 36 months

Abbreviations: CR, complete response; EFS, event-free survival; GEM, Grupo Español de Mieloma; GIMEMA, Gruppo Italiano Malattie Ematologiche dell'Adulto; HOVON, Hemato-Oncologie voor Volwassenen Nederland; IFM, Intergroupe Francophone du Myèlome; nCR, near CR; NR, not reported; ORR, overall response rate; OS, overall survival; PETHEMA, Programa para el Estudio de la Terapéutica en Hemopatía Maligna; PFS, progression-free survival; TAD, thalidomide, doxorubicin, and dexamethasone; TD, thalidomide and dexamethasone; VAD, vincristine, doxorubicin, and dexamethasone; VBMCP/VBAD, vincristine, carmustine, melphalan, cyclophosphamide, prednisone/vincristine, carmustine, doxorubicin, and dexamethasone; Vel, bortezomib; Vel/Dex, bortezomib and dexamethasone; VGPR, very good partial response; VTD, bortezomib, thalidomide, and dexamethasone; PAD: bortezomib, doxorubicin and dexamethasone.

Novel agents given as consolidation after ASCT

Before the availability of novel agents, the first approach to consolidate a response achieved with a first ASCT was the use of a second ASCT procedure in a tandem fashion. Currently, novel agents are being tested soon after ASCT to improve further the quantity and quality of the responses. The most striking report involving novel agents during consolidation has been that of the Italian group. Ladetto *et al*. treated 39 patients who achieved at least VGPR after ASCT and who had an available molecular marker based on the immunoglobulin heavy-chain rearrangement with four courses of VTD.[28] Response was assessed by qualitative nested PCR and RQ-PCR using tumor-clone–specific primers. Immunofixation complete responses increased from 15% after ASCT to 49% after VTD. Molecular remissions (MR) were observed in 3% of patients after ASCT and in 18% after VTD. With a median follow-up of 42 months after consolidation, at the time of the report in 2010, no patient in MR has relapsed. This study is the first to document the occurrence of persistent MR in a proportion of MM patients treated with ASCT followed by consolidation therapy, which is "novel-agent" based. Other trials studying the impact of novel agent-containing consolidation regimens after ASCT are ongoing. One of the most important ones will be conducted in the United States (BMT/CTN study), comparing, after a single ASCT prepared by Mel200, either no consolidation, or four cycles of a triplet combination of RVD, or a second ASCT prepared by Mel200. Another important international, prospective trial, which has been designed to assess the RVD regimen in combination with or without ASCT (IFM/DFCI 2009 trial), will also examine the impact of two cycles of RVD given as consolidation after ASCT.

Novel agents and maintenance strategy

Before the availability of novel agents, several trials tested chemotherapy as maintenance treatment without any positive results, and preliminary findings regarding the efficacy of α interferon in this indication could not be confirmed. Thalidomide was the first novel agent to become available. Several phase 2 studies demonstrated the feasibility of thalidomide maintenance therapy and suggested that it may improve OS.[29–30] Four phase 3 randomized studies have been completed and all of them could show a significant benefit in terms of response and PFS with thalidomide maintenance treatment, while OS was improved in two out of four trials.[31–34] Despite these findings, thalidomide is not being widely used as maintenance therapy, presumably reflecting concerns about cumulative toxicity. Notably, peripheral neuropathy observed with thalidomide is related to the duration of treatment and is cumulative. Lenalidomide, the second oral IMiD available at present, is not neurotoxic, and is currently considered as the best candidate for use as maintenance therapy. The results of two randomized trials, which have tested this novel agent after ASCT, were reported during the 2010 meeting of the American Society of Clinical Oncology.[35,36] In the IFM2005-02 study, 614 patients younger than 65 years with non-progressive disease after a first line ASCT were randomized to receive consolidation with lenalidomide (25 mg/day, 21 days/month, for 2 months) followed by maintenance with either placebo (arm A) or lenalidomide (10 to 15 mg/day until relapse, arm B).[35] Preliminary results showed that maintenance treatment with lenalidomide improved the 3-year PFS from randomization (primary end-point of the study): 34% in arm A versus 68% in arm B ($p<10^{-6}$). The 2-year survival was similar in both treatment arms (95%). The CALGB group reported the results of a similar phase 3 randomized trial of lenalidomide or placebo following HDT and ASCT (CALGB100104 study).[36] Four hundred and eighteen patients younger than 70 years with non-progressive MM were randomized 2–3 months post-ASCT to receive either placebo (n = 208) or lenalidomide 10 mg/day (n = 210) until progression. The study was prematurely stopped because of the superiority of the lenalidomide arm, with a 58% reduction in risk of progression as compared with placebo, and a median time-to-progression (TTP, primary endpoint) not reached on the lenalidomide arm versus 25.5 months on the placebo arm ($p = 0.0001$) at a median follow-up of 14 months. A longer follow-up is needed to assess the impact of lenalidomide maintenance on survival and potential late-effects. Bortezomib has also been investigated as maintenance treatment after HDT in a large phase 3 trial comparing VAD or PAD prior to ASCT, with maintenance using thalidomide (50 mg daily) on the VAD arm, or bortezomib 1.3 mg/m^2 twice a month on the PAD arm for 2 years.[26] There was a trend for a longer duration of response in the maintenance phase using bortezomib. Nevertheless, the IV formulation and the risk of neurotoxicity are not in favor of this compound in this indication.

Incorporating novel agents at each step of the procedure: the optimal strategy

The use of novel agents clearly improves the results of HDT at each step of the procedure. Induction therapy is now based on highly active triplet combinations yielding high CR/VGPR rates, which are important prognostic parameters for long-term outcome. Use of novel agents post-ASCT in a consolidation phase may improve the CR rate, as well as the depth of response leading to molecular CR. Following consolidation, maintenance strategies using oral drugs with a low-toxicity profile, such as lenalidomide, are able to prolong the duration of response and PFS. Taken together, it is now possible to design what could be the best option for patients with newly diagnosed MM fit for HDT: induction consisting of a triplet combination with novel agents, Mel200 plus ASCT, consolidation using additional cycles similar to those used during induction, followed by a maintenance phase using an oral IMiD. What can we expect using this ambitious program regarding duration of response? Barlogie and colleagues have already enrolled 303 patients onto the complex and lengthy total therapy 3 protocol incorporating novel agents during the induction, consolidation, and maintenance phases.[37,38] The 5-year estimates of OS and EFS are 72% and 69%, respectively. Nevertheless, many issues, such as toxicity, late-effects, the choice of salvage therapy at the time of relapse, tumor clone selections, how to overcome poor-risk cytogenetics, risk-adapted strategies, remain open.

Using ASCT as salvage treatment after novel agent-based frontline treatment without ASCT

Combination of two novel agents with dexamethasone, RVD, developed by the group of the Dana–Farber Cancer Institute as part of frontline treatment of de novo MM patients looks very promising.[21] In a prospective phase 1/2 trial, 66 patients received eight 3-week cycles of RVD, and responding patients could proceed to maintenance treatment or ASCT (28 patients). The combination was highly effective, with a PR rate of 100% and with 74% achieving at least VGPR in the phase 2 portion of the study. The median follow-up was relatively short at the time of the report (21 months), but the estimated 18-month PFS and OS for the combination treatment with/without ASCT were 75% and 97%, respectively. A post-hoc landmark analysis showed a low-risk of progression after 1 year regardless of ASCT status. Although preliminary, these results are of major interest since a combination of novel agents without ASCT seems to challenge the outcome achieved in patients treated with ASCT. In this situation, ASCT could be used as a salvage therapy at the time of relapse. These findings form the basis for an international prospective study planned to assess this RVD combination with or without ASCT, followed by maintenance treatment (IFM/DFCI 2009 trial). This comparison is now the key issue in the treatment of newly diagnosed patients eligible for ASCT. Other prospective international phase 3 studies designed to compare early versus late ASCT in the context of novel agents are ongoing.

Treatment of patients older than 65 years

Until 2007, frontline chemotherapy with melphalan and prednisone (MP) was considered as the standard of care in the treatment of elderly patients with MM. Recently, several prospective randomized studies comparing MP with the same combination plus new agents, such as thalidomide (MPT) or bortezomib (MPV), clearly showed that MPT and MPV were superior to MP in terms of progression-free and overall survival. Melphalan-prednisone-lenalidomide (MPR) is currently compared with MP in one prospective trial and will also probably be superior to MP. Lenalidomide plus low-dose dexamethasone is a promising combination. Thus, at least four highly active new treatment options are now available to treat elderly patients with MM. The role of maintenance therapy is now under evaluation in order to prolong the duration of response.

MP regimen

Chemotherapy with melphalan and prednisone (MP) has been used in the treatment of MM since the 1960s, and remained until 2007, the most widely accepted treatment option for elderly patients.[39,40] More complex combinations with alkylating agents have been tested and compared to MP. A meta-analysis of 27 randomized trials has compared MP with various combination chemotherapies and showed that despite a higher response rate with combinations, overall survival was not increased, and MP was considered the standard of care in patients older than 65 years of age.[41] Dexamethasone-based regimens have also shown no survival advantage with MP regimens in elderly patients, and were also associated with more toxic side-effects.[42] Since the median survival with MP is about 3 years, new treatments were needed. The introduction of novel agents, thalidomide, bortezomib and lenalidomide, has changed the scenario. These drugs are now used in combination with MP, and showed superior results in terms of response rates, PFS, and OS as compared with MP. Lenalidomide plus low-dose dexamethasone is also a promising combination.

MP + thalidomide

Six randomized studies have compared the combination of thalidomide and MP (MPT) to MP in elderly patients with newly diagnosed MM.[43–48] These studies consistently reported that MPT resulted in higher overall response rates (57–76 vs. 28–48%) and longer PFS (13–27.5 vs. 10–19 months). However, only three studies demonstrated improved survival with MPT (40–51.6 vs. 27.7–32.2 months), and three studies reported similar OS with MPT (26–47.6 months) and MP (28–45 months). Overall, the data strongly support the use of MPT as the standard of care for elderly myeloma patients. In all studies, the MPT regimen was associated with a significant higher incidence of grade 3 or 4 adverse events, including PN, infections, cardiac toxicity, and thromboembolism. Therefore, antithrombotic prophylaxis is recommended when using MPT. The dosage of thalidomide needs to be reduced in patients less than 75 years of age to minimize toxicity.

MP-bortezomib

The combination of MP + bortezomib (Velcade®) (VMP) has been tested in a phase 1/2 study reported by the Spanish group PETHEMA de novo MM patients.[49] The VMP response rate was 89%, including 32% immunofixation-negative CRs, of whom half of the IF-negative CR patients analyzed achieved immunophenotypic remission (no detectable plasma cells at 10-4 or 10-5 sensitivity). The 1.3 mg/m^2 bortezomib dose level was selected. These interesting preliminary results were the basis of the prospective randomized phase 3 VISTA (Velcade as Initial Standard Therapy) trial comparing MP with VMP.[50] Six hundred and eighty-two patients 65 years of age or older or not transplant eligible with untreated MM were randomized to receive MP for nine 6-week cycles (n=338) or the VMP combination previously described (n=344). The total duration of treatment was 54 weeks in both arms of the study. The primary endpoint was time to progression (TTP), and secondary endpoints including PFS, OS, overall response rate, time to and duration of response, and safety. The median TTP was 24 months in the VMP arm versus 16.6 months in the MP arm, ($p<0.000001$). Responses with VMP were rapid and durable, with a time to response of 1.4 months versus 4.2 ($p<10^{-10}$); response duration in patients with CR was 24 months versus 12.8. Overall survival was also in favor of the VMP arm ($p=0.0078$). Overall survival at 2 years was 82.6% in VMP versus 69.5% in MP. Interestingly, age, creatinine clearance, and cytogenetics did not impact on efficacy. The inci-

dence of peripheral neuropathy, gastrointestinal complications, and herpes zoster infections was higher with VMP. VMP is also considered as another standard of care for elderly patients. In this regimen, the weekly infusion of bortezomib significantly reduces the incidence of PN. When comparing the VMP regimen with the bortezomib, thalidomide, and prednisone (VTP) regimen, there was no significant difference in ORR, but VMP had less adverse events than VTP.[51] The thalidomide plus VMP (VMPT) regimen did result in higher VGPR and CR rates than the VMP regimen.[52] When weekly infusions of bortezomib were used in the VMPT schema, the incidence of grade 3–4 PN was reduced in comparison with the standard biweekly infusion without influencing the outcome. Maintenance with bortezomib after VMP or VMPT as frontline treatment looks promising.

MP-lenalidomide

The combination of melphalan-prednisone and lenalidomide (MPR) at the maximum tolerated dose (0.18 mg/kg melphalan, 2 mg/kg prednisone and 10 mg lenalidomide) achieved an ORR of 81%, 48% VGPR, median time to progression of 28 months in a phase 1/2 study.[53] The most common grade 3/4 adverse events were neutropenia and thrombocytopenia (52% and 24%, respectively at the maximum tolerated dose), febrile neutropenia (9%), vasculitis (9%), and thrombosis/embolism (5%). More than 40% of patients required growth factor support. These results were the basis of the prospective phase 3 randomized trial comparing MPR versus MPR followed by R maintenance versus MP. The primary endpoint of the study is time-to-progression, and preliminary results are in favor of the MPR-R arm.[54]

Lenalidomide plus dexamethasone

Dexamethasone has also been combined with lenalidomide (Revlimid®), (Rev-Dex) for de novo MM patients. Elderly patients represented half the population of the 34 cases enrolled in the phase 2 trial using Rev-Dex.[55] Lenalidomide was given orally 25 mg daily on days 1 to 21 of a 28-day cycle; high-dose dexamethasone was given orally 40 mg daily on days 1 to 4, 9 to 12, and 17 to 20 of each cycle. The overall objective response rate was impressive, 91%, and 55% of patients experienced grade 3 or higher non-hematologic toxicity. Rev-Dex regimen is thus a highly active regimen, but the high-dose dexamethasone combination might be unnecessary, and responsible for detrimental side-effects. To solve this issue, the ECOG group initiated a prospective randomized trial (ECOG E4A03) comparing lenalidomide plus high-dose dexamethasone (40 mg daily, days 1–4, 9–12, 17–20) (Rev/High-dose dex) with lenalidomide plus low-dose dexamethasone (40 mg daily, days 1, 8, 15, 22) (Rev/low-dose dex).[56] Four hundred and forty-five patients, median age 66 years (up to 88 years), were treated, including 233 over the age of 65 years. Results clearly indicated that Rev/high-dose dex was significantly more toxic as compared with Rev/low-dose dex. Infection/pneumonia, fatigue, hyperglycemia, deep vein thrombosis, and cardiac ischemia were more frequent with the Rev/high-dose dex schedule. Overall, non-hematologic toxicity of any type grade greater than or equal to 3 was found in 52% of patients receiving Rev/high-dose dex compared with 34% of patients receiving Rev/low-dose dex ($p<0.001$). A pre-planned interim analysis showed that early deaths were also significantly more frequent in the Rev/high-dose dex arm, and an independent data monitoring committee recommended release of survival results and switching patients to Rev-low dose dex arm. The 2-year survival was superior in the group of patients receiving the Rev/low-dose dex regimen (87% versus 75%), and this survival benefit was maintained in the subgroup of patients aged over 65 years (82% vs. 67%). Of note, the increased mortality in the Rev/high-dose dex arm was due to both disease progression, as well as increased toxicity. Overall, Rev/low-dose dex (Rd) was found highly active in newly diagnosed elderly patients with MM, more tolerable and manageable as compared with Rev/high dose dex. Thus, Rd can also be considered a standard of care, especially in the US. In the US, lenalidomide is not approved as part of frontline therapy, and the results of the prospective study comparing Rd versus MPT as initial therapy in patients older than 65 years of age are eagerly awaited.

Table 4. Novel agents as primary treatment in elderly patients.

	CR + PR (%)	CR (%)	Median PFS (months)	Median OS (months)
MPT (Phase III)[43-48]	57–76	2–16	13–27.5	26–51.6
VMP (Vista)[50] bortezomib twice-weekly	71	30	21.7	3-year: 54%
VMP (Gimema)[52] bortezomib once- weekly	81	24	27.4	3-year: 80%
VMPT-VT (Gimema)[52] bortezomib once-weekly	90	42	37	3-year: 85%
VMP-VT/VP (Pethema)[51]	80	20	34	3-year: 74%
MPR (Phase I/II)[53]	81	24	28	not reported
MPR-R (Phase III)[54]	77	16	31	not reported
Lenalidomide - low-dose Dexamethasone (ECOG)[56]	89	22	25	2-year: 82%

Conclusion

During the last 3 years, results of several phase 1/2 or phase 3 trials have clearly shown that the prognosis and the survival of de novo MM in elderly patients have been markedly improved. MP is no longer the reference treatment and should be abandoned. At least four highly active new treatment options are available, MPT, VMP, MPR, and Rd, with different toxicity profiles (Table 4). The goal of future trials will be to determine the best treatment strategy in this group of patients and to tailor, if possible, therapeutic options, taking into account distinct variables, such as tolerability, efficacy, cytogenetics, or comorbidity. The role of maintenance therapy in this group of patients also needs further evaluation.

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Diagnosis and management of childhood myelodysplastic syndrome and juvenile myelomonocytic leukemia

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A B S T R A C T

Myelodysplastic and myeloproliferative disorders are rare in children. Contemporary classification includes three main groups: myelodysplastic syndrome (MDS), juvenile myelomonocytic leukemia (JMML), and the myeloid leukemias of Down syndrome (ML-DS).

Separating MDS from AML may be a challenge and biological features rather than an arbitrary cut-off in blast count may be more important in distinguishing MDS from AML. The presence of genetic aberrations in the Ras signal transduction pathway in most patients with JMML has contributed major insight into the pathogenesis of JMML and facilitates the diagnosis. Hematopoietic stem cell transplantation is the treatment of choice for MDS and JMML. Reduced AML therapy is very successful in ML-DS. JMML in infants with Noonan syndrome often regresses spontaneously.

Introduction

Myelodysplastic and myeloproliferative disorders are rare in children and in many aspects different from the diseases in adults, requiring a pediatric approach to their diagnosis and management.[1-2] Contemporary classification includes three main groups; myelodysplastic syndrome (MDS), juvenile myelomonocytic leukaemia (JMML), and the myeloid leukemias of Down syndrome (ML-DS).

MDS is the only one of the main subgroups in children that has a partial overlap with the spectrum of disease in adults. However, there are significant differences between MDS in children and adults (Table 1). There is no sharp age distinction between adult and childhood MDS, and young adults with MDS share many of the features of childhood MDS and may benefit from similar management aiming at curing the patient with MDS, which is often not realistic in adults.

There are very few protocol-based studies on pediatric MDS. Most studies on MDS and JMML have been performed by the European Working Group of MDS in childhood (EWOG-MDS) (www.ewog-mds.org).[3-8]

Classification

The WHO classification from 2001[9] recognized JMML, previously termed juvenile chronic myeloid leukaemia (JCML) or chronic myelomonocytic leukaemia (CMML), as a separate entity, but the classification of MDS did not acknowledge the special features of MDS in children. A pediatric approach to the WHO classification separated myelodysplastic and myeloproliferative disorders in children into three main groups: JMML, MDS, and ML-DS (Table 2).[1]

MDS is subdivided into refractory cytopenia of childhood (RCC), RAEB and RAEB-T. The change in nomenclature from RA to RCC reflects that anemia is not a prerequisite for the diagnosis.[4] The revised WHO classification from 2008[10] keeps JMML separate and recognizes ML-DS and RCC as unique groups. Children with more than 2% blasts in the peripheral blood (PB) or 5% in the bone marrow (BM) are classified as RAEB using the same criteria as in adults. RAEB-T is kept for selected patients but it is emphasized that the diagnosis cannot rely on a single blast count but must be a comprehensive evaluation of clinical features, natural course, morphology, immunophenotype, and cytogenetics.

Epidemiology

Combined population-based data from Denmark and British Columbia (BC) in Canada showed an annual incidence of MDS of 1.8 and of JMML of 1.2 per million children, corresponding to a total of 6% of all hematological malignancies in children (Table 3).[11-12] Data from the UK suggest a lower incidence of MDS of 0.8/million (Table 3).[13]

The male/female distribution in pediatric MDS is equal, with a median age at presentation of 6.8 years.[11-14] This is in contrast to the male predominance in JMML and a median age at presentation of 1.8 years.[15]

Down syndrome has been reported in 25% of those with a morphologic diagnosis of MDS but is no longer included in the

Table 1. Major differences between MDS in children and adults.

	Children	Adults
Incidence / year/ million	1-2	>30
RA with ringed sideroblasts	< 2%	25%
Cytogenetic aberrations	50%	40%
-7/del(7q)	30%	10%
-5/del(5q)	1-2%	20%
Mutation of *NRAS*	rare	common
Hypermethylation	> 50%	> 50%
Main aim of treatment	curative	palliative

Table 2. Diagnostic categories of myelodysplastic and myeloproliferative diseases in children according to the pediatric approach to the WHO classification[1] and the revised WHO classification.[10]

Myelodysplastic Syndrome (MDS)

- Refractory cytopenia (RCC) (PB blasts <2% and BM blasts <5%)
- Refractory anemia with excess blasts (RAEB) (PB blasts 2-19% or BM blasts 5-19%)
- RAEB in transformation (RAEB-T) (PB or BM blasts 20-29%) / AML with myelodysplasia-related changes (PB or BM blasts >20%)

Myeloid proliferations related to Down syndrome (DS)

- Transient abnormal myelopoiesis (TAM)*
- Myeloid leukaemia of Down syndrome (ML-DS)*

*characterized by *GATA1* mutations

Myelodysplastic / Myeloproliferative Disease

- Juvenile myelomonocytic leukaemia (JMML) clinical diagnosis of NF1 or mutation in *PTPN11*, *RAS*, or *CBL* in 85%

series of MDS, significantly changing the distribution of MDS subtypes.[16]

MDS occurs with increased frequency in inherited BM failure disorders. The risk is highest in Fanconi anemia, dyskeratosis congenita, and severe congenital neutropenia (SCN). Myeloid leukemia develops in a large fraction of patients with Fanconi anemia during childhood or early adult life. The risk varies according to genetic subgroup and associated abnormalities.[17] The traits of Fanconi anemia may be subtle and the diagnosis should always be considered, even in adults. The cumulative incidence of MDS/AML in dyskeratosis congenita may be as high as reported in Fanconi anemia.[18] Screening of telomere length and telomerase mutations in sibling donors is advised to prevent potentially fatal graft failure.[19] The 15-year cumulative risk of MDS in SCN is 15%.[20] The risk of MDS is highest in patients with a poor response to G-CSF.

Myelodysplastic syndromes

Pathophysiology

MDS is a clonal disease arising in a progenitor cell restricted to myelopoiesis, erythropoiesis and megakaryopoiesis, but the initiating events have remained

Table 3. Annual incidence of hematological malignancies in children 0-14 years. Combined data from Denmark 1980-1991 and British Columbia 1982-1996[11,12] and data from UK 1990-1999.[13]

			Denmark and BC	UK
	N	%	incidence per million	incidence per million
ALL	815	79	38.5	nd
AML[1]	115	11	5.4	5.8
MDS[1]	38	4	1.8	0.8
Myeloid leukaemia of DS	19	2	0.9	0.6
JMML	25	2	1.2	0.6
CML	13	1	0.6	0.5
PV/ET[2]	3	0	0.1	nd
Total	1027	100	48.6	–

[1]*Excluding Down syndrome (DS).*
[2]*PV: polycythemia vera; ET: essential thrombocythemia.*

obscure, in children as in adults. Mutation in the tumor-suppressor gene *TET2* was recently identified in 20% of adult patients with various myeloid disorders including MDS.[21] However, *TET2* mutations are not seen in JMML[22] and have not yet been studied in pediatric MDS.

Inherited disorders with DNA repair defects like Fanconi anemia or acquired mutations in genes maintaining genetic stability may result in a mutator phenotype predisposing to MDS.[23] Subsequent events, e.g., mutations in proto-oncogenes like *RAS*, *TP53,* or *WT1*, and karyotypic changes like monosomy 7, may be part of a final common pathway of disease progression.[24]

Clinical and laboratory features

The presenting features in almost all cases of MDS are those of pancytopenia. Single lineage cytopenia may occasionally be the presenting characteristic. In a few cases the cytopenia is an incidental finding during a routine work-up. Not all children with RCC have anemia, but macrocytosis (elevated MCV) is a characteristic finding.[4] Fetal hemoglobin (HbF) is frequently moderately elevated. WBC is low to normal. Leukocytosis is generally not a feature of MDS, and in the case of increased WBC the diagnosis should be reconsidered. Some patients present with moderate hepatosplenomegaly but most have no organomegaly.

The BM cellularity varies but hypocellular RCC is more common in children than in adults.[4] Both PB and BM display characteristic dysplastic features with megaloblastic erythropoiesis, bizarre small or unusual large megakaryocytes, and dysgranulopoiesis.[25] The characteristic dysplastic features are suggestive of MDS but not diagnostic.[1] Interobserver variation in the evaluation of dysplasia exists[26] and centralized review is recommended.[25]

Cytogenetics

An abnormal karyotype is found in 55% of children with advanced primary MDS and in 76% with secondary advanced MDS.[27] Monosomy 7 is the most com-

mon cytogenetic abnormality in childhood MDS, seen in 25% of all patients.[27] Trisomy 8 and trisomy 21 are the most common numerical abnormalities after monosomy 7. Constitutional trisomy 21 is clinically obvious when present, whereas constitutional trisomy 8 mosaicism may be clinically silent and should be tested for when trisomy 8 is found in the BM.[28]

Monosomy 7, as the only cytogenetic aberration, is not an unfavorable feature in childhood MDS,[3,29-31] whereas structural complex abnormalities are associated with a very poor outcome.[27] Monosomy 7 is associated with a shorter time to progression in children with RCC.[4] Favorable cytogenetic aberrations, identified in adults as -Y, 20q- and 5q-, are so infrequent in children that they are of no practical importance.[5]

AML specific translocations, e.g., t(8;21)(q22;q22), t(15;17)(q22;q12), or inv(16)(p13q22), should be considered as AML regardless of the blast count.[10]

Immunophenotype

Flow cytometry immunophenotyping does not have the same diagnostic yield in MDS as in acute leukemia. Few data on immunophenotype characteristics of MDS in children have been reported.[32] No consensus is available on standard protocols and techniques of flow cytometry in childhood MDS.

Refractory cytopenia versus aplastic anemia

A trephine biopsy is fundamental for the evaluation of a child with suspected aplastic anemia or MDS. Careful sequential morphologic studies are necessary to establish the correct diagnosis. The typical biopsy in MDS shows hypoplasia, scarcely scattered granulopoiesis, patchy islands of immature erythropoiesis, and micromegakaryocytes.[2] Immunohistochemical studies may be helpful in demonstrating a high expression of p53 and a low expression of survivin in MDS, compared with patients with non-clonal BM failures.[33]

Separating MDS from AML

AML is the major differential diagnosis of advanced MDS. There are significant differences in clinical features, cytogenetics and response to therapy between MDS and AML,[34] reflecting fundamental biologic differences,[35] thus making the morphologically based classification a surrogate marker for the distinction between biological entities. Blast count in a single specimen is insufficient to differentiate MDS from AML. Biological features rather than any arbitrary cut-off in blast count may be more important in distinguishing MDS from (chemosensitive) AML.[36]

Prognosis and natural course

Children with RCC and RAEB or even RAEB-T may show a long and stable clinical course without treatment. Blood transfusions may only be required infrequently and severe infections are rarely seen. The condition may smolder with unchanged cytopenia for months or even years. In a series of 67 children with primary RCC, four died from complications of pancytopenia prior to therapy or progression and 20 progressed to more advanced MDS at a median of 1.7 years from presentation.[4] RCC with monosomy 7 is associated with a higher risk of progression, and once progression has occurred, the outcome is inferior even after HSCT.[4]

The International Prognostic Scoring System (IPSS) for MDS weighed data on BM blast count, cytopenia and cytogenetics and separated patients into four prognostic groups. Children show more poor risk features than adults, but only thrombocytopenia and BM blasts >5% correlate with poor survival in children.[5] Overall the IPSS provides little diagnostic information in children but identifies a very small group (7%) of the patients with low-risk disease and a very favorable outcome.[5] Adolescence and complex cytogenetics are associated with a poorer outcome.[27]

Treatment

Myeloablative therapy is the only treatment option with a realistic curative potential. Immunosuppressive therapy with antithymocyte globulin and cyclosporine in children with hypoplastic RCC results in complete or partial response in 70%, with overall and failure-free survival rates at 3 years of 90% and 60%, respectively.[37-38] The long-term outcome of immunosuppressive therapy in MDS is not known.

AML type chemotherapy

Conventional intensive chemotherapy without HSCT is unlikely to eradicate the primitive pluripotent cells involved in MDS and associated with a complete remission rate of less than 60%, treatment-related mortality rate between 10 and 30%, many relapses, and an overall survival rate of less than 30%.[14,31,34,39] However, a few studies have reported outcomes in MDS patients not significantly different from those in AML, especially in patients with RAEB-T or AML following MDS.[31,39,40] Children with monosomy 7 diagnosed as AML have a poor response to induction chemotherapy, as in MDS patients, but in contrast to MDS those who responded well to chemotherapy had an outcome similar to other AML patients.[41]

Hematopoietic allogeneic stem cell transplantation

HSCT is the therapy of choice for virtually all forms of MDS in childhood. Myeloablative therapy with busulfan, cyclophosphamide, and melphalan has cured more than half of children with MDS both after matched family donor (MFD) and matched unrelated donor (MUD) HSCT.[42-43]

Stage of disease has a significant effect on relapse and outcome following HSCT, with a very low relapse rate in RCC. In children with RCC and absence of profound cytopenia, postponement of HSCT with a watch–and-wait strategy may be justified, especially in patients with a normal karyotype.[4] A fludarabine based reduced-intensity conditioning regimen in 19 children with RCC and normal karyotype resulted in an overall survival and DFS at 3 years of 84% and 74%, respectively, comparable to those of patients treated with myeloablative HSCT.[44]

It remains unknown whether AML-type induction chemotherapy prior to HSCT for advanced MDS can reduce relapse and thus improve DFS. Data from EWOG-MDS on children with primary advanced MDS showed no benefit of intensive AML-type therapy preceding HSCT.[43] Small series of patients transplanted as

first line therapy have shown survival of 65-70%.[35,45] Considering the significant morbidity and mortality of induction chemotherapy and the high rate of TRM following HSCT, highest in adolescents,[43] most children with MDS may benefit from HSCT as first line therapy, sparing the toxicity related to induction chemotherapy. Children without a matched donor and progressive disease should be considered for haploidentical HSCT.[46]

Relapse following HSCT is associated with a very grave outcome. Successful withdrawal of immunosuppressive therapy and donor leukocyte infusions in early relapse have occasionally been reported.[47-48]

Myeloid leukemia and Down syndrome

Individuals with Down syndrome (DS) have a more than 150-fold increased risk of myeloid leukemia during the first five years of life.[49] The recognition of the unique biological features of the *GATA1* mutated myeloid malignancy has resulted in consensus about the term *myeloid leukemia of Down syndrome* (ML-DS),[1,10] and it is no longer relevant to talk about MDS or AML in young children with DS. The rare occurrence of myeloid leukemia in older DS children (4 years or older) may be different from ML-DS being *GATA1* negative and a higher risk of relapse.[50-51]

Transient abnormal myelopoiesis

Increased WBC with circulating megakaryoblasts, often accompanied by anemia and thrombocytopenia, may be seen in up to 10% of newborns with DS.[52] The percentage of blasts is often higher in blood than in BM. The condition is referred to as transient abnormal myelopoiesis (TAM), transient leukemic reaction, or transient myeloproliferative disorder. The presentation is indistinguishable from leukemia and some have therefore favored the name transient leukemia.[52-53]

Generally no chemotherapy is indicated in TAM; however, in those with progressive hepatic or pulmonary problems or a very high WBC, a short course of low-dose cytarabine may be very effective.[54] ML-DS develops 1-3 years later in about 20% of those who have recovered from TAM. The risk is higher in those with acquired clonal cytogenetic abnormalities[53] or persistently elevated *WT1* expression.[55]

Pathobiology

Leukemia in children with trisomy 21 mosaicism selectively involves the trisomic cells, indicating that the additional chromosome 21 is the first hit in the multistep process leading to leukemia. Patients with TAM and ML-DS have an acquired mutation in the *GATA1* gene.[56] The *GATA1* gene encodes a transcription factor essential for the normal erythroid and megakaryocytic differentiation, in accordance with the selective involvement of these two lineages in ML-DS.[57]

GATA1 mutation found in TAM may be present in 3-4% of newborns with DS and normal hematology.[58] The mechanisms of the spontaneous regression of TAM remain unexplained but may be associated with the natural switch of hematopoiesis from fetal liver to BM (59). A large proportion of those with TAM and about 1% (> 100 fold increased risk) of DS without abnormal hematology in the newborn period develop myeloid leukemia.[49]

Clinical and laboratory features

Isolated thrombocytopenia is often the presenting feature of ML-DS. Platelet count and WBC are lower at diagnosis than in non-DS patients, in contrast to the very high WBC seen in TAM. In most cases the blast cells have morphologic and antigen features of megakaryoblasts, although other morphological variants may occur. Many patients have a relatively indolent course, characterized by a period of thrombocytopenia and dysplasia, with relatively few blasts in the BM.

Cytogenetics

Numerical aberrations, mainly trisomy 8 and an extra chromosome 21 (tetrasomy 21), are the most common acquired cytogenetic abnormalities.[60] The recurrent structural aberrations seen in AML are not found in ML-DS.

Treatment

In contrast to TAM, ML-DS is fatal if untreated but responds well to AML treatment, with a very favorable outcome. ML-DS cells with GATA1 mutation are very responsive to cytarabine and anthracycline.[61-62] Several groups have reported long-term survival in DS patients well above 80%.[63-67] DS children are at a low risk for relapse and due to the high risk for treatment related toxicity, they benefit from less time-intensive therapy, allowing recovery prior to initiation of the next chemotherapeutic course.[65,68]

Juvenile myelomonocytic leukemia

JMML is a unique pediatric disorder, the pediatric equivalent of what the FAB group termed CMML. JMML was previously named juvenile chronic myeloid leukaemia (JCML), recognizing the distinction from CML occurring in older children and adults.

Clinical and laboratory features

Patients present with pallor, fever, infection, bleeding or symptoms from the organomegaly.[15] Elevated WBC with absolute monocytosis, anemia, and thrombocytopenia are almost universal. WBC at presentation exceeds 50 x 10^9/L in 30% and is above 100 x 10^9/L in 7%.[15] Increased fetal hemoglobin (HbF) is a main characteristic of JMML, with the notable exception of those with monosomy 7, who almost all have normal HbF for their age.[15]

Cytogenetics

Monosomy 7 (mostly as the sole abnormality) is present in 25-30% of JMML, 10% have other aberrations, and 60% show a normal karyotype.[15] Data from the EWOG-MDS did not show any major clinical differences between JMML in patients with and without -7.[15]

Differential diagnoses

JMML may mimic infections and immunodeficiency, delaying the diagnosis. On the other hand infections,

inborn errors of metabolism and immunodeficiency may cause monocytosis and organomegaly and represent diagnostic pitfalls. A diagnosis of JMML, especially in infants, should therefore be made with caution.[69] A period of observation is recommended in cases without clear-cut features. Several viral infections mimicking JMML have been reported.

The international consensus on current diagnostic criteria of JMML includes molecular genetics as a mandatory part of the work-up, as incorporated in the EWOG-MDS 2006 protocol (www.ewog-mds.org). Blood film appearance is characteristic and often more helpful in diagnostics than BM morphology, where monocytosis often is much more discrete.

Pathophysiology

In vitro studies indicating that a defect in the GM-CSF signal transduction pathway plays a major role in the pathogenesis of JMML led to studies of the Ras signal transduction pathway. Members of the Ras family of signalling proteins regulate cellular proliferation by cycling between an active guanosin triphosphate (GTP)-bound state (Ras-GTP) and an inactive guanosine diphosphate (Ras-GDP)-bound state. *RAS* point mutations causing high constitutive Ras-GTP levels are noted in 25% of JMML patients.[70]

Children with neurofibromatosis type 1 (NF1) have an increased risk of malignant myeloid disorders, especially JMML.[71] About 15% of children with JMML carry the clinical diagnosis of NF1.[15] The *NF1* gene functions as a tumor-suppressor gene, and loss of the normal *NF1* allele has been noted in leukemic cells of NF-1 patients, with loss of heterogeneity (LOH) as the second event.[71-73] *NF1* and *RAS* mutations are mutually exclusive in JMML patients, indicating that one abnormality is sufficient to activate Ras.

Recent studies of *CBL* in the subset of JMML patients without mutations in *RAS* or *PTPN11* identified mutations in 40% corresponding to 10-15% of JMML patients overall and no *CBL* mutations in the JMML samples with known mutations in *RAS* or *PTPN11*.[74-76] The identification of homozygous C*BL* mutations in JMML suggests that *CBL* is a new tumor suppressor gene and indicates that *CBL* may have a role in deregulating the Ras pathway in JMML. *CBL* mutations in JMML have so far all been germline mutations associated with developmental delay.[77] With somatic *PTPN11* mutations in 35%, *RAS* gene mutations in 25%, germline *NF1* gene mutation in 10-15% and *CBL* mutation in another 10-15%, mutually exclusive abnormalities of the Ras signalling pathway are identified in 85% of the JMML patients.

Natural course and prognostic factors

JMML is in most cases a rapidly fatal disorder if left untreated. Low platelet count, age above 2 years, high hemoglobin F, and high BM blast count at diagnosis are the main factors predicting a short survival.[5,15,78] Multivariate analysis demonstrates a presenting low platelet count as the strongest factor predicting a poor survival. Non-transplanted children presenting with a platelet count < 33 x 10^9/L have an almost 100% mortality within the first year from diagnosis.[15,79] Blastic transformation is infrequent with JMML and most untreated patients die from organ failure due to infiltration of the leukemic cells.

The prognostic value of genetic subtype is not yet established.[80-81] Gene expression may separate JMML into subgroups with distinct prognosis.[82] A high-methylation phenotype characterizes an aggressive biologic variant and is an independent strong molecular predictor of relapse.[83]

Infants with Noonan syndrome (NS) may show a JMML-like myeloproliferative disorder (NS/MPD) with spontaneous regression.[84] NS/MPD is diagnosed during the first few months of life, often during the first weeks. In the majority the hematological abnormalities gradually resolve but normalization may take several months or even years, especially the monocytosis and splenomegaly, which may persist for several years.[85-86]

NS/MPD has striking parallels with the transient leukemia/TAM of newborns with Down syndrome. Unlike the *GATA1* mutation in TAM, the NS/MPD has no somatic molecular marker and there is no documented effective therapy in those NS patients with an aggressive course.

Following the identification of *PTPN11* germline mutation in 50% of patients with NS, studies in non-NS JMML showed somatic *PTPN11* mutations in 35%.[87]

The *PTPN11* mutations found in JMML have a stronger SHP-2 activation than the mutations in NS, whereas the mutations in NS/MPD have an intermediate gain of function effect.[6,88] It is presumed that the strong activation resulting from the *PTPN11* mutation in JMML is incompatible with life when occurring as a germline mutation.

Treatment

Intensive chemotherapy is mostly unsuccessful in JMML because of an increased risk of treatment-related death, a low rate of true remissions and long-term survival less than 10%.[15,34,79] The evaluation of the efficacy of JMML therapy is hampered by the lack of uniform criteria of response and divergent responses in hepatosplenomegaly, white cell, and platelet count as well as the fact that about 20% of patients observed without therapy show response.[89] Purine analogs, etoposide, and cytarabine as single agents are associated with the best response rates for white cell count and spleen size.[89]

Busulfan-based myeloablative allogeneic SCT results in overall survival in more than half the patients after both family and unrelated donor HSCT[7,90-92] or cord blood.[93] Younger age at HSCT and male sex predict for improved survival.[7] Disease recurrence remains the major cause of treatment failure. Reduced intensity and duration of GvHD prophylaxis may significantly contribute to successful leukemia control[7] and both acute and chronic GvHD are associated with a lower risk of relapse.[7,90-91]

Older age, female sex, increased percentage of HbF, and blast percentage in the BM above 20% predicted the occurrence of relapse in univariate analysis.[7]

Monosomy 7 is associated with an outcome comparable to or even better than that of patients with normal karyotype.[7,13]

Relapse occurs early, at a median of 2-4 months from

transplantation,[7,91] and generally within the first year. Early detection of donor cells by increasing mixed chimerism may be successfully eradicated by reducing ongoing immunosuppressive therapy.[94] Donor lymphocyte infusion (DLI) in JMML relapse is largely unsuccessful.[95] A second or even a third transplantation gives a relatively high chance of survival.[8]

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Central nervous system leukemia in childhood and adolescent acute lymphoblastic leukemia: How to prevent? How to treat?

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer in children. The prognosis of children with ALL has progressively but dramatically improved over the last forty years. A 5-year event-free survival (EFS) of 80-85% is currently observed in the developed countries. The major contributors to this result are the risk-adapted therapies, the central nervous system (CNS)-directed therapy and the improvement of supportive care, administered by dedicated teams using clinical research-grade protocols. This review will focus on the problem of CNS in childhood ALL.

Why a focus on CNS and acute lymphoblastic leukemia?

It is known from older studies that in the absence of any prevention, a 50% incidence of CNS relapse is observed[1]. Currently this rate has been reduced to less than 5% through the introduction of cranial irradiation, intrathecal chemotherapy with methotrexate alone or in combination with other drugs (cytarabine and steroids), and systemic administration of chemotherapies which penetrate the CNS (high-dose methotrexate, dexamethasone, and high-dose cytarabine) or deprive the blood and secondarily the CSF of an essential amino-acid (L-asparaginase). Nevertheless, strategies to prevent CNS relapse must first avoid over-treatment (resulting in potential sequaelae) or under-treatment (resulting in CNS and/or marrow relapse). Second, CNS relapses may now represent as high as 30 to 40% of first relapses, particularly in the low and intermediate-risk ALLs.[2-4]

What are we talking about when saying "CNS"?

In fact ALL cells can invade meninges and, more rarely, the nerve roots or the brain itself. Their migration to these compartments is not fully understood. The leukemic cells can migrate from the skull marrow into the subarachnoid space via the bridging veins and enter the cerebrospinal fluid (CSF) via the choroid plexus. They can directly infiltrate the leptomeninges via osseous lesions of the skull. They can invade cerebral parenchyma via brain capillaries. Leukemic cells can also grow along nerve roots and invade the subarachnoid space. Very rarely in ALL, multiple solid central nervous system tumors composed of leukemic cells are found. Leukemic cells can also enter the CNS via a local CNS hemorrhage when the circulating blood contains blasts, particularly in situations where leucostasis is described (mainly cases of ALL with a very high leucocyte count). Finally, leukemic cells can be introduced at the time of the first lumbar puncture, especially when traumatic.

How to diagnose initial CNS leukemia?

Clinical signs (see Table 1)

Only cerebral palsies are taken as possible direct diagnostic signs of CNS involvement (CNS3 status, cf. section 4). Some cooperative groups consider cranial-nerve palsy by itself a criterion of CNS3 (e.g., Children's Oncology Group, St-Jude, EORTC, FRALLE). Others accept these palsies (or a cerebral mass) only if they are associated with the presence of blasts after cytocentrifugation (BFM group).[5] More recently, palsies or the presence of a cerebral mass were also accepted by this group as sole evidence of CNS involvement (trial AIEOP-BFM ALL 2000, personal communication).

Radiological signs of CNS involvement in ALL

MRI is known to be far more sensitive in the detection of leptomeningeal tumor spread than CT. Findings may include an abnormal MR appearance of the subarachnoid space on pre-contrast imaging. If the subarachnoid space is not of appropriate signal intensity on T1, T2-weighted and, above all, FLAIR images, the possibility of leukemic meningitis should be raised. Abnormal meningeal enhancement, in the cisterns or along the pial surface of the brain or spinal cord, is a good sign of leukemic meningitis. This type of enhancement is, however, non-specific. Non-malignant meningeal enhancement may be observed following diagnostic or therapeutic lumbar puncture. Dural spread of leukemia is best detected radiologically as abnormally thickened and brightly

Table 1. Clinical signs of possible CNS involvement.

Increased intra-cranial pressure	Visual disturbances	CNS palsies	Other
Headaches, vomiting, papilledema, lethargy, seizures, coma	Diplopia, blurred vision, blindness, photobia	VII, VI	Myelopathy, auditory abnormalities, vertigo, ataxia, hallucinations, nystagmus, bulimia

enhanced dura on post-contrast images.

In rare cases of ALL, images compatible with unique or multiple solid central nervous system tumors, in fact consisting of leukemic cell aggregates, are found.

Examination of the CSF after lumbar puncture

Lumbar puncture (LP): considering the negative impact of a traumatic LP (cf. section 6), adequate conditions should be met when performing the first lumbar puncture: experienced doctor, deep sedation, platelet count above 50.000/mm^3 (> 100.000/mm^3 for some authors), immediate first age-adjusted intrathecal injection of chemotherapy. Moreover, the use of the smallest needle possible is recommended to decrease the leak of CSF and prevent post-puncture headache. Finally, prone position for at least 30 minutes would be a way to increase the intra-ventricular concentration of chemotherapy.[6]

CSF cyto-morphology: despite being viewed as the gold standard, there are issues related to CSF cytology:
- Linked to the technique: amount of available CSF, simple or double cytocentrifugation, use of medium or fetal calf serum or bovine serum albumin, speed and length of centrifugation, type of staining, number of cells on the slides[7,8]
- Linked to the cytopathologist: experience and skill are obviously needed.

Additional techniques: they may help to distinguish normal lymphocytes from leukemic cells in cases with difficult morphology. The three main techniques are terminal deoxynucleotidyl transferase staining, flow cytometry (FC) and PCR-based techniques. A recent study has compared the performance of FC and cytomorphology in patients with CNS hematological malignancies: leukemic cells were found in the CSF at diagnosis by FC in 44 patients (73%) versus 19 (32%) by cytomorphology.[9] Questionably, four samples were positive by cytomorphology while negative by FC.[9] The development of PCR as a diagnostic technique based on the detection of clonal rearrangements of the immunoglobulin/T-cell receptor genes has been proposed as a sensitive way to detect CSF involvement. Few studies have yet explored this aspect in ALL.[1-13] Pine *et al*. have used a sensitive RQ-PCR and have found PCR positive samples in three out of 23 CNS1 pts.[1] Scrideli *et al*. reported on 37 pts without traumatic LP.[1] With an unknown sensitivity, 17 PCR positive samples were identified vs. only two by morphology. Interestingly, the patients with positive PCR samples had a worse outcome than the patients without CNS PCR positivity.[12] These preliminary results need to be confirmed in large prospective studies.

How to classify CNS disease?

Patients who have a non-traumatic diagnostic lumbar puncture may be placed into one of three categories according to the number of white blood cells/μL and the presence/absence of blasts on cytospin, as follows:
- CNS1: Cerebrospinal fluid (CSF) that is negative for blasts after cytospin, regardless of WBC count.
- CNS2: CSF with fewer than five WBC/μL and cytospin positive for blasts.
- CNS3: CSF with five or more WBC/μL and cytospin positive for blasts.

Patients with an initial traumatic LP (≥10 erythrocytes/μL) should be classified in two further categories:
- Traumatic LP with blasts (TLP+): ≥10 erythrocytes/μL plus blast after cytospin.
- Traumatic LP without blasts (TLP-): ≥10 erythrocytes/μL with no blast after cytospin.

To determine whether a patient with a traumatic lumbar puncture (with blasts) should be considered as CNS3, the Children's Oncology Group (COG) uses an algorithm relating the white blood cell and red blood cell counts in the spinal fluid and the peripheral blood: If CSF WBC/RBC is two times greater or more than the blood WBC/RBC ratio, the patient is considered to have CNS disease at diagnosis.

Who are the children and adolescents at risk of initial CNS involvement?

All cooperative groups report a 2 to 3% similar incidence of CNS3 pts (see Pui and Howard[14] for review). This mean number, nevertheless, does not reflect the heterogeneity of the disease, children with standard risk B-lineage ALL having the lowest incidence (~1-2%), or infants and patients with T-ALL having the highest (~10%). It should be noted that some B-lineage ALL with poor-risk cytogenetics are also associated to a higher incidence of CNS3 status (~5%) (Table 2).[15-17]

Who are the children at risk of CNS relapse?

There is an obvious overlap between risk factors of initial CNS involvement and risk factors for CNS relapse (see "table 2" and "table 3").
- Compared with patients classified as CNS1 or CNS2, children with ALL who present with CNS disease at diagnosis (i.e., classified as CNS3 patients) are at a higher risk of treatment failure (both within the CNS and systemically).[14] The adverse prognostic significance associated with CNS2 status, initially reported by the St-Jude group,[18] has been questioned[5,19] and may

Table 2. Initial CNS involvement and CNS relapse in patients with ALL and poor-risk cytogenetics.

	Number of pts (%) with CNS involvement at diagnosis	Number of pts (%) with isolated CNS relapse after CR
Infants*	44/405 (10.8%)	23/445 (5.2%)
Philadelphia Chromosome	11/275 (4%)	16/267 (6%)
Hypodiploidy < 45 chromosomes	8/130 (6.1%)	10/130 (7.7%)

**80% of infants have an MLL gene rearrangement induced by a translocation involving 11q23.*
From Pieters R et al.,[13] Arico M et al.,[16] Nachman J et al.[17]

Table 3. Risk factors associated with CNS relapse in children and adolescents with ALL.

- Blast in the CNS
  - CNS3 (CSF with 5 or more WBC/µL and cytospin positive for blasts and/or clinical/radiological signs)
  - Traumatic lumbar puncture with blasts after cytospin
  - CNS2 +/-
- Hyperleucocytosis
- T-Cell ++ if WBC>100.000/mm^3
- t(1;19)/ E2A-PBX1
- Infants -t(4;11)/MLL-AF4
- t(9;22)/BCR-ABL
- hypodiploidy < 45 chromosomes
- Gene polymorphisms (Vitamin D receptor start site, Thymidylate synthase)
- High IL-15 expression
- CCR7 expression (T-ALL model in mice)
- Suboptimal asparagine depletion

be overcome by the application of more intensive intrathecal therapy, especially during the induction phase and/or systemic treatment with CNS penetration, i.e., dexamethasone, high-dose methotrexate.[5,20,21] A traumatic lumbar puncture (≥10 erythrocytes/µL) (TLP) that includes blasts at diagnosis appears to be associated with increased risk of CNS and systemic relapse and indicates an overall poorer outcome.[5,22,23] It should be avoided as much as possible (Section 3). A Japanese group has proposed delaying the first lumbar puncture at D8 of steroid prophase.[24] A standard 2.9% incidence of CNS3 was found, but a very low 0.8% of TLP was shown.[24] After an initial TLP, many protocols recommend at least reinforced intrathecal treatment during induction.

It must be emphasized that if all groups report an overall similar 2 to 3% incidence of CNS3 pts, the incidence of CNS2 pts (2.5-21%) and the incidence of traumatic lumbar puncture (2.5-12%) are highly variable between groups, likely reflecting the existence of many conflicting factors and the heterogeneity of clinical and biological practice (Table 4).[5,20-23]

– T-cell ALL, especially if a high leucocyte count (>100.000/mm^3) is encountered, is associated with a high risk of CNS and systemic relapse in children and adolescents, explaining why many groups still recommend irradiation of this subgroup.[14,25]
– Children with pre-B-ALL and the t(1;19) translocation have also a higher risk of CNS relapse.[26]
– High-risk cytogenetic features such as MLL rearrangement in infants, hypodiploidy and t(9;22) are associated with a higher incidence of CNS relapse in the 5 to 10% range (Table 3).[15-17]
– Other biologic features have recently emerged as risk factors for CNS relapse:
  - A higher expression of interleukin-15 was found in children with ALL and initial CNS involvement compared to children without. A high expression was also predictive of CNS relapse in children without initial CNS involvement.[27]
  - Certain host gene polymorphisms have been proposed as potential factors to describe the heterogeneity in the risk of CNS relapse, particularly through their role in drug disposition or transport (e.g., methotrexate): They include the thymidilate synthase 3/3 genotype for low risk-ALL and the vitamin D receptor start site and intron 8 genotypes for high-risk ALL.[28] They also include polymorphisms of the genes coding for the Glutathione S-Transferase P1 and P-glycoprotein. These proteins are implicated in resistance to a variety of chemotherapeutic agents and are involved in the blood-brain barrier. Some of these polymorphisms were associated with a reduction of the CNS relapse risk in intermediate or high-risk ALL in a recent German study.[29] All these data nevertheless must be confirmed in large prospective studies before a possible clinical use.
  - It is to be anticipated that homing and adhesion molecules will be important players in the prediction of CNS involvement or relapse risk. Interestingly, in a mouse model, the chemokine receptor CCR7 seems to be an essential adhesion signal required for the targeting of leukemic T-cells into the CNS. Indeed, silencing of either CCR7 or its chemokine ligand CCL19 specifically inhibits

Table 4. Distribution of CSF involvement and traumatic LP at diagnosis in selected studies.

	CNS1 and non TLP[a] n(%)	TLP-n (%)	"CNS1" TLP-[b] included n(%)	CNS2 n(%)	CNS3 n(%)	TLP+[c] n(%)	Total TLP n(%)
St-Jude Total therapy XI/XII[22] (546 pts)	336 (61.5)	54 (9.9)	390 (71.4)	80 (14.6)	16 (2.9)	60 (11)	114 (20.8)
St-Jude Total therapy XV*[20] (498 pts)	?	?	359 (72)	102 (20.5)	9 (1.8)	28 (5.6)	? ?
BFM 95*[5] (2012 pts)	1605 (79.77)	111 (5.51)	1716 (85.28)	103 (5.12)	58 (2.88)	135 (6.7)	246 (12.2)
DCOG*[23] ALL-7/ALL-8 (526 pts)	304 (58)	39 (7)	343 (65)	111 (21)	10 (1.9)	62 (12)	101 (19)
EORTC 58881**[21] (2025 pts)	?	?	1866 (92)	50 (2.46)	49 (2.4)	60 (2.9)	? ?

[a] *TLP: traumatic lumbar puncture.*
[b] *TLP-: traumatic lumbar puncture without blast after cytospin.*
[c] *TLP+: traumatic lumbar puncture with blasts after cytospin.*
* *Definition of TLP: ≥ 10 erythrocytes / µl in the CSF.*
** *Definition of TLP: ≥ 100 erythrocytes / µl in the CSF.*

CNS infiltration. Furthermore, murine CNS-targeting by human T-ALL cells depends on their ability to express CCR7.[30]

– Factors related to sub-optimal treatment (e.g., suboptimal exposure to asparaginase) will be considered in the next paragraph.

How to prevent CNS relapse?

– Cranial irradiation historically has been the first method of prevention since the 1960s. Nevertheless, its use must be carefully weighed due to the numerous and severe complications associated with it: increased risk of secondary tumors (meningiomas, malignant CNS tumors, thyroid cancers and other carcinomas), neuro-cognitive defects, growth hormone deficiency and other endocrinopathies. Currently most protocols restrict the use of CNS irradiation to the 2 to 20% of the patients envisaged to be at higher risk of CNS relapse, at a dose ranging from 12 to 18 gy.[14] Unfortunately, there may be no safe dose: a 12 gy irradiation has resulted in a projected cumulative incidence of second neoplasms of 1.7% at 15 years in a population of 1,779 children treated in the BFM studies (11 observed tumors).[31] Even the low doses (1-2 gy) used in the 1950s for tinea capitis have increased the incidence of brain tumors, thyroid cancers and others.[32] Three cooperative groups have reported very interesting results while completely omitting CNS prophylactic or curative irradiation (Table 5).[20,21,33] Moreover, a BFM-based study in Israel suggests that extended intrathecal therapy may allow the replacement of radiotherapy without apparent damage in the population of patients with T-cell ALL and good early response to prednisone, whatever the white blood cell count.[34] Thus omission of prophylactic cranial therapy in childhood and adolescent ALL is a feasible goal to achieve, even if fully exhaustive mid-term and long-term studies focusing on the impact of replacement therapies (systemic treatment intensity, prolonged intrathecal therapy, high-dose dexamethasone, high-dose methotexate) are still lacking.

– Intrathecal therapy: despite the estimate that only 10% of the dose injected after lumbar puncture reaches the lateral ventricles, a successful CNS prophylaxis is achieved in the vast majority of the patients.[14] The three major drugs which are used intrathecally in various combinations are methotrexate, cytarabine and hydrocortisone. Variations between protocols include the nature of the first IT (mainly cytarabine in the US, versus methotrexate in Europe), simple (methotrexate) versus triple IT for continuation of the therapy directed at the CNS, total number of ITs (ranging from 12 to 25 ITs), and administration or not of ITs during maintenance treatment. These variations are partly linked to the three other main orientations of the CNS treatment: no high-dose methotrexate and no irradiation, high-dose methotrexate and no-irradiation, high-dose methotrexate and CNS irradiation. A recent randomized study (CCG-1952) has compared simple (methotrexate alone) versus triple IT in children with standard-risk ALL.[35] It was found, paradoxically, that if a reduction of CNS relapse was shown, an increase of bone marrow and testis relapses was documented.[35] Indeed the 6-year cumulative incidence estimates of isolated CNS relapse were 3.4% +/- 1.0% for triple ITs and 5.9% (SE: 1.2%) for simple IT (p = .004). Because the salvage rate after bone marrow relapse is inferior to that after CNS relapse, logically the 6-year overall survival rate for children assigned to receive triple ITs was 90.3% (SE: 1.5%) versus 94.4% (SE:1.1%)

Table 5. Results of published protocols omitting CNS irradiation.

	n	number of CNS3 pts (%)	EFS of all cohort	EFS of CNS3 pts	CI[a] of isolated CNS relapse	CI of any CNS relapse
EORTC 58881[21] (1989-1996)	2025	49 (2.4)	69.6 % (SE:1%) at 8 years	68.3 % (SE:6.2%) at 8 years	3.57% (SE:0.42%) at 8 years	7.6% (SE:0.6%) at 8 years
COG ALL-9[33] (1997-2004)	859	21 (2.4)	81% (SE:1%) at 5 years	67% (SE:10%) at 5 years	?	?
St-Jude Total Therapy XV[20] (2000-2007)	498	9 (1.8)	85.6% (SE:5.7%) at 5 years	43.2% (SE:23%) at 5 year	2.7% (SE:0.8%) at 5 years	3.9% (SE:1%) at 5 years

[a]*CI : Cumulative incidence.*

for IT methotrexate (p=0.01). It thus appears that triple IT therapy improves pre-symptomatic CNS treatment but does not improve overall outcome in standard-risk ALL.

A liposomal sustained release formulation of cytarabine has been recently proposed.[36-42] The cytarabine in this formulation has a prolonged half-life (100-263 h) versus 3-4 hours for the free drug.[36] It seems effective but toxicity can be associated with its use, including arachnoiditis and central CNS neurotoxicity, particularly if high-dose cytarabine or high-dose methotrexate are used concomitantly.[42] Trials are ongoing in adult and pediatric protocols to define its real efficacy and safety profile.

– Systemic chemotherapy is of paramount importance to prevent CNS relapse.
  - Intensity of the treatment: CCG-105 was the first randomized study to document the importance of systemic treatment in the prevention of CNS relapse.[43] Indeed, for intermediate-risk patients less than 10 years of age, IT methotrexate with an intensified systemic regimen provided a CNS prophylaxis comparable to that provided by cranial radiotherapy, whereas older patients had fewer systemic relapses if they received CNS irradiation.[43]
  - High-dose methotrexate (HD-MTX): despite its extensive use, the benefits of HD-MTX seem more pronounced on the prevention of hematological relapse than on CNS relapse in a meta-analysis.[44] One French randomized study suggested an advantage of HD-MTX (4 cycles of 8 g/m^2) in intermediate-risk B lineage ALL, the benefit being seen in good early responders to chemotherapy in terms of reduction of bone marrow and extra-medullary relapse.[45] The current 5 g/m^2 dose used in many groups is suggested to result in consistently cytotoxic concentrations in the CSF(> 1 micromolar). An adequate exposure to methotrexate (≥ 36h) before beginning the rescue by folinic acid is mandatory, as well as keeping this rescue to the minimum.
  - High-dose cytarabine: less-used in ALL than in AML or Burkitt's lymphoma, no definite proof of its usefulness exists. No benefit of intermediate or moderate high-dose has been shown in medium-risk ALL in the BFM-95, nor in EORTC 58881 studies.[46,47]
  - Dexamethasone versus prednisone: the possible superiority of dexamethasone is still controversial. Some trials have documented this superiority, particularly in terms of CNS relapse, comparing 6 mg/m^2/d of dexamethasone to 40 mg/m^2/d of prednisone or 10 mg/m^2/d to 60 mg/m^2/day during induction therapy.[48-50] Others comparing 8 mg/m^2/day of dexamethasone to 60 mg/m^2/day of prednisone did not find a difference.[51]
  - Thiopurines: Three randomized trials have compared 6-Thioguanine (40 mg/m^2/day) and 6-Mercaptopurine (60 mg/m^2/day).[52-54] Despite an advantage in terms of CNS relapse reduction, an unacceptable increase of veno-occlusive disease was documented.[53,54]
  - Optimal administration of L-Asparaginase: two trials have demonstrated the role of L-Asparaginase in CNS relapse prevention. Indeed the EORTC group and the DFCI group have randomized the administration of Erwinia asparaginase versus native E.Coli asparaginase at the same dose and at the same rhythm.[55,56] In both trials an excess of CNS relapses was documented in the Erwinia asparaginase arm. This excess was documented only in the non-irradiated patients in the DFCI protocol (no irradiation in the EORTC protocol).[55,56] Despite the lack of formal proof, this was most probably due to the shorter half-life of Erwinia, resulting in a lesser asparagine depletion both in plasma and CNS.
  - Dasatinib for ALL with Philadelphia chromosome: it has been recently documented that 11 clinically evaluable patients with CNS disease responded to dasatinib, a more potent tyrosine kinase inhibitor than imatinib.[57] Complete responses, defined as either disappearance of leukemic blasts from CSF or radiologic findings in magnetic resonance imaging, were observed in 7 patients. Four of these were achieved with dasatinib monotherapy. The CNS penetration of dasatinib, a more potent tyrosine kinase inhibitor, seems considerably higher than that achieved by imatinib.[57]
  - Thiotepa: A US trial has studied the possible role of thiotepa for CNS relapse management, using an upfront therapeutic window.[58] At the 65 mg/m^2 dose (one IV infusion), a complete clearance of blasts at D8 was seen in 4 out of 9 patients with B-precursor ALL.[58]

Finally, all these items point to the importance of systemic treatment in the prevention of CNS relapse in ALL.

An analysis of ten trials performed in the 1990s, involving 15,222 patients, was recently conducted by Pui and Howard.[14] It showed that the variable association of all these methods of CNS relapse prevention cur-

rently results in a less than 5% cumulative incidence of CNS relapse at 5 years (from 0.8% to 5%).[14]

How to treat initial CNS involvement or CNS relapse?

1. CNS3 pts

This problem has been evoked in the previous section (see also "Table 5"). The importance of the systemic treatment for these patients must be emphasized again, particularly in protocols trying to omit irradiation. As an example, the CNS 3 pts, treated without CNS irradiation in the EORTC 58881, received either 9 or 10 courses of HD MTX (5 g/m^2), depending on their other risk-criteria.[21] Their prognosis in that trial conducted in the beginning of the 1990s did not differ from the one of the CNS1 pts (8 year EFS 68.3 % versus 69.7%).[21] Also, in the DCOG ALL-9 study, considering CNS3 as a high-risk criterion per se, a 5-year EFS of 67% was observed, with an overall survival of 80%.[33] These results compare favorably to those of the BFM-95 (5-year EFS: 50%), obtained with the use of a 18gy cranial irradiation after 4 courses of HD-MTX (5 g/m^2).[5]

2. CNS relapse

As CNS relapses are rare and first line treatments are evolving, no gold standard exists. A recent study from the UKALL MRC group, reviewing the outcome of 5,564 children with ALL, treated from 1985 to 2001, has shown a marked trend towards a decrease in combined relapses, with a progressive shift towards later relapses (≥ 30 months).[59] Although isolated relapses declined, the proportional incidence and timing of relapses remained unchanged. CNS relapses in the UK MRC studies thus represent 18% of all relapses and are very early relapses (i.e. CR1 < 18 months) in roughly 37% of the cases.[59]

The main points to be considered when tailoring the treatment of a child with CNS relapseare the length of first remission (less than 18 months or not), the immunophenotype (T-cell ALL versus BCP-ALL), the characteristics of the CNS relapse (isolated or combined), the presence of minimal disease in the marrow, and a history of previous CNS irradiation. An additional prognostic factor found in B-lineage ALL is the NCI classification, with standard-risk NCI patients having the best prognosis after an isolated CNS relapse (even if there is some correlation between length of CR1 and NCI risk).[58,59]

Due to this heterogeneity, to small numbers and sometimes to a long recruitment period, it is not surprising to find variable outcomes across the studies (Table 6).[58-63]

Some common principles can nevertheless be accepted:

- To delay cranial or cranio-spinal irradiation for 6 to 12 months allows initial intensification of systemic chemotherapy. This has led to second EFS rates of 70% to 80% in children with isolated CNS relapse.[58,61,62]
- To reduce as much as possible the dose of irradiation, investigators of the Children's Oncology Group have proposed that patients with an initial remission duration of <18 months receive 24gy cranial and 15gy spinal irradiation (4-year EFS: 52%), while those with a longer initial remission receive only 18gy cranial irradiation at 12 months of treatment while not compromising the 4-year EFS of 77.7%.[58] These good results are observed in children with B-lineage ALL who did not receive cranial irradiation during initial treatment. As in first line, some groups are trying to eliminate CNS irradiation, at least in late isolated CNS relapses (Beshuizen A, Erasmus, Rotterdam, as cited by Pui and Howard[14]).

To intensively treat children with very early CNS relapse (first remission <18 months) and/or those with T-cell ALL. Interesting results have been reported with hematopoietic stem cell transplantation.[64,65,66]

- Albeit logical, the predictive value of submicroscopic marrow disease evaluation at the time of CNS relapse and its potentially decisional role is yet to be demonstrated in prospective studies.[67]
- To envisage differently the treatment of children with prior cranial irradiation. This includes in particular the use of intra-ventricular chemotherapy through a reservoir and possibly innovative drugs like liposomal cytarabine (cf. section 7).

Conclusions

Despite steady progress, the problem of CNS in ALL is still a matter of research in terms of understanding, precise diagnosis, prophylaxis and treatment of relapse. CNS irradiation is to be given up in the vast majority of the children, if not all. This is likely to be a major step forward in decreasing the therapy burden of children with ALL.

Table 6. Results of selected studies for isolated CNS relapse of childhood ALL.

	n	Era	OS of all cohort %(SE)	EFS of all cohort %(SE)	EFS if CR1 <18 months %(SE)	EFS 18<CR1< 30-36 %(SE)	EFS CR1 >30-36 %(SE)
Winnick *et al.*[60]	120	1983-1990	NA	46(7)	NA	NA	NA
Ribeiro *et al.*[61]	20	1983-1989	NA	71 (11)	NA	NA	NA
Ritchey *et al.*[62]	83	1990-1993	NA	71.1(5.3)	46.2(3)	83.3(5.3)	
Barredo *et al.*[58]	76	1996-2000	NA	77.2(5.3)	49.4 (11.1)	77.7 (6.4)	
Tallen *et al.*[63]	35	1990-1995	NA	NA	33 (14)	47(12)	50(25)
Krishnan S *et al.*[59]	307	1985-2001	46·3(5.7)	NA	NA	NA	NA

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Symptomatic thromboembolism in children: update 2011

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A B S T R A C T

Apart from acquired thrombophilic risk factors, inherited thrombophilias (IT) have been established as risk factors for venous thromboembolism (VTE) in adults. In children with idiopathic VTE and in pediatric populations in which thromboses were associated with medical diseases, IT have been described as additional risk factors. Follow-up data for VTE recurrence in children suggest a recurrence rate between 3% (neonates) and 21% (idiopathic VTE). Apart from underlying medical conditions, recently reported systematic reviews on pediatric VTE and stroke have shown significant associations between factor V G1691A, factor II G20210A, protein C-, protein S- and antithrombin-deficiency, even more pronounced when combined IT were involved. The pooled odds ratios (OR: single IT) for VTE onset ranged from 2.4 for the factor II G20210A mutation (cerebrovascular occlusion) to 9.4 in children with antithrombin deficiency (venous VTE). In addition, the pooled OR for persistent antiphospholipid antibodies/lupus anticoagulants was 6.6 for children with cerebrovascular occlusion and 4.9 for pediatric cases with venous VTE. The factor II G20210A mutation (OR: 2.1), protein C- (OR: 2.4), S- (OR: 3.1), and antithrombin deficiency (OR: 3.0) also played a significant role at recurrence. Based on these data, screening and treatment algorithms must be discussed.

Introduction

Venous thromboembolism (VTE) is a rare disease that was increasingly diagnosed and recognized in pediatrics in the past decade, usually as a secondary complication of primary underlying diseases such as sepsis, cancer, congenital heart disease, elevated endogenous testosterone or after therapeutic interventions such as central venous lines (Table 1).[1-13] Pediatric VTE is a severe disease for which long-term outcomes include lack of thrombus resolution in 50% of cases and (apart from central line associated thrombosis in children with malignancy) the development of post-thrombotic syndrome in greater than one third of patients.[8-11] Within the entire childhood population, neonates are at the greatest risk for VTE (5.1/100,000 live births per year in Caucasian children),[1,2,6,14] with a second peak in incidence during puberty and adolescence. The annual incidence of venous events was estimated to be 0.07 to 0.14 per 10,000 children, or 5.3 per 10,000 hospital admissions of children and 24 per 10,000 admissions of neonates to neonatal intensive care units.[1,6,12-14]

Currently, the results of single studies on the risk of VTE onset and recurrence associated with inherited thrombophilia (IT) are contradictory or inconclusive, mainly due to lack of statistical power. Apart from acquired thrombophilic risk factors, such as lupus anticoagulants and the antiphospholipid syndrome,[15-17] antithrombin-, protein C-, or protein S-deficiency, the mutations of coagulation factor V (G1691A), and factor II (G20210A) have been established as risk factors for VTE events in adults.[18-23] In children with idiopathic VTE[38] and in pediatric populations in which thromboses were associated with underlying diseases, IT has been described as an additional risk factor.[24-58] Follow-up data for VTE recurrence in children are available from few reports and suggest a recurrence rate of approximately 3% in neonates and 21% in children with idiopathic VTE. [7,9,12,13,38,48,49,51,54,56]

Thrombotic locations in pediatric populations

In neonates the most commonly reported VTEs are renal veins thrombosis,[1,6,42,49,51] vena caval occlusion, and thromboembolic stroke, mainly of venous origin.[6,13,14] In addition, central line associated VTEs have been reported.[2-4,13,14] Further locations of childhood thromboembolism are cerebral venous thrombosis,[39,41,43,45,47,56,57] and portal or mesenteric vein thrombosis.[27,46] Purpura fulminans, a life-threatening event characterized histologically by microvascular thromboses in the dermis followed by perivascular hemorrhage, has been reported in neonates with congenital absence of protein C or protein S, or the presence of homozygous or heterozygous factor V G1691A mutation.

Imaging methods

Duplex sonography, venography, computed tomography and magnetic resonance (MR) imaging can be used to diagnose VTE

Table 1. Examples for acquired risk factors associated with pediatric thromboembolism.

Perinatal diseases	Birth asphyxia
	Respiratory distress syndrome
	Infants of diabetic mothers
	Neonatal infections
	Necrotizing entercolitis
	Dehydration
	Congenital nephrotic syndrome
	Polycytemia
Medical interventions	Central lines (arterial or venous)
	Surgery
	Renal transplantation
	Immobilization
	Plaster casts
	Extracorporeal membrane oxygenation
Acute diseases	Trauma
	Sepsis
	Dehydration
	Acute rheumatic diseases
	Neprotic syndrome
	Acute lymphoblastic leukemia
Chronic diseases	Malignancies
	Renal diseases
	Cardiac malformations
	Chronic rheumatic diseases
	Metabolic diseases
Drugs	E. coli asparaginase
	Prednisone
	Coagulation factor concentrates
	Heparins
	Antifibrinolytic agents
	Oral contraceptives
	Oral contraceptives & testosterone

in children. However, venography in combination with Doppler ultrasound is mandatory to confirm suspected thromboses in the upper venous system.[59] MR imaging and MR angiography are recommended to confirm the diagnosis of thromboembolic ischemic stroke. Ventilation/perfusion scan or MR angiography are suitable methods for diagnosing pulmonary embolism in children.

Role of inherited thrombophilias in pediatric VTE

The distribution of IT varies in different countries with respect to the population background and the number of patient/controls investigated.

In three recent systematic reviews and meta-analyses, including observational studies in pediatric patients with deep venous VTE and cerebrovascular occlusion (cerebral venous thrombosis and stroke), more than 70% of patients had at least one clinical risk factor.[60-62] The pooled odds ratios (OR) showed statistically significant associations between factor V G1691A; factor II G20210A; protein C-, protein S-, or antithrombin-deficiency; elevated lipoprotein (a); combined IT and the presence of lupus anticoagulants/antiphospholipid antibodies and VTE onset (table 2).[62,63] For second VTE events, statistically significant associations with recurrence were found for protein S- or antithrombin-deficiency, the factor II variant and combined IT.[60] The pooled OR (single IT) for VTE onset ranged from 2.4 for the factor II G20210A mutation (cerebrovascular occlusion) to 9.4 in children with antithrombin deficiency (venous VTE: Table 2). In addition, the pooled OR for persistent antiphospholipid antibodies/lupus anticoagulants was 6.6 for children with cerebrovascular occlusion and 4.9 for pediatric cases with venous VTE.[61,62] The factor II G20210A mutation (OR: 2.1), protein C- (OR: 2.4), S- (OR: 3.1), and antithrombin deficiency (OR: 3.0) also played a significant role in recurrent VTE (Table 2). Interestingly, in all three pediatric meta-analyses (observational studies), age at first thromboembolic onset, publication year or study country did not play a significant role on the odds ratios obtained, thus data are valid for the entire pediatric population aged neonate to 18 years.

Table 2. Pooled odds ratios and 95% confidence intervals [in brackets] for genetic traits and persistent antiphospholipid antibodies associated with a first VTE onset in children and in pediatric patients with recurrent VTE (Young *et al*. 2007[60] & Kenet *et al*. 2010[61] and 2011[62]).

Inherited thrombophilia	OR (cerebrovascular occlusion) fixed-effects model [95% CI]	OR (deep venous thrombosis) fixed-effects model [95% CI]
First VTE onset		
Protein C-deficiency	9.3 [4.8-18.0]	7.7 [4.4-13.4]
Protein S-deficiency	3.2 [1.2-8.4]	5.8 [3.0-11.0]
Antithrombin-deficiency	7.1 [2.4-22.4]	9.4 [3.3-26.7]
Factor V G1691A	3.3 [2.6-4.1]	3.6 [3.8-4.8]
Factor II G20210A	2.4 [1.7-3.5]	2.6 [1.6-4.4]
Lipoprotein (a)	6.5 [4.5-8.7]	4.5 [3.3-6.2]
LA/aPL	6.6 [3.5-12.4]	4.9 [2.2-10.9]
> Two inherited ITs	11.9 [5.9-23.7]	9.5 [4.9-18.4]
VTE recurrence		
Protein C-deficiency	–	2.4 [1.2-4.4]
Protein S-deficiency	–	3.1 [1.5-6.5]
Antithrombin-deficiency	–	3.0 [1.4-6.3]
Factor V G1691A	–	1.4 [0.4-1.2]
Factor II G20210A	4.3 [1.1-16.2]	2.1 [1.01-3.5]
Lipoprotein (a)	–	0.8 [0.5-1.4]
≥ Two inherited ITs	–	4.5 [4.5-6.9]

Abbreviations: OR: Odds ratio; IT: inherited trait; LA/aPL: lupus anticoagulants/antiphospholipid antibodies; VTE: venous thromboembolism.

Inherited thrombophilia screening in children

Follow-up data for VTE recurrence in children are available from few reports, suggesting a recurrence rate between 3% in neonates and 21% in children with idiopathic VTE.[7,9,12,13,38,48,49,51,54,56,64,65] However, in the pediatric age group, it still remains a controversial issue as to whether children with thrombosis or offspring from thrombosis-prone families benefit from screening for IT.[64,66-74]

Based on the data obtained from the recent meta-analyses and systematic reviews, at least the symptomatic propositus should be screened in a specialized coagulation unit for prothrombotic defects,[60-63] such as antithrombin-, protein C-, or protein S-deficiency. Furthermore, since the rate of combined IT associated with a first symptomatic onset is not negligible in the pediatric population, especially when the family history is positive for thrombosis,[64] non-major risk factors such as the factor V G1691A mutation or the prothrombin G20210A variant should be included in a screening program. Since a second VTE after a first episode of spontaneous VTE, i.e., thrombosis in the absence of further secondary causes, has indicated a subgroup of pediatric patients suffering from combined prothrombotic risk factors to be at high risk of recurrent thrombosis,[38] the latter approach to search for multiple risk factors is stressed.

With respect to the Mendelian theory of inheritance, approximately 50% of siblings of a symptomatic propositus suffering from a combined prothrombotic defect carry one single risk factor, while 25% carry two or more gene mutations/polymorphisms. Thus, based on the fact that an effective primary prophylactic anticoagulant therapy is available in risk situations, IT screening programs must be individually discussed in selected non-symptomatic siblings and further first degree family members in high risk families such as antithrombin-, protein C-, or protein S-deficiency carriers, or in cases in which combined IT are identified.

Treatment modalities

Adult patients with a first thrombotic episode will receive oral anticoagulation for at least three months after venous thrombosis, six to 12 months when the deep venous thrombosis was idiopathic, and at least six to 12 months after pulmonary embolism.[75] Decisions on extending anticoagulant therapy are individually based on the perceived risks of VTE recurrence and anticoagulant-related bleeding. Whether long-term continuation of anticoagulant treatment should be considered after a first VTE in carriers of a thrombophilic trait is still a matter of debate.[75] In children with a first VTE, randomized therapeutic trials are missing and treatment guidelines are mainly adapted from adults.[76,77] More than in adults, the prolonged use of anti-coagulant treatment in a physically active age group must be weighed against the risk of bleeding. Data from the meta-analyses mentioned above will help physicians to decide, along with parents and patients, in which cases a prolonged anticoagulant treatment may be justified,[60-62,77] or practically, if pediatric patients additionally carrying genetic trait with chronic conditions associated with VTE might require secondary anti-coagulant in high risk situations, e.g., post-operatively, prolonged immobilization, or dehydration. The findings based on the analyses of risk increase underscore the hypothesis that in cases in which the temporary risk factors recur, the risk of a second VTE in children is probably less likely if IT is not present.[56,60]

Until results from randomized trials are available for anticoagulant treatment and duration of symptomatic index patients, children with clinically- and imaging-proven VTE are treated according to recommendations based on small-scale studies in children and guidelines adapted from adult patient protocols.[76,77] Unfractionated heparin, low-molecular weight heparins, and vitamin K-antagonists are the commonly used antithrombotic drugs, whereas newly developed antithrombotic agents such as argatroban, bivalirudin or fondaparinux are under discussion and are increasingly administered in small pediatric clinical trials.[76-79] Keeping in mind the low level of evidence for treatment-related recommendations in the pediatric population, it must be pointed out here that there is an urgent need for each pediatric patient with an early VTE manifestation to receive secondary anti-coagulation prophylaxis in clinical risk situations prone to thromboembolism, for example, catheterization, dehydration, hematologic malignancies,[38,80-82] or prolonged immobilization, respectively. Since randomized treatment trials are lacking, doses and durations of secondary anti-coagulation should be individually adapted to patient's risk.

Conclusions

Apart from a risk stratification in children with VTE based on clinically-acquired circumstances along with acquired and genetic thrombophilic risk factors, future evidence-based prospective anticoagulant/antithrombotic treatment trials including newly available anticoagulants are mandatory.[79] In addition, a worldwide pediatric expert consensus on standardized clinical outcome measurements, imaging methods to be used, and determination of follow-up intervals is urgently requested. The effective prevention of thrombosis-related complications in children, such as the post-thrombotic syndrome, will considerably reduce the burden and the costs associated with the disease.[83]

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New insights in congenital dyserythropoietic anemias

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A B S T R A C T

Dyserythropoiesis appears to be a qualitative and a quantitative defect of erythropoiesis and occurs in a wide range of diseases embracing a number of conditions that primarily affect the nucleus or the cytoplasm of erythroblasts or the environment in which erythropoiesis takes place. This could be an acquired or an inherited defect; it may include dyserythropoiesis as the principal (congenital dyserythropoietic anemias, CDA) or a secondary characteristic (thalassemia syndromes; unstable hemoglobins or thiamine- responsive anemias). One of the main problems of the CDAs is their heterogeneity, both at clinical and genetic levels. In general, the features of dyserythropoiesis, in terms of ineffective erythropoiesis, should be demonstrated by several criteria: bone marrow evaluation by light microscopy, ultrastructural features, assessment of red cell production and destruction, and studies of iron metabolism. Certainly the main assessment method, and one which is easy to perform, is light microscopy. Biochemical approach (such as in CDA II) or a molecular approach (such as in CDA I or II) are also indicated. Mapping and cloning showed that these conditions have different molecular mechanisms that induce disturbances of cell maturation and of cell division during erythropoiesis.

Cloning of codanin 1 and Sec23b genes allowed for the molecular characterization of these two conditions. Moreover, cases with clinical and morphological features of CDA , both type I and II, and without mutations in these genes clearly demonstrated genetic heterogeneity. The molecular basis of several cases was recently obtained by characterization of forms due to eKLF-1 and GATA-1 defects.

The congenital dyserythropoietic anemias (CDAs) comprise a group of rare hereditary disorders that are characterized by ineffective erythropoiesis as the predominant mechanism of anemia and by distinct morphological abnormalities of erythroblasts in the bone marrow. Diagnosis has to exclude other congenital anemias such as the thalassemia syndromes, certain types of pathological hemoglobins or hereditary sideroblastic anemias, megaloblastic anemia due to vitamin deficiencies or myelodysplastic syndromes.[1,2]

The term "dyserythropoiesis" was first used by Crookston *et al.*[3] (for cases later classified as CDA II) and by Wendt and Heimpel[4] (for cases later classified as CDA I). The working classification (CDA I, II and III) mainly due to Heimpel studies is still used in clinical practice. There are, however, families that fulfill the general definition of the CDAs, but do not conform to any of the three classical types (Table 1). These classifying criteria could explain part of the genetic heterogeneity of these conditions, since dyserythropoiesis appears to be a morphologic criteria common to several (inherited and acquired) conditions. Recent identification of several causative genes could help in reclassifying these disorders (Table 1).

Ineffective erythropoiesis in combination with moderately shortened red cell lifespan, particularly in type II, are common features. A dyserythropoietic anemia could be suspected in presence of symptoms and signs of increased hemoglobin turnover, such as mild jaundice, and low or absent haptoglobin, as in hemolytic anemia, with a reticulocytosis that does not correspond to the degree of anemia. The bone marrow is always hypercellular, exclusively due to a pronounced increase of erythroblasts, with increased erythropoietic/granulopoietic ratio (normal reference values 0.3 – 1.0). All types of CDA share a high incidence of cholelithiasis and distinct iron loading. As in other forms of anemia with ineffective erythropoiesis, this is due to up-regulation of iron resorption, mediated by hepcidine[19]. Extramedullary hematopoiesis presenting as paravertebral bulks may be observed in all types of CDA.

CDA type III was reported in 1962 in a Swedish family accounting for 34 patients. This observation allowed for the mapping of the gene on chromosome 15. Very few cases are present in literature and this impeded further molecular insights. This review will deal with new insights on the two most frequent forms of CDA: type I and II.

CDA type I

Until August 2009, 169 cases from 143 families with CDA I were recorded in the literature, and hence this condition appears approximately 3 times less frequently than CDA II. Most families presenting were Western European and Arab, but single cases were also reported from the USA, India,

Table 1. Characteristic features of different types of congenital dyserythropoietic anemias.

CDA type	I	II	III familial	III sporadic	Variants
Inheritance	Autosomal-recessive	Autosomal-recessive	Dominant	Recessive/X-linked	Autosomal-recessive
Cases reported	~150	~370	3 families	<10	>20
Morphology	Abnormal chromatin structure, chromatin bridges	Binuclearity of mature erythroblasts	Giant multinucleated erythroblasts	Giant multinucleated erythroblasts	CDA I-like CDA II-like others
Gene	*codanin* 15q (15.1.3)	SEC23B 20p (11.2)	15q (21-25)	*eKLF1 / GATA1*	unknown
Associated dysmorphology	Skeleton, others	Variable, rare	B-Cells Retina	variable	CNS others

Japan, and Australia.[5]

The clinical picture includes anemia, sometimes with neonatal appearance, jaundice, splenomegaly, hepatomegaly, frequent and diverse dysmorphisms, predominantly affecting the digits (particularly syndactyly in hands or feet, absence of nails or supernumerary toes)[1-6] and a progressive build up of iron overload. The degree of anemia is variable not only between families, but for unexplained reasons (modifier genes) may also be different among siblings.[6] Most patients have life-long anemia with hemoglobin concentration between 7 and 11 g/dL. Occasionally, there are severe cases requiring transfusion in-utero[7] or immediately after birth, and regular blood transfusions during childhood and adolescence.[6] On the other side there are patients with only low-borderline hemoglobin but distinct macrocytosis. The anemia is usually macrocytic with MCV between 100 and 120 fl, but may be normocytic in childhood.

Light microscopy of bone marrow demonstrates 30 to 60% of early and late polychromatic erythroblasts showing characteristic and partially bizarre abnormalities of nuclear shape and size and of chromatin structure, but proerythroblasts and immature basophilic erythroblasts usually appear normal. There are large polyploid cells, and a minority of cells are bi- or multinucleated; in contrast to CDA II, nuclei are of different size and stain. The hallmark of CDA I is incompletely divided cells with thin chromatin bridges between pairs of erythroblasts, which may also be seen between two nuclei in a single cell. Electron microscopy studies demonstrated that heterochromatin is denser than normal and forms sharply delineated clumps with small translucent vacuoles, giving rise to the description of "Swiss cheese appearance", and cytoplasm may penetrate through widened pores of the nuclear envelope.[8]

The gene responsible for CDA I (*CDAN1* gene) was mapped to the long arm of chromosome 15 between 15q15.1q15.3 by homozygosity mapping in four Bedouin families with a high degree of consanguinity. Similar results were reported in patients from Europe and the Near East. The *CDAN1* gene was successively cloned with 28 exons spanning 15 Kb encoding a protein named codanin-1. In nine unrelated patients of European, Bedouin and Asian origin, different point mutations were detected.[9,10] Approximately 90% of patients with a bone marrow suggesting CDA I have codanin gene mutations. Interestingly, families with definite phenotype of CDA I in which no mutation of the CDAN1-gene could be found suggested either a promoter defect or a mutation in another gene[11] (genetic heterogeneity). These observations, associated with the exclusion of linkage with chromosome 15 in several families, suggested the presence of a second disease locus.[11]

The vast majority of patients with confirmed diagnosis of CDA I showed mutations of at least one allele from exons 6 to 28 within *CDAN1*, and more than 30 unique mutations have been identified so far (Table 2). Interestingly, no homozygotes or compound heterozygotes for null-type mutations have been identified, supporting an earlier notion that codanin-1 may have a unique function and may be essential during development.

The mutated gene (*CDAN1*) encodes a ubiquitously expressed protein (codanin-1) of unknown function.[9] This protein is still not well characterized, but it seems to be related to chromosome structure and it must be involved in mitotic process. Tamary *et al.* have previously shown that codanin-1 is a direct transcriptional target of the E2F1 transcription factor and that the levels of codanin-1 increase during S-phase and decrease during mitosis.[12] Furthermore, they conducted a yeast two-hybrid screen using a human bone marrow library and found that codanin-1 binds to Asf1a. Asf1 (anti silencing function) is a H3/H4 histone chaperone involved in the chromatin structure dynamics by its role in nucleosome assembly and disassembly. Coimmunoprecipitation experiments confirmed that histone chaperone Asf1a is a direct binding partner of codanin-1. They suggested that binding of codanin-1 inhibits dissociation of histones from Asf1a, which therefore cannot be deposited onto DNA.[13]

Very recently, another contribution attempts to define the role of codanin-1 in pathophysiology of CDA type I. Renella *et al*. investigated localization, distribution and interactions of codanin-1 in CDA I patients and generated a murine knock-out model for *CDAN1*. They found no gross differences between normal and patient samples both in the amounts of histone proteins or various

Table 2. Mutational spectrum of *CDAN1/CDAN2*

Gene	Exon	Nucleotide change[c/*]	Legacy name[a/b]	Protein domain affected	Ref. for published mutations
CDAN1 (53 cases)					
	missense				
	2	c.156 C>G	F52L		Tamary, 2005 Br J Haematol
	6	c.1078 T>C	F360L		Heimpel, 2006 Blood
	12	c.1796 A>G	N599S		Dgany, 2002 Am J Hum Genet
	14	c.2015 C>T	P672L		Dgany, 2002 Am J Hum Genet
	14	c.2062 C>T	R688W		Tamary, 2005 Br J Haematol
	14	c.2069 T>C	V690A		Ru, 2008 Ann Hematol
	14	c.2092 G>A	E698K		Dgany, 2002 Am J Hum Genet
	14	c.2140 C>T	R714W		Dgany, 2002 Am J Hum Genet
	14	c.2173 C>T	R725W		Heimpel, 2006 Blood
	15	c.2248 G>T	G750C		Heimpel, 2006 Blood
	19	c.2602 T>A	F868I		Dgany, 2002 Am J Hum Genet
	19	c.2605 G>A	V869M		Dgany, 2002 Am J Hum Genet
	20	c.2650 A>G	T884A		Tamary, 2005 Br J Haematol
	24	c.3107 C>A	S1036F		Dgany, 2002 Am J Hum Genet
	24	c.3124 C>T	R1042W		Dgany, 2002 Am J Hum Genet
	24	c.3128 A>T	D1043V		Dgany, 2002 Am J Hum Genet
	24	c.3194 G>A	R1065Q		Heimpel, 2006 Blood
	26	c.3389 C>T	P1130L		Dgany, 2002 Am J Hum Genet
	nonsense				
	14	c.2044 C>T	R682X		Dgany, 2002 Am J Hum Genet
	18	c.2539 C>T	Q847X		Tamary, 2005 Br J Haematol
	23	c.2992 C>T	R998X		Dgany, 2002 Am J Hum Genet
	27	c.3547 C>T	Q1183X		Heimpel, 2006 Blood
	splicing				
	8	c.1367+1 G>A			Tamary, 2005 Br J Haematol
	12	c.1860+5 G>A			Tamary, 2005 Br J Haematol
	21	c.2868+2 insCCG			Heimpel, 2006 Blood
	28	c.3558 del -10 to +31			Heimpel, 2006 Blood
	frameshift				
	6	c.1117_19 delGTT	V373del		Ahmed, 2006 Blood
	12	c.1789_1791 delGAG	E597del		Heimpel, 2006 Blood
	23	c.3024 insTT	G1008Gfs23X		Heimpel, 2006 Blood
	24	c.3145 insT	S1049Ffs25X		Heimpel, 2006 Blood
CDAN2 (129 cases)					
	missense				
	2	c.40 C>T	R14W	Segment 1	Schwarz, 2009 Nat Genet.
	2	c. 53 G>A	R18H		Schwarz, 2009 Nat Genet.
	2	c.197 G>A	C66Y	Zink finger	Fermo, 2010 Blood Cells Mol Dis
	4	c.325 G>A	E109K		Schwarz, 2009 Nat Genet.
	7	c.716 A>G	D239G	Trunk	Schwarz, 2009 Nat Genet.
	8	c.938 G>A	R313H		Schwarz, 2009 Nat Genet.
	8	c.953T>C	I318T		Schwarz, 2009 Nat Genet.
	9	c.1043 A>C	D348A		Bianchi, 2009 Hum Mutat.
	10	c.1157 A>G	Q386R		Schwarz, 2009 Nat Genet.
	11	c.1254 T>G	I418M	β-sheet	Russo, 2010 Am J Haematol
	11	c.1307 C>T	S436L		Russo, 2010 Am J Haematol
	12	c.1385 A>G	Y462C		Schwarz, 2009 Nat Genet.
	13	c.1489 C>T	R497C		Schwarz, 2009 Nat Genet.
	13	c.1508 G>A	R503Q		Iolascon, 2010 Haematologica
	14	c.1571 C>T	A524V	Helical	Schwarz, 2009 Nat Genet.
	14	c.1588 C>T	R530W		Schwarz, 2009 Nat Genet.
	14	c.1589 G>A	R530Q		Iolascon, 2010 Haematologica
	14	c.1654 C>T	L552F		Russo, 2010 Am J Haematol
	15	c.1685 A>G	Y562C		Iolascon, 2010 Haematologica
	15	c.1733 T>C	L578P		Russo, 2010 Am J Haematol
	15	c.1735 T>A	Y579N		Russo, 2010 Am J Haematol
	16	c.1808 C>T	S603L		Bianchi, 2009 Hum Mutat.
	16	c.1832 G>C	R611P		Russo, 2010 Am J Haematol
	16	c.1858 A>G	M620V		Russo, 2010 Am J Haematol
	17	c.1968 T>G	F656L	Gelsolin	Iolascon, 2010 Haematologica
	18	c.2101 C>T	R701C		Bianchi, 2009 Hum Mutat.
	18	c.2129 C>T	T710M		Amir, Acta Haematologica in press
	19	c.2166 A>C	K723Q	Segment 6	Iolascon, 2010 Haematologica
	20	c.2270 A>C	H757P		Russo, 2010 Am J Haematol

Table 2. Mutational spectrum of *CDAN1/CDAN2*

Gene	Exon	Nucleotide change°/*	Legacy name[a/b]	Protein domain affected	Ref. for published mutations
	nonsense				
	3	c.235 C>T	R79X	Zink finger	Schwarz, 2009 Nat Genet.
	5	c.367 C>T	R123X	Segment 2	Russo, 2010 Am J Haematol
	5	c.568 C>T	R190X	Trunk	Bianchi, 2009 Hum Mutat.
	6	c.649 C>T	R217X		Schwarz, 2009 Nat Genet.
	7	c.790 C>T	R264X		Schwarz, 2009 Nat Genet.
	8	c.970 C>T	R324X		Schwarz, 2009 Nat Genet.
	9	c.1015 C>T	R339X		Russo, 2010 Am J Haematol
	10	c.1201C>T	R401X	Segment 3	Schwarz, 2009 Nat Genet.
	14	c.1603 C>T	R535X	Helical	Russo, 2010 Am J Haematol
	14	c.1648 C>T	R550X		Iolascon, 2010 Haematologica
	14	c.1660 C>T	R554X		Bianchi, 2009 Hum Mutat.
	splicing				
	2	c.221+31 A>G		Zink finger	Russo, 2010 Am J Haematol
	3	c.279+3 A>G			Russo, 2010 Am J Haematol
	6	c.689+1 G>A		Trunk	Schwarz, 2009 Nat Genet.
	9	c.1109+1 G>A			Russo, 2010 Am J Haematol
	9	c.1109+5 G>A			Russo, 2010 Am J Haematol
	19	c.2149-2 A>G		Gelsolin	Russo, 2010 Am J Haematol
	frameshift				
	3	c.222-817_366+4242del	Q75EfsX7	Zink finger	Schwarz, 2009 Nat Genet.
	5	c.387(del G)	L129LfsX26	Trunk	Russo, 2010 Am J Haematol
	5	c.428_428delAinsCG	D143AfsX35		Bianchi, 2009 Hum Mutat.
	9	c.1063delG	D355IfsX8		Schwarz, 2009 Nat Genet.
	16	c.1821delT	H608IfsX7	Helical	Bianchi, 2009 Hum Mutat.
	16	c.1857_1859delCAT	I619del		Russo, 2010 Am J Haematol
	17	c.1962-64 (delT)	T654TfsX13	Gelsolin	Iolascon, 2010 Haematologica
	19	c.2150 (delC)	A717VfsX7		Russo, 2010 Am J Haematol

° CDAN1: The nucelotides are numbered from the A of the ATG initiation codon (ENST00000356231). All mutations have been revised according to the rules of nomenclature of HGVS database (http://www.hgvs.org/mutnomen/).

** CDAN2: The nucelotides are numbered from the A of the ATG initiation codon (ENST00000377475).*

[a] CODANIN: Accession number: Q8IWY9 (UniProtKB/Swiss-Prot).

[b] SEC23B: Accession number: Q15437 (UniProtKB/Swiss-Prot).

epigenetic marks of histone tails, suggesting that histone signatures involved in maintenance of chromatin structure and epigenetic regulation, are globally maintained in CDA I.[14]

Using a hybridoma technique, they demonstrated codanin-1 distribution in both nucleus and cytoplasm of cells in normal primary human erythroblasts. This localization pattern was unchanged in CDA I erythroblasts.

No differences were found in the distribution patterns of RNA-polymerase-II, PML, nucleophosmin, HP1β and HP1γ between patients and control erythroblasts. However, the localization of HP1βα, a key component of heterochromatin, was found to be markedly perturbed. HP1α accumulates in the Golgi apparatus of CDA I but not normal erythroblasts. They confirmed that the abnormal localization of HP1α in CDA I patients is confined to the intermediate erythroblast maturation stage, where the characteristic ultrastructural chromatin pattern of CDA I is observed. Furthermore, they suggested that an abnormality in codanin-1 could be responsible for the aberrant localization of HP1α.

Interestingly, by confocal immunofluorescence, they also found codanin-1 co-localizes partially with SEC23B, the protein mutated in CDA II, suggesting a molecular link between the two major types of CDA.

As expected by the molecular studies in human patients, the total absence of codanin-1 is lethal. Renella *et al.* generated the first murine *Cdan1* gene-trap model demonstrating its widespread expression during embryonic development. *Cdan1*$^{gt/gt}$ homozygotes die in-utero before the onset of primitive erythropoiesis, suggesting that *Cdan1* has other critical roles during embryogenesis.

Another unsolved problem in CDA type I was the bone-marrow response of interferon administration. Literature data demonstrate that interferon remains an effective treatment of anaemia of CDA I for a long period, without any noticeable side-effects.[15] Furthermore, its effects on iron status parameters raise the question of its use even in patients moderately anemic but with a marked iron overload. Increased expression of GDF-15, which represents a negative regulator of hepcidin, seems to be characteristic of this type of anemia and related to increased apoptosis on bone marrow erythroblasts.[16]

CDA type II

CDA type II is an autosomal recessive disorder also known as hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS).[4] The severity of anemia varies from mild to severe. Diagnosis of CDA II is usually made later in life compared with CDA I, because symptoms can be mild.[17,18] Patients may come to attention because of the anemia combined with jaundice (90% of cases), splenomegaly (70%), or hepatomegaly (45%). CDA II patients with Gilbert syndrome had a greater tendency to form gallstones than those without.[19] The evolution of CDA II is

doomed, once again, by iron overload and biliary complications, and in about 20% of patients by liver cirrhosis secondary to iron overload. In certain cases, the presence of posterior mediastinal or paravertebral masses consisting of extramedullary hemopoietic tissue could be present.[20-26] The anemia of CDA II is normocytic, hemoglobin levels are somewhat lower in children than in later life, ranging to 8 and 11 g/dl with a normal MCV, and the peripheral blood shows basically aniso-poikilocytosis without specific types of poikilocytes, with basophilic stippling of cells and few, occasionally binucleated, mature erythroblasts. Relative reticulocyte counts are normal or moderately increased. The bone marrow is hypercellular with erythroid hyperplasia but, in contrast to CDA I, is of normoblastic appearance with a large number of binucleate (10%-35%) and rarely multinucleate late polychromatic erythroblasts.[27] On electron microscopic examination, vesicles of endoplasmic reticulum (ER) containing proteins normally found in this organelle, calreticulin, glucose regulated protein (GRP78) and protein disulfide isomerise,[28-31] appear to be running beneath the plasma membrane in this disorder.

Erythrocytes of CDA II patients lyse in acidified serum (Ham test) because of an IgM class antibody that recognizes an antigen present on CDA II cells but absent on normal cells. The test checks whether red blood cells become more fragile when they are placed in mild acid. Usually, this test is done to diagnose paroxysmal nocturnal hemoglobinuria (PNH), although it is increasingly being replaced by a flow cytometry. The technical difficulty of this test, and the fact that cross-testing of more than 30 normal sera is needed to obtain a reliable result, has undermined its usefulness.[1]

In addition to the tendency to lyse in acidified sera, red blood cells of CDA II patients agglutinate in sera containing anti-i antigens. This test is sensitive but not specific, and results cannot be compared among different institutions because of the different anti-i sera used.

The diagnostic hallmark of CDA II is the analysis of erythrocyte membrane proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by identifying the narrower band size and faster migration of band-3 and band-4.5 proteins.[32-35] Exceptional cases that do not show the characteristic SDSPAGE pattern have been reported, but it is recommended that these should be considered CDA II-like conditions. Research on the abnormalities found in CDA II red blood cells has yielded several additional tests such as western blotting analysis of endoplasmic reticulum proteins of red blood cells (GRP-78, calreticulin, PDI).

Genome-wide linkage analysis localized the disease gene (*CDAN2*) to a 5-cM region of chromosome 20q11.2[36] but only recently has the causative gene for CDA II been identified.[37] The study was epoch-making because it not only identified the responsible gene, but also showed that the gene defects directly led to production of binucleated cells, which is a hallmark of CDA type II. It has been demonstrated that short hairpin RNA (shRNA)-mediated suppression of SEC23B expression recapitulates the cytokinesis defect. Knockdown of zebrafish Sec23b also leads to aberrant erythrocyte development. The gene encoding the secretory COPII component SEC23B was mutated CDA type II. The secretory pathway in eukaryotic cells is critical for membrane homeostasis, localization of proteins within cells and secretion of extracellular factors. During a budding reaction, cytoplasmic coat proteins (COPs) are assembled on a membrane surface, capture cargo molecules and polymerize into a cage sculpting different-sized cargo vesicles.[38-39] So far, severe mutations in genes encoding COP II components have been assigned to human genetic disorders. *SAR1B* defects cause chylomicron retention disease, Anderson disease and Marinesco-Sjogren syndrome[40], and *SEC23A* is mutated in cranio-lenticulo-sutural dysplasia.[41]

A recent study on 42 patients with CDA type II showed a correlation between the mutations and various biological parameters. In this study, patients were divided into two groups: (1) patients with two missense mutations and (2) patients with one nonsense and one missense mutation. Compound heterozygosity for a missense and nonsense mutation tended to produce more severe clinical presentations than homozygosity or compound heterozygosity for two missense mutations. Homozygosity or compound heterozygosity for two nonsense mutations were never found, and it was supposed to be lethal.[42]

In a recent study, we characterized the allelic distribution of *SEC23B* gene mutations found in CDA II patients from Italy compared to those found in international cases. Until now, 53 different causative mutations have been described (Table 2). We substantially confirmed our previous reports[42-43] as the most representative mutations in both cohorts were R14W and E109K. Nevertheless, we also showed that the high recurrence of *SEC23B*-R14W mutation among the Italian CDA II patients is due to a founder effect, while the most frequent variant E109K showed a common genetic background among European patients (Russo R. *et al.*, in submission 122-11- EJHG).

Very recently *Sec23b* deficient mice (*Sec23b gt/gt*) from ES cells with a genetrap cassette inserted into the last intron of *Sec23b* was generated. *Sec23b gt/gt* mice die at birth with destruction of the exocrine pancreas. Interestingly, 8 to 12-week-old lethally irradiated C57BL/6J recipients transplanted with fetal liver cells from either wildtype or *Sec23b gt/gt* showed no haematological findings.[44] These data demonstrate that *Sec23b* deficient humans and mice exhibit disparate phenotypes, apparently restricted to CDA II in humans and a prominent neonatal pancreatic insufficiency in mice.

Others congenital dyserythropoietic anemias

In the past, several genes have been already described associated to congenital dyserythropoietic anemias. One of these is the transcriptional factor GATA-1. Mutations in *GATA*-1 have been found to have important clinical significance, and are directly linked to deregulated formation of certain blood cell lineages. Five are rare syndromes caused by defects in GATA-1 gene expression or a malformed protein product. These disorders are: X-linked thrombocytopenia (XLT), X-linked thrombocytopenia with thalassemia (XLTT), congenital erythropoietic porphyria (CEP), transient myeloproliferative disorder (TMD) and acute

megarakaryoblastic leukemia (AMKL) associated with trisomy 21, and anemia associated with the production of GATA-1s.[45] GATA-1 has two zinc fingers essential for normal function. The C-terminal finger is necessary for DNA binding. The N-terminal finger mediates interaction with FOG-1, a cofactor for GATA-1.

A family with X-linked dyserythropoietic anaemia and thrombocytopenia due to a substitution of methionine for valine at amino acid 205 of GATA-1 was described.[46] This highly conserved valine is necessary for interaction of the amino-terminal zinc finger of GATA-1 with its essential cofactor, FOG-1 (for friend of GATA-1). They showed that the V205M mutation abrogates the interaction between Gata-1 and Fog-1, inhibiting the ability of GATA-1 to rescue erythroid differentiation in an erythroid cell line deficient for GATA-1 (G1E). Their findings underscore the importance of FOG-1:GATA-1 associations in both megakaryocyte and erythroid development, and suggest that other X-linked anemias or thrombocytopenias may be caused by defects in GATA1. This mutation most closely resembles those of mice with the 'knockdown' Gata1 mutation.

Another group described a family with a novel single base mutation that results in an amino acid substitution (Gly208Arg) within the highly conserved portion of the GATA-1 N-terminal finger domain, leading to dyserythropoietic anemia and macrothrombocytopenia.[47]

In the 90s a 10-year-old female Danish case was extensively described.[48-51] She showed severe anemia at birth and required repeated transfusion during childhood. The clinical characteristic exhibited by the patient was persistent expression of ε and ζ embryonic globin, an HbF level of 40%, novel intra-erythroblastic and intra-erythrocytic inclusions and deficiency of erythroid proteins CD44 and Aquaporin 1. The marrow aspirate studies revealed active erythropoiesis with some dyserythropoietic features. Blood grouping tests further showed that the child has the very rare Co(a-b-) blood group phenotype and is negative for the high incidence antigen AnWj.[50]

Only recently an erythroid transcriptional factor alteration has been elucidated. It was the case of two patients with a hitherto unclassified CDA in whom a missense mutation in KLF1 has been identified. One of these was a Danish patient previously described. The first patient described showed a similar clinical phenotype of Danish. KLF1 is an erythroid transcription factor, and extensive studies in mouse models have shown that it plays a critical role in the expression of globin genes, but also in the expression of a wide spectrum of genes potentially essential for erythropoiesis.[52-54] The unique features of this CDA confirm the key role of KLF1 during human erythroid differentiation. Sequencing of KLF1 in both patients revealed the presence of de novo c.973G>A (p.E325K) mutation that had never been reported and was not present in 96 regular blood, suggesting it was the disease-causing mutation. The authors suggested that mutations in KLF1 cause a hitherto unclassified CDA.[55]

Finally, in recent months the case of a patient with mevalonate kinase deficiency (MKD) and congenital dyserythropoietic anemia has been described. The clinical phenotype was variable, ranging from the hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) to mevalonic aciduria (MA), a severe metabolic disease. Genomic sequencing of the mevalonate kinase gene revealed compound heterozygosity for a missense mutation previously described in MA (V310M) and a novel missense mutation (Y116H). In contrast, sequencing of *SEC23B* gene revealed no mutations, suggesting that the bone marrow abnormalities were causally related to the MKD.[56]

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Vaso-occlusion in sickle cell anemia

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Introduction

Sickle cell anemia (SCA) is a common inherited congenital hemolytic anemia affecting millions of persons worldwide. SCA is characterized by a predominance of abnormal sickle hemoglobin (HbS) within the erythrocytes, which polymerizes under deoxygenating conditions, leading to deformed (sickled) cells with increased adhesiveness, aberrant membrane composition, and cellular dehydration. These intrinsic defects, coupled with abnormal flow characteristics of sickled erythrocytes, lead to erythrocyte fragility and pathological interactions with the endothelium and reticuloendothelial system; hence, the SCA is a hemolytic anemia with both an intravascular and extravascular component.

SCA is also a chronic inflammatory condition featuring widespread endothelial damage, dysfunction, and activation. The vasculopathy of SCA is now well-characterized but still not completely understood. The intravascular milieu features sickled erythrocytes, adhesive circulating blood cells, hypercoagulability, plasma mediators of inflammation, and products of hemolysis. Together, these factors promote abnormal cellular attachments and interactions with the endothelium, which then alter normal vessel integrity and lead to pathological responses. The processes by which circulating leukocytes, reticulocytes, and sickled erythrocytes adhere to vascular endothelium and the effects of these interactions are increasingly understood using *in vitro* and animal models, which provide an opportunity to discover and test therapeutic interventions.

Clinically, patients with SCA have periodic acute events that reflect abrupt blockage of blood flow within small vessels, leading to distal tissue hypoxia and infarction; this process is usually referred to as "vaso-occlusion", since normal blood flow is impaired. These sporadic events are typically painful and often require medical attention, while some present mainly with respiratory or neurological symptoms. This sickle cell *crisis* is among the more perplexing aspects of SCA, and its optimal clinical management is the subject of numerous current clinical trials. This review focuses on vaso-occlusion in SCA, which refers predominantly to patients with homozygous HbSS. Although patients with other genotypes, such as HbSC and HbS/thalassemia, also have vaso-occlusive events, their pathophysiology is different and less studied and will not be emphasized further in this review.

Nomenclature

The nomenclature surrounding the pain that is so characteristic of SCA has recently been called into question regarding cultural sensitivity and appropriateness. Although the origin and first use of "crisis" for SCA patients is unknown, this word is quite useful and has long been accepted, since it encompasses both the suddenness and severity of the pain. Particularly in discussions involving the lay community, the word is common but can be preceded by descriptors such as "pain" or "painful" or even "acute" to accentuate the symptoms. Among healthcare providers, the word "vaso-occlusive" is added to emphasize the pathophysiology of the pain and to distinguish it from other etiologies, and "vaso-occlusive crisis" has become the recognized VOC acronym. Subsequently, however, the terms "event" and "episode" have been advocated over "crisis", since these are potentially less pejorative and remove any suggestion of psychological overlay or origin. Phrases like "vaso-occlusive event (VOE)" or "painful event" have thus become commonly used to describe SCA pain that requires medication or medical evaluation. The fact that a 2011 sickle cell conference had a highlighted topic titled "Don't take away my Crisis" that features a debate on the merits and sanctity of the word "crisis" in SCA indicates that the issue is still far from resolved. In an attempt to remain neutral yet informative, the current text will simply use the descriptive but admittedly rather bland term "vaso-occlusive event" to describe these sickle-related pain and other acute processes.

Pathophysiology

The topic of sickle cell vaso-occlusion has been extensively studied over the past 40 years, and our understanding of the process has evolved considerably since the original simplistic concept of misshapen sickled ery-

throcytes forming a transient log-jam within small vessels, thereby impeding local blood flow until released. One major advance came 30 years ago when several investigators identified abnormal adhesion between sickle erythrocytes and vascular endothelium,[1-2] indicating that deoxygenation and HbS polymerization had effects beyond changing erythrocyte shape. Elucidation of the critical role of circulating leukocytes in triggering this contact was another major step forward.[3-4] Several recent reviews have highlighted the numerous advances and refinements of the current state of knowledge about vaso-occlusion in SCA since that time.[5-6] Vaso-occlusion still begins with sickled erythrocytes but is now known to be a highly complex process involving frequent abnormal interactions among a wide variety of circulating blood cells, plasma factors, and vascular endothelium. Each major component of this triad contributes to the vaso-occlusive process and the interactions are circular, highly interactive, and often amplifying (Figure 1). Proper understanding of vaso-occlusion, with consequent blockage of blood flow and distal tissue hypoxia, requires recognition and discussion of all aspects of the pathophysiology within this triad.

First and foremost, it should be emphasized that the vaso-occlusion event observed in SCA is unique and does not have another medical equivalent; this fact mandates that the pathophysiology begins and hinges upon the presence of HbS and the sickled erythrocyte. Abundant HbS within the erythrocytes, which leads to intracellular polymerization, morphological shape changes, and abnormal cell-vessel interactions, cannot be overlooked as the principal culprit of the vaso-occlusive process. Newborn infants with SCA have low amounts of HbS (typically 5-25%) and appear to be protected in the early neonatal period due to high amounts of intracellular fetal hemoglobin (HbF). Vaso-occlusive events do not develop clinically until the protective effects of HbF begin to wane, usually in the first few years of life.[7] Conversely, hereditary persistence of HbF provides lifelong protection against clinical events,[8] which further emphasizes the critical importance of the erythrocyte (and its HbS concentration) in the genesis and development of vaso-occlusive events. The sickle erythrocyte is not simply an inert carrier of HbS, however, because the erythrocyte membrane itself is abnormal[9] and coated with adhesive molecules that promote adhesion to the endothelium. Reticulocytes are especially adherent,[10] perhaps because they are less dense and retain surface markers from the marrow that are not removed or "polished" in the absence of a healthy spleen. Sickle erythrocytes express a wide variety of proteins and lipids, including exposed phosphatidylserine, which aberrantly bind to vascular endothelium and the subendothelial matrix as a critical part of the development of vaso-occlusive events.[11-16]

Over time and after repeated abnormal sickled erythrocyte-vessel interactions, these events damage the vascular endothelium and begin a slow but almost inexorable slide toward generalized endothelial injury, dysfunction, and activation. Damaged endothelium cannot properly produce local nitric oxide or detoxify reactive oxygen species, which leads to further endothelial injury through hypoxia/reperfusion events.[17] Circulating endothelial cells (CEC) can be identified in SCA,[18] often

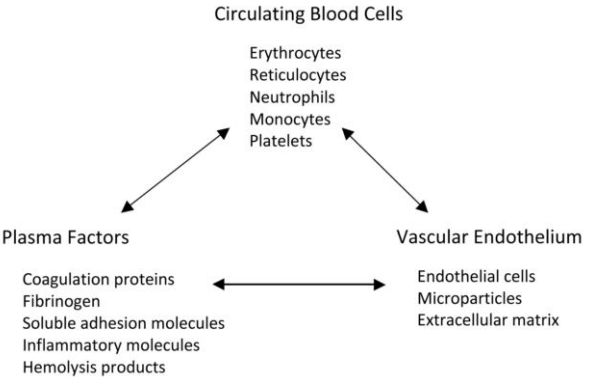

Figure 1. Main blood components participating in the complex interactions of vaso-occlusion in SCA. Although the sickling process begins within erythrocytes, many other circulating blood cells become involved as primary and secondary participants. Plasma factors promote vaso-occlusive and reflect cellular damage and vascular inflammation. The vascular endothelium is progressively damaged and its inflammation and dysfunction leads to additional vaso-occlusion and vasculopathy. These interactions are circular and amplifying.

with an abnormal surface marker profile reflecting widespread vascular damage. Levels of CEC are elevated in steady-state SCA but further increase during acute vaso-occlusion and may reflect disease severity.[19] Microparticles derived from endothelium also circulate in SCA.[20] Activation markers can be detected on these circulating cells and microparticles, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, P-selectin, and tissue factor.[21-24] Not all endothelium is identical, however, and it is likely that intrinsic differences in the vascular endothelial beds themselves are important determinants of the cerebrovascular and pulmonary damage in SCA that begins early in life and could perhaps be as important as physiological fluctuations such as blood flow and oxygenation. This is an area that has not been fully explored but could reveal insights into the pathophysiology of vaso-occlusion and help explain the propensity for damage within certain organs.

Another critical consequence of these abnormal erythrocyte-endothelial interactions is the progressive development of a generalized inflammatory process. As previously mentioned, circulating erythrocytes, and especially younger reticulocytes, have increased levels of adhesion and activation molecules. The total white blood cell (WBC) count becomes elevated early in life,[25] particularly an elevated neutrophil count, along with increased platelets, and these circulating cells feature surface adhesion markers that reflect activation and promote adhesion to endothelial cells.[26-27] Activated $CD11b^+$ monocytes have also been identified in SCA; these circulating cells stimulate endothelium through TNF-alpha and interleukin-1-beta and trigger nuclear factor-kappa B nuclear translocation,[28] which then presumably leads to further endothelial activation. Each of these cells also spawns microparticles that express inflammatory and activation markers.[20,29] Within the plasma itself, a wide variety of soluble non-specific inflammatory markers can be identified, including elevated levels of C-reactive

protein, cytokines, and soluble adhesion molecules like ICAM-1, VCAM-1, and E-selectin.[30-31] Taken together, it is clear that SCA features widespread and chronic inflammation that affects circulating cellular elements, cellular-derived microparticles, and soluble plasma factors that collectively promote vaso-occlusion and endothelial damage.

An additional characteristic feature of SCA that is likely important in vaso-occlusion and organ damage, but relatively poorly understood at this time, is the hypercoagulable state.[32] A variety of factors could contributes to hypercoagulability in SCA, including functional asplenia, reticulocytes and erythrocytes that express adhesion molecules and exposed surface phosphatidylserine, increased numbers of WBC that express increased adhesion markers, activated platelets,[30,33] chronic inflammation, and elevated levels of plasma clotting proteins.[34-35] Owing to a combination of these abnormalities, the coagulation system in SCA is clearly tilted toward the procoagulant state. Whether or not this hypercoagulability initiates cell-endothelial contacts remains unclear, but almost certainly it promotes and propagates the pathophysiology of vaso-occlusion. It should be noted, however, that patients with SCA do not develop deep venous thrombosis or other common manifestations of a hypercoagulable state.

Finally, the contributions of products of hemolysis toward vaso-occlusion must be considered in the unique setting of SCA. Sickled erythrocytes are fragile, and about one-third of the overall hemolysis in SCA occurs in the intravascular compartment.[36] Considerable effort has been directed toward promoting the hypothesis that this intravascular hemolysis, measured specifically by elevated levels of plasma hemoglobin but more often by lactate dehydrogenase (LDH), is itself a direct and critical etiologic factor of vascular endothelial damage in SCA through consumption of nitric oxide, with substantial clinical consequences, including stroke, leg ulcers, pulmonary hypertension, and early mortality.[37-38] However, the merits of these arguments have been recently questioned with regard to their scientific rigor and accuracy.[39-40] Indeed, many other hematological disorders include intravascular hemolysis with similarly elevated levels of LDH (or higher), yet none features the clinical spectrum of SCA. The most likely answer to this apparent paradox is that no other medical condition features intravascular hemolysis so prominently in the setting of generalized inflammation, hypercoagulability, and endothelial dysfunction;[39] hence hemolysis per se is not the etiology of the vasculopathy in SCA but merely a participant. Another alternative explanation regarding hemolysis-based pathophysiology warranting further experimental testing is the possibility that some elevated serum LDH in SCA actually derives from damaged and/or circulating endothelial cells rather than just from erythrocytes; if proven, this observation would suggest that elevated LDH reflects an "effect" rather than a "cause" of the endothelial damage in SCA. Additional possibilities should be explored as well, including the recent proposal that hemolysis-derived pathophysiological characteristics of SCA, such as excessive plasma heme, also contribute to blood cell and endothelial damage.[40-41]

In this complex environment, the process of small vessel vaso-occlusion in SCA thus begins initially with repeated erythrocyte sickling and adhesion-mediated vessel blockage, but over time develops into a unique vasculopathy characterized by activated circulating blood cells, including erythrocytes, reticulocytes, neutrophils, monocytes, platelets, and even endothelial cells. These sticky cells are then coupled with soluble plasma mediators of inflammation, coagulation, and hemolysis, which lead to further adhesion that alters the normal integrity of the endothelium. Perhaps in this setting of such widespread cellular adhesion, damage, and inflammation, the question should not be "Why do patients with SCA have acute vaso-occlusive events?" but instead "Why do patients with SCA not have vaso-occlusive events all the time?" since the microvascular processes are both widespread and continuous. It should be emphasized that vaso-occlusion is ongoing even when not clinically recognizable.

Epidemiology

Most of the data regarding the types and frequency of acute vaso-occlusive events in SCA derive from two large prospective studies, the Jamaican cohort and the US multicenter Cooperative Study of Sickle Cell Disease (CSSCD). These studies have documented that painful events, acute chest syndrome, and stroke are frequent clinical manifestations that lead to substantial morbidity and mortality.[42-44] However, there is considerable phenotypic diversity with regard to vaso-occlusive events; most patients are affected occasionally, but a small percentage of patients suffer from frequent and repeated events.

In the first decade of life, pain occurs at a surprisingly low average frequency of events per year, based on recall of pain severe enough to warrant medical evaluation.[7] Hand-foot syndrome (dactylitis) occurs primarily under age 2 years, while long-bone pain is more common among older children and adults.[43,45] Acute chest syndrome in young patients can have an infectious component but becomes exacerbated by intrapulmonary sickling and vaso-occlusion.[46] Stroke is perhaps the most severe form of acute vaso-occlusion in children with SCA,[47] featuring abrupt interruption of cerebral blood flow, with consequent damage to both grey and white matter.

Among teens and adults with SCA, pain remains a serious and occasionally debilitating part of their disease. A recent multicenter study has documented that sickle-related pain, both acute and chronic, is typically under-reported and diminishes quality of life.[45,48] Acute chest syndrome from acute vaso-occlusion becomes more frequent and more dangerous, often escalating into a life-threatening process characterized by respiratory distress with hypoxia, acute pulmonary hypertension, multi-organ failure, and death.[46] Stroke occurs in adults but is more often hemorrhagic than vaso-occlusive in etiology.[47] In adulthood, chronic vaso-occlusion (or repeated acute events) becomes equally as important as the acute clinical events, with progressive damage to the kidneys, brain, eyes, bones, and other organs that ultimately leads to early mortality.[44]

Prediction

Perhaps the most characteristic feature of acute vaso-occlusion in SCA is its unpredictability of onset and severity. Often the acute episode arises from a relatively stable steady-state. Some painful events have recognizable physiological and environmental triggers, such as dehydration, change in temperature or weather, and infection, but for most patients and most events, there is no identifiable trigger at all. Patients with previous acute vaso-occlusive events tend to have more future events, but that observation is hardly useful for an individual patient. Owing to the remarkable diversity in how vaso-occlusion develops and presents, a better understanding about predicting vaso-occlusive events remains a worthwhile yet elusive goal.

Data from the prospective Cooperative Study of Sickle Cell Disease indicate that low HbF and elevated WBC are the two most important laboratory parameters that influence the number of acute vaso-occlusive events and overall clinical severity.[43-44,49] HbF presumably exerts its powerful influence through lowering intracellular HbS concentration and inhibiting polymerization; the simple phrase "more is better" summarizes the benefits of higher HbF levels. The elevated WBC count is presumably a result of inflammation and cellular damage, but these adhesive leukocytes can also contact endothelium and initiate vaso-occlusion.[50] For these reasons, the WBC should be considered "cause-and-effect" with regard to being a risk factor for acute vaso-occlusive events. It should be noted, however, that a recent large prospective cohort study failed to validate previously published predictors of vaso-occlusive events and clinical severity among children with SCA.[51]

Genetic modifiers have become popular as a potential explanation for the clinical diversity observed in SCA. Specific genetic polymorphisms outside of the beta-globin locus might represent independent risk factors for the development of acute vaso-occlusive events.[52] In most cases, the validity of these results has not been established using independent patient cohorts; furthermore, the clinical utility of such genetic modifiers remains unclear, as well. It is possible that well-validated genetic modifiers of acute vaso-occlusion might eventually lead to a more personalized treatment plan for certain individuals with these polymorphisms, specifically one that encourages early and preventive treatment.

Treatment

The development and use of hydroxyurea for the treatment of SCA should be considered the most important therapeutic advance to date. Hydroxyurea cannot be used as a specific or urgent treatment for an acute vaso-occlusive event, however, so it will be discussed in the section on Prevention.

The time-honored therapeutic interventions for acute painful vaso-occlusive events include hydration, analgesia, and anti-inflammatory medications. Increasing the circulating blood volume helps open blocked capillaries and assists erythrocytes back to oxygenation. Hydration can be provided either by oral or intravenous means, depending on the severity of the event and the patient's ability to tolerate oral fluid intake. Maintaining mild hyponatremia (e.g., serum sodium ~135 meq/mol) provides a slight osmotic gradient favoring water entry into the circulating erythrocytes, thereby lowering their intracellular HbS concentration, but greater degrees of hyponatremia should be avoided. When the vaso-occlusive event involves the lungs, care must be taken to avoid over-hydration that can worsen acute chest syndrome and lead to pulmonary edema and effusions.

Analgesia can be provided using non-narcotic or narcotic medications, depending on severity, previous use, and tolerance of narcotics. Many patients use a combination of non-narcotics and narcotics as out-patient therapy, but the latter category is indicated when patients are hospitalized. Intravenous narcotics such as morphine or hydromorphone are optimally delivered using patient-controlled analgesia methods, which allow continuous infusion with small boosts as needed for improved clinical outcomes.[53] Anti-inflammatory medications such as non-steroidal compounds are commonly used to reduce the clinical symptoms of acute vaso-occlusion. Corticosteroids are effective at reducing inflammation but are often associated with rebound symptoms,[54-55] so are no longer routinely used in the setting of acute vaso-occlusion.

Additional commonly used interventions include incentive spriometry, heat, physiotherapy, or other forms of supportive comfort measures. Together these interventions provide some symptomatic relief but do little to alter the timing or natural course of the vaso-occlusive event. It should be noted that supplemental oxygen is not indicated for painful events unless there is concomitant hypoxemia; this common treatment measure is not warranted and could theoretically reduce the erythropoietic drive.[56-57] Similarly, blood transfusions are not helpful to relieve pain associated with acute vaso-occlusion.

More specific and even targeted therapy should be possible for the treatment of acute vaso-occlusive events, based on our current knowledge of the pathophysiological processes involved (Table 1). However, most investigations to date have been limited due to a relative paucity of testable compounds, lack of a robust clinical research infrastructure, and poor clinical enrollment. Especially for industry-sponsored trials, unrealistic demands for single-agent success have limited efforts to build an armamentarium of treatment options. For

Table 1. Pathophysiological processes involved in the vaso-occlusive event of SCA that might offer opportunities for non-specific or even targeted therapeutic intervention.

Blood Cells	Plasma	Endothelium
Sickling	Adhesion	Adhesion
Adhesion	Inflammation	Inflammation
Hypoxemia	Hypercoagulability	Vasculopathy
Oxidative Stress	Hemolysis	Reperfusion Injury
Hemolysis		Activation

example, improving blood viscosity could theoretically help alleviate acute vaso-occlusion, and published industry-sponsored Phase 1/2 results with polaxmer-188 (Flocor) were encouraging,[58] but no further Phase 3 investigation ensued. Similarly, a novel Gardos channel blocker that improves erythrocyte hydration showed promising cellular effects in a Phase 2 trial,[59] but the industry-sponsored Phase 3 trial was halted when it did not significantly reduce pain when tested as a single treatment modality. An industry-sponsored trial using inhaled nitric oxide was closed prematurely without release of results to date. Without industry backing, however, agents that are off-patent can suffer from lack of sponsorship and interest.

Therapeutic interventions directed against the adhesive properties on circulating leukocytes, endothelial cells, and microparticles offer an exciting new opportunity to treat acute vaso-occlusion. General inhibitors of adhesion such as intravenous immunoglobulin show efficacy in an animal model of SCA,[60] suggesting that interruption of cell-endothelial contacts may be therapeutic. Specific inhibitors of individual adhesion molecules also show promise,[61-62] especially a novel "panselectin" compound that should theoretically provide broad yet specific inhibition of leukocytes and other circulating cells.[63]

Targeted therapy for the endothelium itself reflects the observation that the chronic inflammatory vasculopathy triggers and participates in acute vaso-occlusion. Treatment to reduce the activation state of endothelial cells[24,64] has shown promise in animal models, and direct delivery of nitric oxide could restore vascular tone to help prevent vaso-occlusion.[65] Indeed, treatment of vaso-occlusion may require treatment for the endothelium as well as the erythrocyte,[66] since both are important in the pathophysiology. The idea of multimodal therapy for SCA has been proposed,[67] much like that often required for optimal therapeutic outcomes of treatment for infections or malignancies.

Open clinical trials

The large number of currently open therapeutic clinical trials for acute vaso-occlusion suggests that the treatment armamentarium may improve in the near future. A search for "sickle cell crisis" on the ClinicalTrials.gov website in January 2011 identified a total of 15 open therapeutic clinical trials (Table 2). A variety of Phase 1, 2, and 3 trials are currently enrolling subjects. Treatments range widely from specific (e.g., Adenosine 2A agonist, GMI-1070 anti-panselectin compound, eptifibatide, L-glutamine) to non-specific (e.g., IVIG, fish oil capsules) interventions. Some trials are being conducted in the US, while others originate from Europe; some are industry-sponsored, while others have governmental funding.

The ClinicalTrials.gov website also lists a large number of completed therapeutic trials for acute vaso-occlusion in SCA. While most results have not been provided on the website to date, interventions with widely diverse agents such as fructose, corticosteroids, ketamine, ketorolac, sodium nitrite, aspirin, magnesium pidolate, nitroglycerin, sildenafil, poloxamer 188, stem cell factor, varespladib, nitric oxide, ICA-17043 channel blocker, and far red infrared radiation have all been completed over the past few years.

Prevention

In light of the severe clinical consequences and morbidity associated with vaso-occlusion, as well as the chronic inflammatory state with endothelial dysfunction that develops as a result of repeated vaso-occlusion, it is perhaps obvious that prevention of the process would be preferable to acute intervention. Toward that goal, hydroxyurea is now a well-established oral agent with proven laboratory and clinical efficacy for SCA, ranging from infants to children to teens to adults.[68-71] The primary benefit of hydroxyurea derives from its

Table 2. Active registered clinical trials involving therapeutic intervention for patients with SCA and vaso-occlusive events, typically designed to treat acute pain in the emergency or inpatient setting. All trials registered at clinicaltrials.gov with open recruitment as of January 2011 are listed, along with two studies scheduled to open recruitment soon.

Clinical Trials #	Intervention	Phase	Current Status
NCT01085201	Adenosine 2A agonist	1	Recruiting
NCT01256281	Femoral nerve block	1	Recruiting
NCT00644865	IV immunoglobulin vs placebo	1,2	Recruiting
NCT01033227	Sodium nitrite, open label	1,2	Recruiting
NCT00834899	Eptifibratide vs placebo	1,2	Recruiting
NCT00142051	Inhaled nitric oxide vs placebo	2	Recruiting
NCT01119833	GMI-1070 (anti-panselectin)	2	Recruiting
NCT01197417	Magnesium sulfate vs placebo	2,3	Recruiting
NCT00748423	Inhaled nitric oxide vs placebo	2,3	Recruiting
NCT00999245	High vs Low demand narcotics	3	Recruiting
NCT00313963	Magnesium sulfate vs placebo	3	Recruiting
NCT01179217	L-glutamine vs placebo	3	Recruiting
NCT00874172	Nitrous oxide and nefopam vs usual	3	Recruiting
NCT01202812	Omega-3 fatty acids vs placebo	2	Recruiting soon
NCT00880373	Ibuprofen vs placebo	3	Recruiting soon

induction of HbF, yet it offers additional benefits through a wide range of mechanisms of action, including reduction in the number of circulating reticulocytes and leukocytes, improved erythrocyte shape and deformability, and even local NO production.[72]

Importantly for the pathogenesis of vaso-occlusion, the benefits of hydroxyurea in SCA have been reported for reducing adhesiveness of erythrocytes,[73-76] reticulocytes,[77] leukocytes,[78] and even endothelial cells.[79] Reduction in plasma endothelin-1, a marker of inflammation, has been measured,[80] as well as a number of cytokines, in a small number of patients.[81] While it is tempting to hypothesize that hydroxyurea has a broad salutary influence on adhesion and inflammation, a large prospective study is needed to test this formally. For example, a large panel of plasma inflammatory markers should be measured using newly available multiplex platforms, before any definitive conclusions can be reached about the impact of hydroxyurea therapy on the inflammatory pathophysiology of vaso-occlusion in SCA. A clearer understanding of these non-HbF effects of hydroxyurea will likely increase our enthusiasm for this drug and add to the argument that this potent once-daily oral agent should be offered to most, if not all, patients with SCA.[82]

Conclusions

The pathogenesis and pathophysiology of vaso-occlusion in SCA has been investigated for over 40 years, yet important questions still remain. With recent increased understanding of the complexity of vaso-occlusion, we now have opportunities for targeted therapies to interrupt the process; several key clinical trials with novel agents are currently underway. Ultimately, however, prevention of vaso-occlusion should be viewed as the optimal treatment outcome. Until a better agent or treatment regimen is developed, hydroxyurea should be offered more often and better utilized in this patient population.

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Treatment of viral infections after hematopoietic cell transplantation

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A B S T R A C T

Viral infections are important complications after hematopoietic cell transplantation. Progress has been made in the management of cytomegalovirus and other herpes viruses, as well as influenza. However, for many viral diseases, there are either no or only moderately active treatment options available. This review will provide an overview of currently available treatment options and define areas of need for future development of therapeutics.

Introduction

Viral infections are a major complication after hematopoietic cell transplantation (HCT). Patients are at risk for complications of viruses that reactivate from latency (e.g., herpes viruses), as well as those that are acquired exogenously (e.g., respiratory viruses). Major progress has been made in the management of cytomegalovirus, historically the most deadly of all viruses, but challenges remain with effective management of a large spectrum of viral disease. This review will summarize recent developments in the treatment of viral diseases. Prophylactic strategies are not reviewed here. The reader is also referred to published guidelines that have recently been endorsed by a wide range of international professional societies,[1-4] as well as recently published comprehensive reviews of management options for the most relevant viruses, including cytomegalovirus (CMV), Epstein–Barr Virus (EBV), influenza virus, adenovirus, and respiratory syncytial virus.[5-9] Due to a paucity of randomized trials, many treatment recommendations are based on expert opinion.

Adenoviruses

Adenovirus disease is an emerging problem in HCT recipients. Disease occurs mainly in patients with profoundly reduced T cell function (i.e., following *in vivo* or *ex vivo* T cell depletion) but sporadic cases in non-T cell depleted patients can occur.[10] The outcome is often fatal, especially in the presence of low lymphocyte counts.[10] Treatment options are very limited and consist presently of cidofovir and ribavirin. No randomized trial has been performed and neither drug is approved for this indication. Presently, cidofovir is considered the treatment of choice.[8] (Table 1). Two dose regimens are available, that is, 5 mg/kg once a week or 1 mg/kg three times per week.[11,12] The latter may be less toxic but does not cover CMV. Ribavirin treatment has shown variable results, possibly due to strain-specific susceptibilities. We occasionally use a trial of ribavirin in addition to cidofovir or by itself in salvage situations with close viral load monitoring. Reduction of immunosuppression should be attempted given the strong correlation of lymphocyte recovery with outcome;[8] however, this is often not feasible. A newer compound (i.e., CMX-001, Chimerix Inc.) with potentially higher activity, and an improved toxicity profile is presently under investigation.[13,14] Treatment with donor-derived adenovirus-specific T cells is being studied at several centers.[8]

Respiratory viruses

Respiratory syncytial virus RSV

RSV pneumonia carries a high mortality in highly immunosuppressed HCT recipients. However, recovery from RSV lower respiratory tract disease without specific treatment has also been reported.[15] Unfortunately, treatment recommendations are based almost entirely on non-randomized clinical trials. Only one small randomized trial has been conducted.[16] Thus, most of the recommendations here are based on our experience and some larger published retrospective series. Early treatment of RSV upper respiratory tract infection has been advocated by several experts,[1] especially in the presence of lymphopenia (which is a significant risk factor for progression) or in other high risk situations.[17] This strategy was moderately effective in one large retrospective review.[18]

The data suggest that treatment of early pneumonia (i.e., prior to mechanical ventilation) is associated with improved outcome.

Table 1. Overview of available treatment options for viral infections in HCT recipients*. Prophylactic indications are not included.

	Drugs	Clinical use in HCT recipients	Strength of Evidence	Comments
Adenovirus	Cidofovir, Ribavirin (systemic)	Adenovirus disease Adenovirus viremia: preemptive therapy	Case series	Cidofovir considered first-line drug by most experts; lower dose regimen of cidofovir (1 mg/kg 3x/week not active against CMV); combination of both drug used anecdotally in salvage situations; serial viral load measurements useful to monitor response; reduction of immunosuppression if feasible; T cell therapy being investigated
RSV	Ribavirin (aerosolized, systemic) RSV-specific polyclonal monoclonal antibodies (palivizumab) Pooled immunoglobulin	RSV pneumonia, preemptive therapy for URI Combination therapy (ribavirin and antibody formulations) for RSV pneumonia	Case series, cohort studies	Moderate evidence for use of ribavirin in combination with antibody preparations for pneumonia; RSV-specific preparations appear to be more effective; preemptive use of ribavirin for RSV URI advocated by some, especially in high risk situations (e.g. profound lymphopenia)
HMPV	Ribavirin, pooled immnunogloblin	No data	Case reports	Only in vitro evidence
Parainfluenzaviruses	Ribavirin, pooled immunoglobulin	Parainfluenzavirus pneumonia	Case series, small cohort studies	No apparent effect for pneumonia of either aerosolized ribavirin or the combination with immunoglobulin; some positive reports with oral ribavirin; preemptive therapy not tested; reduction of immunosuppression (reduction of steroid dose) may be effective (see text)
Influenzaviruses	M2-Inhibitors (Amantidine, rimantidine), neuraminidase inhibitors (oseltemavir, zanamivir)	Influenza upper and lower lower respiratory tract disease;	Cohort studies, randomized trials (different settings)	Neuraminidase inhibitors agents of choice; M2-Inhibitors only effective against Influenza A, most isolates now resistant; both groups of drugs extensively studied in immunocompetent subjects; neuraminidase inhibitors appear to have improved toxicity profile, although experience in HCT is limited; combination therapy may be more effective than monotherapy but no systematic evaluation available
Rhinovirus	No treatment available			Pleconaril is active but drrug is not approved
HSV	Acyclovir, valacyclovir, famciclovir	HSV disease	Randomized trials	Ganciclovir, foscarnet and cidofovir have activity as well but are not used first line due to toxicity
VZV	Acyclovir, valacyclovir, famciclovir	VZV disease	Randomized trials	Ganciclovir, foscarnet and cidofovir have activity as well but are not used first line due to toxicity
CMV	Ganciclovir, foscarnet, cidofovir	Preemptive therapy of pp65 antigenemia/DNAemia CMV disease	Randomized trials	Cidofovir not tested in randomized trials; T cell therapy being investigated for refractory disease
CMV (drug resistant)	Drug with different mechanism of action or higher doses of original drug ; leflunomide, maribivir ; CMX-001	Suspected and documented drug resistance	Case reports, case series	In general switch to another drug is recommended if possible; increased doses of the ganciclovir with G-CSF support may work but toxicity is a limitation; combination therapy may also work; reduction of immunosuppresssion is advised
EBV	Rituximab	Preemptive therapy of viremia; PTLD	Case series	No clear evidence that antiviral drugs have an effect; chemotherapy may be needed for PTLD; T cell therapy being investigated
HHV-6	Ganciclovir, valganciclovir, Foscarnet Cidofovir	HHV-6 CNS disease	Case series	Both foscarnet and ganciclovir have been used for the treatment of CNS disease; anti-HHV-6 activity does not appear to be HHV-6 variant-dependent; preemptive use for plasma viremia advocated by some in high-risk settings, but no systematic evaluation of this approach is available
HHV-7	Foscarnet, Cidofovir	No data	No data	Pathogenic role of HHV-7 poorly defined
HHV-8	Ganciclovir, valganciclovir	No data	No data	Role of HHV-8 as a pathogen after HCT poorly defined
BK virus	Supportive care (hydration, pain management, irrigation), cidofovir, leflunomide	Hemorrhagic cystitis, nephritis	Case series	Supportive care critical; low-dose of cidofovir has been used; no systematic evaluation of efficacy available; leflunomide has shown a reduction in viral load (no controls); combination therapy not studied; reduction of immunosuppresssion if feasible
JC virus	Cidofovir, risperidone, IL-2, cytarabine	Multifocal leukoencephalopathy	Case reports	Case studies only, no strong evidence that any of these treatments have an effect
Hepatitis B	Lamuvidine, adefovir, entecavir, HBIg	HBV DNA positive donors pre transplantation HBV DNA positive recipients following donor-derived infection HBV DNA positive recipients (infected before HCT)	Case series, small cohort studies	Choose HBV immune donor if available for recipients with evidence of HBV infection before HCT
Hepatitis C	Pegylated interferon-alpha plus ribavirin	HCV RNA positive donors before HCT Treatment of chronic hepatitis C late after HCT	Case series, small cohort studies	Usually no complications of hepatitis C early after HCT; pegylated interferons can cause cytopenias; interferon alpha may activate chronic GVHD
Parvovirus	Pooled immunoglobulin	Cytopenia, possibly neurologic disease	Case reports	Parvovirus as a cause of series disease recently implicated in other immunosuppressed populations; limited data after HCT

** For details of dosing, details of indications and possible adverse events and limitations the reader is referred to the original literature and peer-reviewed recommendations of professional societies (references are listed in the text).*

For RSV lower respiratory tract disease, the best outcomes have been reported with aerosolized ribavirin while systemic ribavirin alone does not seem to be effective;[19,20] better response rates with systemic ribavirin have been described for treatment of RSV URI; however, small samples sizes limit the strength of the data.[17,21] Whether concomitant intravenous immunoglobulin or RSV-specific immunoglobulin or palivizumab, an RSV-specific monoclonal antibody, is beneficial has not been studied in a randomized fashion. There are some suggestions from uncontrolled data that high-titer antibody preparations or palivizumab is associated with better outcomes than pooled immunoglobulin preparations;[22-24] however, the strength of the evidence is low. Overall, outcome results from different sites are highly variable due to a long list of factors that are likely to affect outcome, including the timing of initiation of therapy, the presence of lymphopenia at the time of diagnosis, presence of co-pathogens (e.g., invasive moulds, CMV), and the use of immunosuppressive agents.

Influenzaviruses

Influenza can cause series disease after HCT. Both seasonal and the recent pandemic H1N1 strain can lead to lower respiratory tract disease and death.[25] Fortunately, antiviral drugs are widely available and largely effective, although drug resistance is a potential problem and may differ between circulating strains.[26] Generally, all influenza cases, both upper and lower respiratory tract disease, should be immediately treated. There is evidence that early treatment is more effective but even treatment that is started after 2 days appears to be effective.[25,27] Treatment aspects have recently been reviewed by Casper *et al*.[6] Perhaps the most interesting question is whether combination therapy (e.g., a combination of neuraminidase inhibitors with M2 inhibitors and ribavirin, or other combinations) is beneficial in the immunocompromised patients. Also, the role of adjunctive use of corticosteroids has been raised by studies that suggested a potential beneficial effect in lower respiratory tract disease.[25,27] These studies were done retrospectively and examined steroids that were given for the treatment of graft versus host disease, however, the results are provocative and should trigger further investigations. Given the high virulence of the pandemic H1N1 strain, more aggressive approaches are urgently needed. Thus, randomized trials of combination therapy, higher dose regimens, and adjunctive use of corticosteroids are needed.

Parainfluenzaviruses PIV

Approximately 10% of patients acquire PIV after HCT.[28] PIV can cause fatal pneumonia and late airflow obstruction, especially following lower respiratory tract disease.[29] One study reported an association of high-dose systemic steroids with progression to lower respiratory tract disease.[30] Aerosolized ribavirin for lower respiratory tract disease was unsuccessful in one retrospective analysis.[30] Oral ribavirin has been used in small series for both upper and lower respiratory tract disease (Table 1).[31] Overall, the data are difficult to interpret due to the retrospective nature of the studies and the small sample sizes. However, we attempt to reduce imnunosuppression and provide oral ribavirin for lower respiratory tract disease.

Human metapneumovirus HMPV

HMPV occurs in up to 5% of HCT recipients and can cause serious lower respiratory tract disease.[32] There is no proven treatment for HMPV disease. Intravenous immunoglobulin effectively neutralizes HMPV *in vitro*, however, its effect *in vivo* is unknown.[33] Ribavirin is active *in vitro*;[33] however, whether this translates into therapeutic benefit in patients is unknown.

Human rhinoviruses HRhV

HRhV is the most common respiratory virus infection after HCT. Most infections are restricted to the upper respiratory tract but serious lower respiratory tract disease can occur occasionally.[34] No specific antiviral treatment exists.

Other respiratory viruses

Several new viruses have been discovered recently, including several coronaviruses, human bocavirus, and the polyomaviruses WU and KI. No clear evidence of disease association has been reported to date;[35] however, studies are ongoing to further examine possible disease associations of these viruses in the HCT setting. No specific antiviral treatment exists for these viruses.

Herpesviruses

Cytomegalovirus

CMV continues to be a challenge, although preemptive treatment strategies have been optimized over the past decade. A recent large multicenter randomized trial showed CMV disease rates of less than 3% with pp65 antigenemia and PCR-guided preemptive therapy during the first 100 days after HCT.[36] In the same trial, a novel drug, maribavir, failed to be effective in preventing CMV antigenemia or DNAemia at a dose of 100 mg twice daily. Treatment problems arise when the patient develops endorgan CMV disease, and include primary refractory disease (mainly with CMV pneumonia), drug resistance, and the inability to use available drugs due to renal insufficiency or neutropenia. A detailed description of current management options has recently been published.[5]

Drug resistance continues to be infrequent but may be a formidable management problem if it occurs. Resistance to ganciclovir is more commonly observed than that to other drugs due to the prominent role this drug plays in CMV management; however, resistance to foscarnet and cidofovir can occur as well. Typically, drug resistance occurs in a setting of prolonged and/or repeated antiviral drug exposure and incomplete suppression of viral load.[5] This scenario is most common in situations of severe immunosuppression and/or low antiviral dose regimens. Management includes molecular testing when resistance is suspected, switching to an alternative antiviral drug, and reduction of immunosuppression if possible. In case of ganciclovir resistance, dose increases with hematopoietic growth factor sup-

port have also been used.[5] Combination therapy has been used anecdotally but no clear assessment can be made of its efficacy.

Organ toxicity is another potential limitation to effective drug treatment. Ganciclovir is well known to cause neutropenia in approximately one-third of HCT recipients. Strategies that have been proposed include holding the drug (only recommended when viral load is completely suppressed), switching to foscarnet, or continuation of ganciclovir with concomitant use of hematopoietic growth factors. Hematopoietic growth factors generally lead to a faster recovery of neutrophil counts when used earlier during the course of neutropenia. We start growth factors when the absolute neutrophil count drops below 1000/mm^3 and start foscarnet concurrently if the patient is still viremic.[5,37] If a switch to foscarnet is not possible, for example, due to renal insufficiency, we would consider initiating growth factors even earlier while the patient is still receiving ganciclovir. It should be noted that none of these strategies have been tested in randomized trials.

Herpes simplex virus and varicella zoster virus

Treatment of HSV and VZV disease has become less common with effective prophylaxis available. Treatment of wild-type disease caused by HSV and VZV is with acyclovir or valacyclovir.[2] Disseminated VZV disease is a potentially life-threatening condition that should be treated aggressively with high-dose intravenous acyclovir. Abdominal VZV disease is a medical emergency. It can present without skin manifestations, is characterized by severe abdominal pain and rapidly rising transaminases, and may be associated with inappropriate antidiuretic hormone secretion.[38] Acyclovir prophylaxis at the appropriate dose and when given for prolonged time, effectively prevents both wild-type VZV and HSV disease; drug-resistant HSV disease was also reduced with this regimen.[39,40] Acyclovir resistant disease typically respond to foscarnet.[41] Cidofovir has been used in selected cases but no larger series have been reported.

EBV, Human herpes viruses 6, 7, and 8

EBV can cause viremia and lymphoproliferative disease (PTLD) in HCT recipients, mainly in patients with severe T cell immunosuppression. Screening for viremia and early treatment with rituximab appears to be beneficial in high-risk settings.[4,9] There is no evidence that antiviral treatment is effective in this setting. PTLD may also be treated with rituximab, but chemotherapy may also be required[9] (Table 1). EBV specific T cell therapy showed promising initial results and is presently being investigated at selected transplant centers.[9] HHV-6 disease can cause encephalitis, which occurs early after HCT in most cases.[42] Recently, HHV-6 has also been independently associated with delirium and neurocognitive decline after HCT.[43] Both ganciclovir and foscarnet have been used in clinical practice but no randomized trial exists (Table 1). Cidofovir also has activity *in vitro* against HHV-6 but larger series on its use in patients have not been reported. We treat HHV-6 disease with prolonged courses of induction dosing (i.e., 3 weeks) followed by maintenance dosing for at total of 4–6 week or until resolution of symptoms and suppression of viral load is achieved. Complications due to HHV-7 and 8 appear to be very rare in HCT recipients.

Polyomaviruses

BK virus

BK virus shedding is highly prevalent early after HCT. Some patients develop BK-associated hemorrhagic cystitis, which is usually associated with high viral load in both urine and blood.[44] BK virus nephritis has also been described. No proven treatment exists. Cidofovir has been used in both low-dose (0.5-0.10 mg/kg/week) and regular dose regimens (5 mg/kg/week) but no conclusive evidence exists that this treatment is superior to supportive care[45] (Table 1). Leflunomide has also been shown to reduce viral load in some studies but not in others,[46-48] however, no systematic controlled evaluation of its therapeutic effect (alone or in combination) has been published. Reduction of immunosuppression is generally recommended in kidney transplant recipients.[44] Whether this approach is effective in HCT recipients has not been systematically examined; however, it seems reasonable to attempt reduction if feasible.

JC virus

JC virus is the cause of progressive multi-focal leukoencephalopathy in immunosuppressed patients. No proven treatment exists. Cidofovir has activity *in vitro*, but a study in HIV infected individuals failed to show therapeutic benefit. No data exists in HCT recipients.[49] Drugs that have been in individual patients with variable success include IL-2, cytarabine, chlorpromazine, or the antipsychotic drugs ziprasidone, risperidone, and olanzapine.[50-52] CMX-001 also shows activity *in vitro*.

Hepatitis viruses

Treatment guidelines for hepatitis viruses are complex. Therefore, treatment should be coordinated with specialists in infectious diseases or hepatology who are familiar with the treatment of chronic viral liver disease.[4] The following summary includes selected aspects of viral hepatitis management from a well-referenced recent summary by an international expert panel that has been endorsed by several professional societies, including the American Society for Blood and Marrow Transplantation, the European Blood and Marrow Transplant group, the Infectious Disease Society of America, the US Center for Disease Control and Prevention among others[4] (Table 1).

Hepatitis B

Management of hepatitis B involves assessment of both the donor and recipient serostatus, often accompanied by viral load determinations, if the donor is HBV.[4] Recipients of HBV DNA positive transplant products should receive post transplant antiviral prophylaxis though 6 months after discontinuation of immunosuppression; if, at the time of harvest, both the donor and

the harvested cells are HBV DNA-negative, alanine aminotransferase (ALT) levels should be monitored monthly in the recipient for the first 6 months. If the ALT levels increase, the recipient should be tested for HBV DNA and treated if positive.

If the recipient has evidence of active HBV infection before HCT (DNA positive), antiviral treatment is recommended before transplantation; a donor with evidence of HBV immunity should be selected if possible.[4]

If the HCT recipient is serologically positive (anti-HBc and anti-HBs), the risk of HBV reactivation is considered low during chemotherapy/conditioning, but higher following prolonged treatment with prednisone for GVHD.[4] In these situations, serum ALT should be monitored, and if levels increase, HBV viral load should be tested and antiviral treatment should be given if HBV DNA load is detectable. Prophylactic antiviral treatment may also be considered for anti-HBc positive and anti-HBs positive recipients before, and for 1 to 6 months after, HCT.[4] Additionally, anti-HBs levels should be monitored every 3 months. Reduction in anti-HBs titer should prompt HBV DNA testing.[4] Patients who lose anti-HBs responses, but have no HBV DNA in the serum, should be vaccinated in an attempt to restore protective levels of anti-HBs.[4] Patients with positive HBV DNA assays should receive antiviral therapy.[4] The duration of antiviral treatment in this setting has not been studied, but a common practice is to continue therapy for at least 6 months following discontinuation of immune suppressive drugs.[4] Rebound HBV replication and clinical hepatitis may follow discontinuation of antiviral treatment and should be monitored by regularly (e.g., biweekly measurement of ALT and HBV DNA).[4]

HCT candidates with evidence of active HBV replication (HBsAg positive and/or HBV DNA positive) should undergo liver biopsy and receive antiviral therapy prior to conditioning, optimally for 3 to 6 months. After discontinuation of antiviral therapy, rebound HBV replication and fulminant hepatitis may occur; therefore, frequent monitoring of liver function and HBV DNA is suggested.[4]

HCT candidates who are positive for anti-HBc but negative for HBsAg and anti-Hbs should be tested for HBV DNA. If HBV DNA is undetectable, the patient should receive HBV vaccination as described before and proceed to HCT.[4] Subsequent monitoring and antiviral treatment should be performed as described for anti-HBc/anti-HBs-positive HCT candidates. If HBV DNA is positive, proceed to HCT with preemptive antiviral therapy as outlined above.[4]

Hepatitis C

Both donors and recipients should be tested for anti-HCV antibodies. Those found to be anti HCV-positive or at high risk for HCV infection should be tested for HCV-RNA. Stem cell donors with detectable RNA should be treated with combination therapy prior to stem cell harvest if feasible, preferably until clearance of viral load.[4]

HCT candidates with evidence of HCV infection should be assessed for evidence of chronic liver disease. A liver biopsy may be warranted in selected situations (iron overload, history of excessive alcohol intake, history of hepatitis C for more than 10 years, clinical evidence of chronic liver disease) to assess the risk of the conditioning regimen. Close discussion with a specialist is needed to decide on the optimal transplant conditioning and antiviral treatment regimens to minimize adverse outcomes.[4]

Hepatitis C infected recipients without chronic liver disease rarely have complications within the first few years after transplantation, but progression to cirrhosis is accelerated late after HCT.[4]

Antiviral treatment should be considered for all chronically infected patients. To qualify for antiviral treatment after HCT, the patient must be in complete remission from the original disease, be more than 2 years after HCT without evidence of either protracted acute GVHD or chronic GVHD, have been off immunosuppression for 6 months, and have normal blood counts and serum creatinine.[4] Treatment should consist of full-dose peginterferon and ribavirin.[4] Toxicities, such as cytopenias, can occur and may require alternative treatment regimens.[4]

Other viruses

Other viruses that can cause disease in HCT recipients include rotavirus, norwalkvirus, parvovirus, enterovirus, and papillomavirus. No specific antiviral therapy is available for these infections.

Summary and conclusion

Progress has been made over the past two decades in the management of some of the most significant viral infections after HCT such as CMV, hepatitis viruses, and influenza virus. However, more and improved treatment options are needed for drug-resistant CMV, adenoviruses, polyomaviruses and respiratory viruses. A persisting challenge is the relatively low frequency of these infections, which makes the conduct of randomized trials extremely difficult. Innovative ways are needed to overcome this difficulty, including the use of multicenter study groups to conduct randomized trials and to perform outcome research using internet-based case report forms to develop larger treatment cohorts that allow multivariable modeling.

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Vaccination and stem cell transplantation

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A B S T R A C T

The fundamental alterations to the immune system that accompany stem cell transplantation (SCT) represent a unique immunological situation presenting challenges and opportunities for successful vaccine development. Vaccines are applied in several distinct settings in SCT. Firstly, they are used to boost or restore immunity against infectious agents, lost or weakened during the transplant period. Secondly, they can be used to generate de novo immunity to infectious agents not previously encountered by transplant recipient or donor. Thirdly, antitumor vaccines are being explored for their ability to prevent relapse or progression of malignant disease. Here, we summarize the immunological changes that accompany various forms of SCT, with particular reference to the limitations placed on vaccination effectiveness, describe the standard approaches to vaccination, and lastly outline some of the exciting new applications of vaccine strategies against malignant diseases.

The immune system in the SCT recipient

Both autologous and allogeneic SCT involve a period of immune deficiency followed by a gradual reconstruction of the cellular and humoral immune system in the post-transplant period. The immunological changes that accompany SCT are reviewed in.[1,2] The immunosuppression that accompanies conditioning regimens causes a profound B and T cell lymphopenia, together with progressive hypogammaglobulinemia and a loss of recipient antigen-presenting cells (APC). Immune recovery is a slower process than hematological recovery. While there is some (protocol dependent) persistence of host B cells and T cells, immune recovery depends on the donor and comes from two sources – (i) the donor CD34 stem cells, which regenerate donor-derived APC, NK cells, and pre-thymic T cells repopulating the thymus and regenerating the peripheral lymphocyte compartment with host-tolerized naïve T cells; (ii) a library of long-lived memory T cells, which confer immunity to a wide range of infective agents, for example, to the cytomegalovirus (CMV) and Epstein–Barr virus (EBV). While even T cell depleted SCT appear to conserve enough residual memory T cells to transfer donor immunity, it should be remembered that umbilical cord blood (CB) SCT coming from an immunologically naïve donor is defective in immunity to a wide range of infectious agents. Lymphocyte recovery is driven by powerful lymphokines, notably IL-15 promoting proliferation of both T and NK cells. While absolute values of T cells approach normal within the first 3 months after SCT, the CD4 counts are much slower to recover, and global T cell function remains deficient for many months. Furthermore, the T cell repertoire, as measured by T cell receptor (TCR) V typing, remains incomplete for months. B lymphocytes and immunoglobulin levels also take 6–12 months to recover. Whether the donated graft is autologous or allogeneic, the slow recovery of T and B cell immune function renders the patient more at risk to infections of all types, as well as to reactivation of endogenous viruses.

Factors affecting recovery of immunity and infection risk after SCT

Many factors affect the speed and completeness of immune recovery and need to be considered when planning vaccination. While some individuals do show more rapid recovery, immune recovery can be delayed by so many factors that it is best to assume that vaccines cannot reliably be protective unless administered 12 months or more from transplant.[2,3]

The type of transplant

While autologous (A) SCT recipients have the potential advantage of immune recovery uncomplicated by graft-versus-host disease (GVHD), these individuals have typically received chemotherapy prior to transplant conditioning, which has compromised their immune status. Thus, ASCT recipients are considered equally at risk as allograft recipients, and it is appropriate to apply the same vaccination schedule common to both transplant types. T cell deplete transplants, and reduced intensity transplants have specific patterns of immune reconstitution but as a general rule, no SCT type reliably achieves full immune recovery before a year from transplant.[2-4]

The recipient

Many viruses, including CMV, BKV, HSV, and VZV, remain latent lifelong in tissues and can reactivate post SCT. Adult recipients tend to have accumulated a more extensive spread of resident viruses than pediatric recipients and consequently have more problems with viral reactivation. Asplenic patients are at much greater risk from capsulated bacteria after SCT.

The donor

Donors already exposed to infectious agents and immune to them provide functioning immune memory to the recipient, which can, in the case of many DNA viruses, be lifelong. Recipients of grafts from donors, including CB donors who have no immunity (to CMV for example) have much greater risk of life-threatening complications from CMV reactivation until the delayed emergence of donor-derived immunity to the virus.[5] Such observations support the strategy of vaccinating the donor prior to transplant in order to transfer immunity with the graft, and this has been used in patients who are Hep B positive. In the case of EBV, Hep A/B and to a lesser extent CMV, the donor may also be a source of infective agent.

Post transplant events and their treatment

Acute and chronic GVHD, and the associated immune suppression used to control these alloresponses delay full immune recovery and extend the risk period for overwhelming infection post SCT.[2–3] Rituximab, used to treat cGVHD and lymphoma, significantly prolongs B cell recovery and antibody production.[6]

What post transplant infectious risks can be prevented by vaccination?

There are some specific risk factors for infections where vaccination can be protective after SCT (Table 1). Vaccination represents only one component of the strategies we use to prevent or treat infection in SCT recipients (Figure 1). The use of vaccines in the context of SCT has important limitations. Firstly, vaccines have been engineered to prevent infections in healthy individuals who are immune competent and can make antibodies to the infectious agent. After transplant, immune function remains compromised for many months, limiting the efficacy of vaccination. Secondly, vaccines have been engineered to generate antibody responses, and a large group of infectious complications post SCT (notably DNA viruses) is mainly controlled by cell mediate immunity, where commercial vaccines have no established role. Lastly, the situation after SCT is complex. Aside from new infections contracted after SCT, infections can be caused by reactivation of infectious agents persisting in the recipient, or by infection from the donor transplant. Thus, for a vaccine to be effective in SCT patients, it would have not only to provide protective levels of antibody to prevent new infections but also boost cell mediated immunity to prevent reactivation.

Table 1. Major infectious agent groups and vaccine options.

Category	Organism	Probability of infection	Vaccine available?	Recommended as routine
VPD*	MMR, DPT	(+)	+	+
	Influenza	++	+	+
	Polio	(+)	+	+
	Hep A/B	++	+	+
Encapsulated bacteria	Pneumo	+++	+	+
	HIB	+++	+	+
	Meningococci	(+)	+	
Persistent DNA viruses	HSV	+++		
	VZV	+++	+	+
	CMV	+++		
	EBV	+++		
	BK	+++		
	HPV	+++	+	+

** Vaccine preventable diseases.*

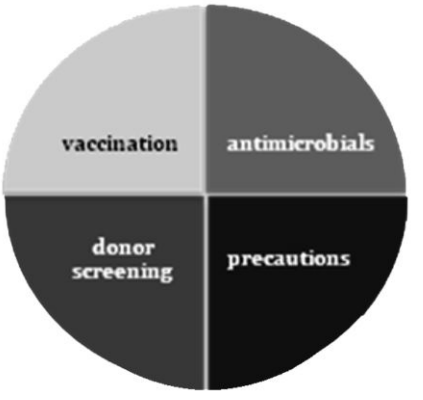

Figure 1. Vaccination is only one component of the strategies to prevent infection in SCT recipients.

Risks of vaccination

Some vaccines (e.g., BCG, Influenza, Polio, VZV, MMR, yellow fever) are available as live and attenuated forms of the infective agent. In individuals with intact immune function, they are safe and effective. The same vaccines in SCT recipients deficient in cellular and humoral immunity may result in disseminated and lethal infection. As a general principal, live vaccines are not given to patients after SCT. In many instances, effective non-living vaccine alternatives are available (Influenza, Polio, VZV). The exception is the combined vaccine for mumps, measles, and rubella (MMR). For this reason, patients are not revaccinated with MMR until 2 years post-transplant and only in the absence of active GVHD. While vaccines can occasionally cause allergic reactions, no data suggest that these adverse effects of vaccines are commoner in SCT recipients.

Immunity to vaccine preventable diseases

Infections usually prevented by vaccination in childhood

A large group of infections are currently prevented by successful vaccination of the healthy population. These

include the childhood infectious illnesses (measles, mumps, rubella, diphtheria) polio, pertussis, tetanus, hepatitis A and B, and influenza. Immunity to these agents is conferred by lifelong production of protective antibodies. Immunity to some agents like tetanus is not permanent and may require vaccine boosts. The rationale for vaccinating SCT recipients is the observation that in the decade after transplant, antibody levels to many of these agents decline without further boosting. Internationally agreed guidelines drawn up by European and American stem cell transplantation groups, the Infectious Disease Society of America and the Center for Disease Control recommend revaccination of all SCT recipients within 2 years of transplant with a full reimmunization schedule, as well as yearly influenza vaccine.[7] While this is a very reasonable strategy, it is of interest to note that the risk of developing new infections from childhood exanthems appears to be extremely rare in post SCT recipients even when not vaccinated.[3] However, it is anticipated that as more transplants using immunologically naïve umbilical cord blood are performed, the risk of new infection from some of these agents will increase.

Influenza virus

SCT recipients remain at particular risk from severe and overwhelming infection with influenza virus for many months after SCT. The variability in influenza strains requires yearly administration of vaccine for the prevalent strain and it is therefore appropriate to vaccinate all SCT recipients yearly (avoiding intranasal live virus vaccines) to prevent seasonal flu.[8] Patients undergoing all forms of SCT (autologous and allogeneic) are at risk from influenza and may not mount functional protective immunity when vaccinated after transplant.[9]

The recent outbreak of new pandemic strain A/H1N1 stressed the importance of having strategies in place for management of these patients. When the new pandemic H1N1 strain spread rapidly around the world, new vaccines were rapidly developed. Very little was known about the safety, immunogenicity, and optimal dosing regimen of 2009 H1N1 vaccine in the immunocompromised host, although several investigators reported efficacy of single dosing in healthy adults and children.[10–13] A number of studies, including work by our group, demonstrated that vaccination against 2009 pandemic H1N1 is associated with an acceptable safety profile in SCT recipients.[14–16] We found that the immune response in allogeneic SCT recipients can be substantially improved by a second dose of vaccine, supporting the European Medicines Agency and the UK DoH guidelines for the administration of two vaccine doses in this group of patients.[14] These data may also apply to vaccination against other viral agents in the immunocompromised host and warrant further studies.

Hepatitis B

Whether transmitted from donor to recipient, reactivating in the recipient, or occurring de-novo, represents an important post-transplant problem because of the propensity of Hep B to cause liver damage. Patients who are Hep B surface and core antigen positive require lamivudine antiviral treatment post SCT. Negative seroconversion – the loss of protective antibody to Hep B – is an indication to revaccinate.[17,18]

Tetanus

Antibodies to tetanus fall after SCT. Revaccination 1-year post SCT is partially effective but immunity may decline within a year.[19]

Immunity to capsulated bacteria[20,21]

Since immunity to capsulated bacteria *pneumococcus*, *haemophilus influenzae type B* (HIB) and to a lesser extent, *meningococcus* depends on circulating antibodies, SCT recipients with defective antibody production are particularly at risk from these infections. Notably patients who have been splenectomized and those with chronic GVHD are especially vulnerable. The problem with pneumococcal vaccines has been the incomplete coverage of all the common antigenic strains of the bacteria. However, the replacement of the earlier 7-valent vaccine (PCV7; Prevnar) by a 13-valent vaccine (Prevnar13), (both Pfizer Inc) is likely to provide more effective protection against pneumococcus in the SCT recipients. Despite the lack of data, the 13-valent vaccine is recommended for use in SCT patients until additional studies have been performed. A clinical trial to assess the efficacy of this 13-valent vaccine in allogeneic SCT recipients is ongoing.

Immunity to DNA viruses after SCT

The DNA viruses CMV, HSV, VZV, EBV, Adenovirus (ADV) BKV, and human papillomavirus (HPV), represent a group of viruses that can reside in the recipient lifelong after primary infection. These viruses generate protective immunity in the immune competent host but can reactivate if immune function falls off, as occurs after SCT. The reactivation of these viruses in an immune compromised host is potentially life-threatening. Unlike the previous categories of infectious agents, immunity is largely cell mediated, and commercial vaccines have not been developed against these viruses. Fortunately, the antiviral agents, acyclovir, ganciclovir, foscarnet, and cidofovir have activity against HSV, VZV, and CMV. Cidofovir has some efficacy against the BK virus and adenovirus. Furthermore, effective adoptive transfer of viral specific T cells can effectively control adenovirus, EBV, and CMV and rituximab can control EBV-related lymphoproliferative disease.[22–24] Two exceptions in this category are VZV and HPV, where effective vaccines exist and are being evaluated in SCT recipients.

VZV

Reactivation of VZV in the form of herpes zoster is a very frequent problem after SCT and while it can be suppressed with continuous treatment with acyclovir or valacyclovir, the virus typically reactivates promptly after discontinuation of antivirals.[25] A VZV vaccine represents a cheaper and more durable alternative to preventing zoster. However, vaccination of recipients with repeated doses of live, attenuated VZV vaccine is only partially protective.[26] An alternative approach is to vaccinate the donor. Limited studies suggest this approach may be at least partially protective in the recipient.[27] Two new inactivated varicella vaccines are under development.

HPV

Squamous cell cancers (SCC) involving the head and neck and cervix are increased after allogeneic SCT and in immunosuppressed individuals. Among SCT recipients, this risk is five times higher in patients with a history of chronic GVHD, and in a recent study, patients receiving immunosuppression for more than 3 years post SCT had the greatest risk of cervical dysplasia.[28] SCC are linked to HPV infection – especially HPV strains 16 and 18 – and HPV vaccination is now approved for prevention SCC and HPV warts in both healthy males and females. Vaccination results in over 99% seroconversion in healthy individuals and is around 95% in immunosuppressed individuals. These observations provide a strong rationale to give HPV vaccine to post transplant recipients to reduce the risk of SCC.[29] Several trials are currently evaluating the HPV strain 6,11,16,18 vaccine given in three doses starting 3–12 months post SCT.

Standard guidelines for vaccination

Collaboration between European, North, and South American groups has produced unified global guidelines for transplant centers.[3,7,30] Their recommendations are summarized (Table 2).

New developments

Improving post transplant immune competence

A clearer understanding of the dynamics of immune recovery post SCT and the cytokines that control lymphocyte recovery reveals several strategies that could be used to improve immune responses to vaccines. The first few weeks after SCT represent a period of powerful lymphoproliferative stimuli driven by IL-15 and IL-7 in particular, and promoting T cell recovery. Lymphocytes stimulated by vaccine at this stage post SCT expand preferentially. It may, therefore, be possible to rapidly induce immunity to infectious agents by early vaccination after SCT. Vaccine responses might be further enhanced by treatment with IL-15 or IL-7, which will soon be available for clinical trials. Another strategy being explored is the use of denileukin difitoxin (Ontak ©), which targets the CD25 IL-2 receptor and can be used to block CD4+ CD25+ FoxP3+ regulatory T cells to enhance antigen-specific proliferation.[31] However, immunostimulatory strategies will need to be applied cautiously in allogeneic SCT because of the risk of inducing GVHD.

Vaccinating the patient pre-transplant

Patients with multiple myeloma are severely immune-compromised and fail to make antibody responses to common vaccines.[32] Autologous SCT only worsens the patient's already compromised immune system and increases the risk of post transplant infection. Influenza can be fatal in these patients, and post transplant vaccination is not always protective. A recent study showed that immunity to influenza in myeloma ASCT recipients can be significantly improved by adoptive transfer of T cells, previously stimulated by pre-transplantation multivalent flu vaccine, followed by a vaccine boost 2 weeks later.[33] This strategy of combining pre-and post transplant vaccination boosting with reinfusion of vaccine-primed lymphocytes could be more generally applied to boost immunity to a variety of infective agents or tumor antigens before autologous SCT.

Tumor vaccines

As our ability improves to control infectious problems and other transplant-related complications after SCT, the problem of relapse of malignant disease becomes an increasingly significant cause of post transplant treatment failure. Lessons learned in protecting patients with vaccines against infectious agents have relevance for developing effective tumor vaccines. There is currently only limited experience combining anti-tumor vaccines with SCT but combination of leukemia-specific vaccines with SCT promises to increase the immune competence of the graft to control and eradicate residual malignant disease.[34]

Table 2. Post transplant vaccine schedule drawn up by an international consortium (adapted from[3,7]).

Vaccine	Recommendation*	Time post-SCT	Doses	Comments
Non living vaccines				
Pneumococcal conjugate	+	3–6 months	3-4	
Tetanus Diphtheria Pertussis	+	6–12 months	3	D T response improved if donor is vaccinated
H Influenzae conjugate	+	6–12 months	3	
Meningococcal conjugate	varies by region	6–12 months	1	
Inactivated polio	varies by region	6–12 months	3	
Recombinant Hep B	varies by region	6–12 months	3	
Inactivated influenza	+	yearly	1-2	
Live vaccines				
Measles	seronegative adults	24 months	1-2	given together -
Mumps	+			avoid if active
Rubella	+			GVHD

**Depends on national guidelines.*

Summary

Patients undergoing hematopoietic stem cell transplantation lose immune memory of exposure to infectious agents and vaccines accumulated through a lifetime, and are therefore advised to undergo re-immunization. Internationally agreed guidelines on vaccination have been drawn up by European and American stem cell transplantation groups, the Infectious Disease Society of America and the Center for Disease Control. However, a number of variables may affect the recipient's response to the vaccine such as type of transplant, stem cell source, conditioning, the use of T-cell depletion and donor immunization and need to be considered when planning vaccination. With our increasing understanding of immune recovery and factors that determine a successful vaccine response, it is likely that a constant update of current recommendations are needed.

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Late complications after hematopoietic stem cell transplant in adult patients

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A B S T R A C T

More than 40,000 HSCT are performed yearly worldwide. Therefore, the number of long-term survivors, free of the disease is continuously increasing. Despite improved prognosis, long-term outcome may be impaired by transplant associated morbidity and mortality. Long-term survivors can present a variety of malignant and non-malignant complications, impairing physical and psychological performance, normal integration in family and social life, and quality of life. Conditioning regimen and chronic graft-versus-host disease are key risk factors in the development of late effects. With increasing follow-up new types of late effects have emerged. Awareness on long-term effects after HSCT is crucial to provide adapted pre-transplant counseling, and recommendations for post-transplant screening, prevention and early treatment.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for defined malignant and non-malignant hematological disorders.[1] Overall survival has improved substantially, and the number of long term survivors is continuously increasing.[2] Thus, the general health status and quality of life in the long-term has become a major issue in the 21st century. Still, HSCT remains associated with late treatment-related morbidity and mortality. Compared to general population, late mortality is increased after HSCT.[3,4] Complications can appear within the first year, and other events will become apparent only years or even decades after HSCT. Late complications can be separated into delayed events, late events, and very late events.[5] Most of current literature is orientated toward late effect after allogeneic HSCT and very few involved patients after autologous HSCT only. Only main complications will be summarized here and limited to adulthood; readers can find extended reviews elsewhere.[6] Main key issues in late effects after HSCT are summarized (Table 1).

Non-malignant late effects

Non-malignant late effects are heterogeneous in their presentation and clinical manifestation. Any organ can be the target, and frequently multiple causes are involved.[7]

Chronic Graft-versus-Host disease

Chronic GVHD is the most common and clinically significant problem affecting long-term survivors. Up to 60% of patients receiving HLA-identical sibling grafts and 70% of those receiving alternative donor develop chronic GVHD. Chronic GVHD is the leading cause of non-relapse mortality more than 2 years after allogeneic transplantation.[8] Chronic GVHD is associated with decreased quality of life, impaired functional status, and ongoing need for immunosuppressive medications.[7] The incidence of chronic GVHD is increasing because of increasing use of alternative donors, older recipient age, and use of peripheral blood cells as the graft source. Even if reduced intensity conditioning seems to decrease the incidence and severity of acute GVHD, there is little evidence that chronic GVHD is reduced in this setting.[2,9]

Many excellent reviews have been published recently on chronic GVHD and its clinical consequences.[10–13] This review focuses on late effects, which arise predominantly as a result of chronic GVHD and its treatment.[14] Often it is not possible to determine whether chronic GVHD itself or its treatment is responsible for complication.

Chronic GVHD causes profound immune dysfunction,[13] and most chronic GVHD deaths are attributable to infection. Defects in mucosal integrity, immunosuppressive medications, and reduced number and function of mature T and B cells contribute to the high fatality rate from bacterial, fungal, and viral pathogens. Functional asplenia with an increased susceptibility to encapsulated bacteria, particularly pneumococcal infection, is common. According to the European guidelines, vaccination against Streptococcus pneumonia and Haemophilus Influenza are strongly recommended and vaccination against Varicella Zoster can increase survival.[15] Patients are also at high risk for Pneumocystis carinii pneumonia.

Although classical, these late infections have been poorly reported in the literature.

Table 1. Key issues.

- Due to the increasing number of long-term survivors after HSCT, the health status in the long-term is becoming a major issue
- Long-term survivorship includes malignant and non-malignant late effects, physical and psychological performance, social integration, sexuality, and quality of life
- Each type of post-transplant late effect has its own characteristics in respect of time of appearance, clinical presentation, prognosis and main risk factors involved
- The conditioning regimen and graft-versus-host disease play a key role in the development of late effects
- In very long-term survivors, new type of late effects may emerge
- Pre-transplant counseling on potential late effects is mandatory, and discussion on the currently available methods to preserve fertility has to be addressed.
- Regular screening allows early detection of late effects, and initiation of adequate preventive and therapeutic measures
- Counseling on self examination for early cancer detection, healthy life style, and social reinsertion is an important component of the follow-up of the long-term aftercare
- HSCT is a life-long commitment between the long-term survivor and the transplant team

In a retrospective study of the Hospital Saint Louis, Paris, 196 long term survivors were included.[16] Median follow-up was 8 years. Thirty patients died after the first year, with infections being one of the leading causes. Late severe bacterial and fungal infections occurred in 30 and 8 patients, yielding to an 8-year cumulative incidence of 15 and 4%, respectively. The majority of viral infections were hepatitis C and Varicella-zoster virus (8-year cumulative incidence: 10 and 27%, respectively). Three risk factors for late severe bacterial infections were CMV status (positive recipient, negative donor), irradiation-based conditioning regimen, and extensive chronic GVHD. Extensive chronic GVHD was the only risk factor for non-HCV viral infections. The Karolinska group in Sweden reported 44 patients dying from an infectious complication from a cohort of 688 consecutive patients surviving more than 6 months without relapse.[17]

Endocrine complications

Endocrine dysfunctions after HSCT are frequent and may be related to the underlying disease, the primary treatment, and the conditioning regimen used for HSCT. These deficiencies are either primary, involving the endocrine organ, or secondary/tertiary, involving the hypothalamic-pituitary axis. A comprehensive review on late endocrinal complications after HSCT in children has recently been published.[18]

Thyroid function

Thyroid dysfunction is classically a frequent late complication after HSCT (5–58% patients), the majority being diagnosed within the first 2–3 years after transplantation.[19] Total body irradiation (TBI) delivered as single-dose instead of fractionated TBI was a major risk factor. Today, subclinical compensated hypothyroidism with elevated TSH- and normal fT4-levels is the most common form of thyroid dysfunction and occurs in 7 to 37%. In a subgroup of these patients, TSH will normalize spontaneously. Early treatment with L-thyroxin is therefore controversial.

Fertility and gonadal failure after HSCT

Gonadal damage by irradiation is dependent on age, dose, and fractionation schedule of TBI. Ovaries are more vulnerable to irradiation and chemotherapy than testes are.[20] Busulfan is one of the most gonadotoxic chemotherapeutic agents. The age at transplantation is of major significance: indeed the younger the age, the better the chances for gonadal recovery. In female patients, hypergonadotropic hypogonadism is almost the rule, with elevated serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In pre- and post-pubertal female recipients, conditioning with cyclophosphamide alone is usually associated with only minor effects on gonadal function and fertility, with a high probability of recovery.

Reduced intensity conditioning is expected to have a sparing effect on gonadal function and reduce the risk of infertility, particularly when HSCT is performed in childhood for non-malignant diseases. In adult patients, the situation is more complex. Indeed, HSCT with reduced intensity conditioning is often preferred for selected patients, those with older age and increased comorbidity at time of transplantation. So far, data on late effects and particularly on gonad function after reduced intensity conditioning are still lacking.

Up to 90% of adult female patients require sex-hormone replacement therapy after HSCT. This replacement therapy can be interrupted every 1–2 years to evaluate spontaneous recovery.[7,21] In male recipients, endocrine dysfunction of the testis is less pronounced. Testosterone levels are usually normal since the Leydig cells are more resistant to chemotherapy and irradiation than the Sertoli cells are. Sex-hormone replacement will not be necessary in most male patients despite reduced or absent spermatogenesis. The absence of spermatozoa in the semen is a common long-term sequel in male patients receiving chemotherapy and irradiation prior to HSCT and as pre-transplantation conditioning.[22] Azoospermia is less frequent in patients conditioned with Busulfan and cyclophosphamide (50%) and uncommon when treated with cyclophosphamide alone (10%).[23] However, with increasing follow-up time, even when conditioned with standard dose TBI, male recipients surviving more than 10 years, younger than 25 years at HSCT, and apparently without chronic GVHD have a reasonable likelihood of spermatogenesis.[24] Nevertheless, recovery of a normal spermatogenesis as defined by WHO guidelines remains unlikely. Reduced fertility due to low sperm count and poor sperm mortality can be circumvented by assisted reproduction. The question on semen cryopreservation prior to treatment should nevertheless always be addressed.

The definitive proof of recovered fertility would be successful pregnancy in females or fatherhood in males. A number of pregnancies following HSCT have been observed.[25,26] The Bone Marrow Transplant Survivor

Study used a mailed survey to assess the magnitude of compromise in reproductive function and investigate pregnancy outcomes in 619 women and partners of men treated with autologous (n = 241) or allogeneic (n = 378) HSCT. Thirty-four patients reported 54 pregnancies after HSCT, of which 46 resulted in live births. No conception was associated with older age at HSCT (≥30 years), female sex, and TBI. Miscarriage or stillbirth were not more frequent when compared with age-matched siblings.[25] These results are in contradiction with previous reported data showing an increase in spontaneous abortions and miscarriages, pre-term delivery, and low birth weight.

Patients must be informed of the potential fertility damage. Currently, available strategies to preserve fertility should be discussed, and if possible encouraged: cryopreservation of sperm, testes, oocytes, ovaries, and embryos. Female cancer survivors have limited clinical options for fertility preservation. Embryo banking is a proven method but requires both available sperm and several weeks of preparation. The oocyte banking has been hampered by poor oocyte survival, fertilization, and resulting pregnancy rates. Recently, however, there have been more encouraging reports of the outcomes of oocyte freezing. Ovarian tissue banking has been successful in restoring fertility in animal models and in at least one human case. National legislation can be an obstacle for application of some of these procedures. In male recipients, cryopreservation of spermatozoa is an established option to preserve sperm, and allows insemination after HSCT. Sperm cryopreservation should be encouraged.

Noninfectious respiratory tract complications

Late-onset noninfectious pulmonary complications involving both, the airway and lung parenchyma are frequent after HSCT, appearing usually between 3 months and 2 years post-transplant; however, functional consequences may persist for years. The most common delayed pulmonary complications include bronchiolitis obliterans (BO), bronchiolitis obliterans organizing pneumonia (BOOP), and idiopathic pneumonia syndrome (IPS).[27] Restrictive and obstructive flow patterns and gas transfer abnormalities are common after HSCT. Asymptomatic presentation with abnormal functional tests is observed in 20% of the cases. Strictly speaking most of these complications are not late events.

Cardiac and cardiovascular complications

Long-term survivors of HSCT are at risk for cardiac and cardiovascular late effects.[28] However, publications on long-term cardiac complications after HSCT are scarce. After autologous HSCT only 2.4%, and after allogeneic HSCT, 3% of the late deaths were due to cardiac toxicity.[4,29]

In a prospective multicenter EBMT study, cardiac function before and yearly up to 5 years of follow-up was evaluated in 119 children treated with allogeneic HSCT. The 5-year cumulative incidence of cardiac impairment was 26%. All children were asymptomatic. TBI alone and TBI together with pre-transplant anthracycline administration were significant risk factors for reduced cardiac function. So far, clinically relevant cardiac failure after HSCT appears to be rare but cardiac complications will occur as very late events.

For a long time, cardiovascular diseases were not considered to be related to HSCT. Only single cases of fatal stroke or coronary heart disease after HSCT have been reported. However, in retrospective single center study, including 265 long term survivors, the cumulative incidence of an arterial event, such as cerebro-vascular, coronary artery, and peripheral artery disease was 22% at 25 years after allogeneic HSCT.[30] It was higher after allogeneic than after autologous HSCT, supporting the hypothesis that the alloreactive transplant is involved in the atherosclerotic process. In a subsequent multicenter study of the EBMT, including 548 long-term survivors after allogeneic HSCT, the established cardiovascular risk factors (hypertension, dyslipidemia, diabetes, smoking, physical inactivity) were associated with higher risk of cardiovascular complications post-transplant.[31] It is not surprising that patients with cardiovascular risk factors develop cardiovascular diseases. However, there is now evidence that the risk of arterial hypertension,[32] impaired glucose tolerance, dyslipidemia, abdominal obesity, and the clustering of cardiovascular risk factors, called metabolic syndrome[33] is increased after allogeneic HSCT, even in patients off immunosuppressive treatment. The reason for the high prevalence of cardiovascular risk factors after allogeneic HSCT is not well understood. It could be the consequence of prolonged and intensified immunosuppressive treatment, post-transplant endocrine dysfunction or leptin resistance.[33]

Thus, the cardiovascular risk factors could be responsible for a premature atherosclerosis in transplanted patients who already present vascular endothelial injury due to the conditioning and endothelial GVHD. Regular screening for established cardiovascular risk factors should be included for all patients. Counseling for heart healthy life style (stop smoking, regular exercise, maintaining healthy weight, dietary counseling) and early treatment of cardiovascular risk factors have become an essential part of the long-term management.

Liver complications

The most common causes of hepatic dysfunction after HSCT are acute and chronic GVHD, veino-occlusive disease, iron overload, and viral infections.[34] In the same patient, several causes of liver disease might coexist.

During the first decade chronic HCV hepatitis is often asymptomatic with fluctuating transaminase levels. However, patients surviving more than 10 years after HSCT are at higher risk of chronic hepatitis, and for the development of earlier cirrhosis as compared with HCV infected patients without HSCT.[35] Among 3721 patients, 31 presented cirrhosis, 23 with portal hypertension and 1 with hepatocellular carcinoma. The hepatic disease was the cause of death in 13 of them. HCV infection was present in 81% of patients with cirrhosis compared to 45% of controls.

Liver disease is seen in 90% of patients with chronic GVHD. Liver GVHD typically presents as cholestasis. The decision to treat liver GVHD is based on the severity of the GVHD and the involvement of other organs.

Iron overload due to multiple transfusions and increased iron absorption are common in long-term survivors transplanted.[36] Iron overload can be assessed by measuring serum ferritin. MRI is a very sensitive, non

invasive method to assess iron overload. Despite a clear correlation existing between iron overload and persistent hepatic dysfunction, the clinical consequences of therapeutic iron depletion in transplant recipients have not been extensively evaluated.

Late complications of bones and joints

Avascular necrosis of bone AVN

The published incidence of AVN varies from 4% to over 10% in the largest series. The mean time from transplant to AVN is 18 months.[37–39] Pain is usually the first sign. Early diagnosis can rarely be made using standard radiography but MRI is the investigation of choice. The hip is affected in over 80% of cases with (bilateral involvement in 60% cases). Other locations include the knee, wrist, and ankle. Symptomatic relief of pain and measures to decrease the pressure on the affected joints are of value, but most adult patients with advanced damage will require surgery. The probability of total hip replacement following a diagnosis of AVN is approximately 80% at 5 years. While short-term results of joint surgery are excellent in the majority (>85%) of cases, long term follow-up of the prostheses is needed in young patients who have a long life expectancy. Studies evaluating risk factors for AVN have clearly identified steroids (total dose and duration) as the strongest risk factor. The second major risk factor is TBI, the highest risks being associated with receipt of single doses of 10 Gy or higher or more than 12 Gy in fractionated doses. In a recent series involving more than 1300 patients at the City of Hope, the cumulative incidence of AVN at 10 years was 4% after autologous HSCT, 6% after allogeneic sibling donor HSCT, and 15% after unrelated donor HSCT.[40]

Osteoporosis

HSCT can induce bone loss and osteoporosis via the toxic effects of TBI, chemotherapy, and hypogonadism.[41,42] Osteopenia and osteoporosis are both characterized by a reduced bone mass and increased susceptibility to bone fracture. The incidence and clinical course of bone density abnormalities following HSCT have been studied in two large series.[43,44] In both, the cumulative dose and number of days of glucocorticoid therapy and the number of days of cyclosporine or tacrolimus therapy showed significant associations with loss of bone density. Non-traumatic fractures occurred in 10% of patients. Using WHO criteria, nearly 50% of the patients have low bone density, a third have osteopenia, and roughly 10% have osteoporosis, 12–18 months post transplant. The true incidence and morbidity rate of osteoporosis in very long term HSCT survivors have recently been assessed in a prospective study of the Hospital St Louis (Paris) in 135 patients. The cumulative incidence of steroid-induced osteoporosis was 52% at 2 months (more frequent in men and in children) but only 30% showed osteoporosis at 1 year. Preventive measures of osteoporosis must include sex-hormone replacement in patients with gonadal failure; the efficacy of new treatments for osteoporosis in long-term survivors of HSCT still requires evaluation.

Ocular complications

The two most common late ocular complications after HSCT are cataract formation and sicca syndrome. Cataract formation is closely related to TBI.[45,46] The probability to develop cataracts is dependent on the total radiation dose, the number of radiation factions, and the dose rate. At 10 years, the cumulative incidence of cataract lies between 4–10% without TBI, 30–50% with fractionated TBI, and 60–100% with single dose TBI. In four randomized studies, the incidence of cataract was significantly higher for patients conditioned with cyclophosphamide and TBI as compared with those treated with cyclophosphamide and Busulfan.[47] The only treatment for cataracts is to surgically remove the lens. Today, cataract surgery is a low-risk procedure and improves visual acuity in more than 95%.

Keratoconjunctivitis sicca syndrome is usually part of the sicca syndrome, including xerostomia, genital involvement, and dryness of the skin. All manifestations are closely related with chronic GVHD,[11] which may lead in its more extensive form to a Sjögren-like syndrome. In a retrospective study of the EBMT, the actuarial probability to develop sicca syndrome in long-term survivors was 21% at 15 years, 38% for patients with, and 10% for patients without GVHD.[46] Dry eye is the most frequent clinical manifestation occurring in 40 to 76% of the patients. Other ocular manifestations include painful eyes, photophobia, and difficulty, and corneal ulceration. Medical therapy consists primarily of lubricants and anti-inflammatory agents. Preservative-free artificial tears are the mainstay of therapy. In refractory cases, topical corticosteroid or cyclosporine may be effective. Systemic therapy of GVHD has been shown to be effective, particularly when there are other GVHD manifestations.

Oral complications

Late oral complications are strongly associated with chronic GVHD[48] and conditioning with TBI. Painful mucosal ulcers hinder normal food ingestion. Saliva plays a major role in maintaining oral health. Abnormal salivary composition and reduced salivary flow can be the consequence of TBI and/or chronic GVHD.[49] In long-term survivors, salivary gland dysfunction is associated with increased risk of dental caries. A poor oral hygiene favored by oral pain is an additional predisposition to dental caries. Maintaining oral and dental health is critical. Basic oral care include brushing with soft toothbrush twice a day, the use of fluoride-containing toothpaste, daily flossing between teeth and under bridge, the use of remineralizing solutions, and avoidance of sugar containing brewages.

Malignant complications

It has been common to distinguish four types of malignant complications after allogeneic HSCT: late relapse of the primary disease, secondary leukemia, lymphomas and post-transplant lymphoproliferative disorders, and secondary solid tumors.

Secondary leukemia

Usually this refers to leukemia of donor cell origin. Although described more than 2 decades ago, most of

the first reports likely described relapse of the original leukemia because the "donor" origin of the leukemia was based on classic cytogenetics of leukemic cells in a transplant setting with donor/recipient sex-mismatch, in which the cell origin was demonstrated on sex chromosome. However, today it is recognized that loss of the Y chromosome and duplication of the X chromosome does occur in leukemic cells. Thus nowadays, only molecular proof of the donor origin can only be accepted using PCR of VNTRs as the most widely used tool. Using these stringent criteria, leukemias or malignancies of donor cell origin, although rare, do exist.[50] A further complexity came also recently from a study by the Seattle group showing that malignancies could also be transmitted through the graft either at a premalignant stage or as a minimal clone undetectable before transplantation.[51]

Lymphomas and post-transplant lymphoproliferative disorders

Patterns of post-transplant lymphoproliferative disorders (PTLD) have been recently assessed among 26,901 patients who underwent allogeneic HSCT. PTLD developed in 127 recipients, with 105 (83%) cases occurring within 1 year post-transplant. In multivariate analyses, PTLD risks were strongly associated with T-cell depletion of the donor marrow, ATG use, and unrelated or HLA mismatched grafts. Significant associations were also confirmed for acute and chronic GVHD. The increased risk associated with unrelated or HLA mismatched donors was limited to patients with T-cell depletion or ATG use.[52]

Today, prospective monitoring of EBV activation and early treatment intervention with Rituximab has dramatically changed these features. While risk factors may remain the same, the true incidence of EBV-reactivation and evolution to overt PTLDs remain essentially unknown. Furthermore, the potent iatrogenic consequence in term of infection due to long-lasting Rituximab-induced B-cell lymphopenia warrants further studies. Late-onset B-cell neoplasm and Hodgkin's disease have been described but remain exceptional.[53]

Solid tumors

Several studies[32,54–62] have reported that survivors of HSCT have an increased risk of developing new solid cancers with the risk rising among long-term survivors from 2% to 6% at 10 years after transplantation. Several factors contributed to this increase, including total body irradiation TBI, which has been a mainstay of the preparative regimens for allogeneic HCT until recently, primary disease, male sex, and pre-transplantation therapy. However, with longer follow-up, solid tumors after Busulfan cyclophosphamide have been recently reported (especially lung cancers).[2,63] Chronic GHVD and immunosuppressive therapy have also been shown to contribute to excess risk, particularly for squamous cell carcinomas of the buccal cavity and the skin.[58] Young age at transplantation has been reported to be a strong risk factor in some, but not all, previous studies.

The largest studies today included multi-institutional cohort of 28,874 allogeneic transplant recipients with 189 solid malignancies. Overall, patients developed new solid cancers at twice the rate expected based on general population rates (observed-to-expected ratio 2.1; 95% confidence interval 1.8–2.5), with the risk increasing over time; the risk reached three-fold among patients followed for 15 years or more after transplantation. New findings showed that the risk of developing a non-squamous cell carcinoma following conditioning radiation was highly dependent on age at exposure. Among patients irradiated at ages under 30 years, the relative risk of non-squamous cell carcinoma was nine times that of non irradiated patients, while the comparable risk for older patients was 1.1. Chronic GVHD disease and male sex were the main determinants for risk of squamous cell carcinoma.[64]

However, even in this largest cohort the very long-term survivors are still few and some solid tumor type might be under-estimated. This is especially true for breast carcinomas that tend to develop very late after transplantation. The EBMT and the Seattle group thus sought to determine the risk among 3337 female 5-year survivors who underwent an allogeneic transplantation.[59] Fifty-two survivors developed breast cancer at a median of 12.5 years following HSCT. Twenty-five-year cumulative incidence was 11.0%, higher among survivors who received TBI (17%) than those who did not receive TBI (3%). Hazard for death associated with breast cancer was 2.5.

Altogether, these data indicate that allogeneic transplant survivors face increased risks of solid cancers, supporting strategies to promote lifelong surveillance among these patients.

Quality of life, sexuality, and social integration

There are many studies available on the quality of life after HSCT but results are rather heterogeneous.[65–68] Quality of life is generally perceived as satisfactory to good in the majority of patients after HSCT. Whereas some studies support the observation that with time quality of life continues to improve others do not. Some patients with more advanced disease report better quality of life after transplant than patients with less advanced disease. While quality of life is subjective and reflects the patient view, health status is assessed by the physician. One of the major drivers of health status is chronic GVHD, and this observation may or may not be concordant with the patient's view.[43,69] Coping strategies of some patients with late complications, such as chronic GVHD, may provide them with the ability of perceiving quality of life as much better than their treating physician.[69,70]

Chronic fatigue in otherwise asymptomatic patients is a common complain after allogeneic and autologous HSCT. There is a great diversity in the prevalence reported, which may reflect sample heterogeneity, time of assessment since HSCT, type of treatment and population at risk. Behavioral interventions, as for instance, individually tailored aerobic exercises, are associated with sustained improvement of severe fatigue.

Several studies have addressed the issue of sexuality after HSCT and have in general found that libido was diminished, more so in women.[71] Sexual dysfunction may be the consequence of hormonal abnormalities, genital problems, and psychological stress. Some of these dysfunctions may be corrected by hormonal sub-

stitution but even with optimal substitution a negative impact generally persists. In female patients with chronic GVHD, pelvic examination is an important part of care. Both the patients and their partners require counseling to address marital difficulties as early as possible and to avoid additional trauma.

Most long term survivors (approximately 90%) are reported to have good to excellent performance status.[69] Social integration is related to family and friends and to the work/school environment.[66,69,70] According to the literature, approximately 60–90% of HSCT survivors return to work with higher rates in office and intellectual work than in a physically demanding environment.[66,69] There is a wide social gap according to prior social status. Available resources vary much between countries with very different social systems.

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Tissue factor pathway inhibitor

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A B S T R A C T

The tissue factor pathway inhibitor (TFPI) pathway represents an essential negative regulatory mechanism on thrombin generation in plasma. TFPI is a multimodular Kunitz-type inhibitor that first needs to interact with activated factor X before inhibiting the complex of tissue factor and factor VIIa, the physiological activator complex of blood coagulation. More recently, it has become clear that vitamin K-dependent protein S acts as a cofactor for TFPI in stimulating the initial interaction with factor Xa. TFPI and protein S form a complex in plasma, which likely stabilizes TFPI, and as a result, protein S and TFPI covariate in plasma in health and disease. As TFPI and protein S are constitutively active in plasma, an effective anticoagulant barrier against procoagulant activity is provided. Both protein S and TFPI deficiencies are associated with an increased risk for venous thrombosis, and part of the thrombotic risk associated with protein S deficiency might well be explained by accompanying low levels of TFPI.

Blood coagulation

Blood coagulation proceeds through a tightly regulated cascade system that is triggered by a small stimulus causing activation of a coagulation zymogen into an active enzyme by selective peptide bond cleavages. Subsequently, the activated enzyme with its designated cofactor will activate the next coagulation factor, which again assembles with a cofactor and activates the following coagulation factor and so on, until by sequential amplification at each step, a burst of thrombin is generated.

The initiation event occurs when tissue factor comes into contact with blood. Traces of activated factor VII (FVIIa) in blood plasma bind to tissue factor generating the extrinsic tenase complex (TF/FVIIa), which subsequently activates zymogens factor X (FX) and IX (FIX). FXa and its cofactor FVa then form the prothrombinase complex (FVa/FXa) that is the central prothrombin converting complex of the blood coagulation (Figure 1). The initial thrombin generation by prothrombinase, however, is not enough to polymerize fibrinogen effectively but governs major positive feedback reactions through activation of platelets and factors XI, VIII, and V. The activation of FXI and FVIII by thrombin together with FIX activation by FXIa and extrinsic tenase marks the propagation of coagulation, leading to additional prothrombinase and thrombin formation sufficient to generate a fibrin clot (Figure 1).

Natural anticoagulants: restoring the balance

Procoagulant responses are limited to the site of injury by the presence of several inhibitors and negative feedback systems. Inhibition of active coagulation enzymes by the serine protease inhibitors (serpins) antithrombin (AT),[1] heparin cofactor II,[2] and α_1-antitrypsin (α_1AT)[3] eliminates free enzymes from plasma, preventing downstream activation of coagulation (Figure 1). In addition, the tissue factor pathway inhibitor (TFPI) regulates the initiation of coagulation by inhibiting both factor Xa (FXa) and the phospholipid-bound complex of tissue factor and factor VIIa (TF-FVIIa).[4] Lastly, traces of thrombin bound to endothelial cell receptor thrombomodulin loose their procoagulant properties and can activate (endothelial cell protein C receptor-bound) protein C. Subsequently, APC will inactivate cofactors FVIIIa[5] and FVa[6] of blood coagulation with the help of its non-enzymatic cofactor protein S,[7] shutting down the intrinsic tenase complex and the prothrombinase complex, calling a halt to thrombin generation, and restoring the haemostatic balance.[8–10]

Tissue factor pathway inhibitor TFPI

TFPI is a 276 amino acid glycoprotein from the family of Kunitz-type inhibitors. It consists of a negatively charged N-terminus, three consecutive Kunitz-domains, and a positively charged C-terminal tail (Figure 2).[11] TFPI contains several N-linked and O-linked carbohydrate chains, which add approximately 10 kDa to the average amino acid backbone mass of 31,932 Da. TFPI is mainly synthesized in endothelial cells,[12] and each of its structural elements have separate functions in the mechanism of anticoagulant action of TFPI. It was reported that Ser2 in the positively-charged N-terminus is involved in phosphorylation of TFPI,[13] that the first Kunitz-domain binds to

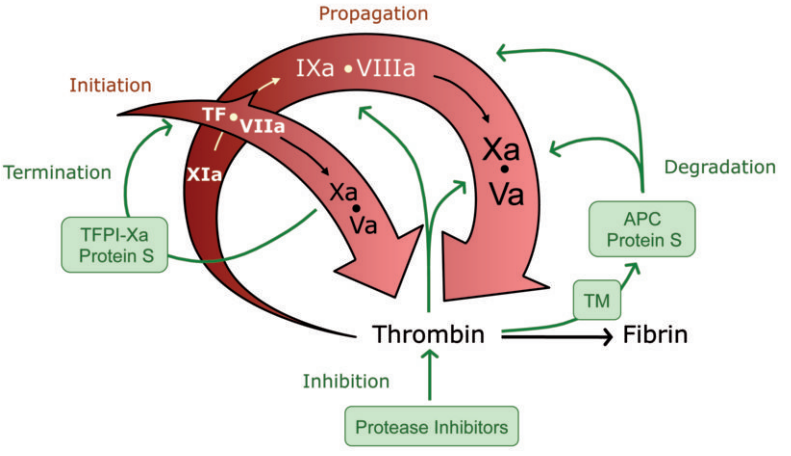


Figure 1. Blood coagulation and its regulation. *Initiation*: Clotting is initiated by a tissue factor exposed to plasma that subsequently recruits factor VIIa (VIIa) from the circulation. The TF/FVIIa complex activates factor X (Xa) and factor IX (IXa). Xa and factor Va form the prothrombinase complex that generates the first traces of thrombin. *Propagation*: A small amount of thrombin is not yet sufficient to generate fibrin but instead offers a positive feedback by activation of platelets, factors XI (XIa); more V (Va), and VIII (VIIIa). XIa generates more IXa that together with VIIIa, form the intrinsic tenase. The intrinsic tenase IXa/VIIIa generates more Xa, which together with Va, form more prothrombinase, resulting in a burst of thrombin generation and a subsequent fibrin clot. *Termination*: As soon as traces of Xa are generated, extrinsic initiator TF/VIIa is shut down by a TFPI/Xa/protein S-dependent process. As a result, clotting now becomes dependent on the propagation loop. *Inhibition*: Downstream enzyme activity is inhibited by protease inhibitors to prevent systemic coagulation activation. *Degradation*: Thrombin/thrombomodulin (TM) activates protein C that proteolytically inactivates Va and VIIIa in a protein S-dependent manner, restoring the hemostatic balance.

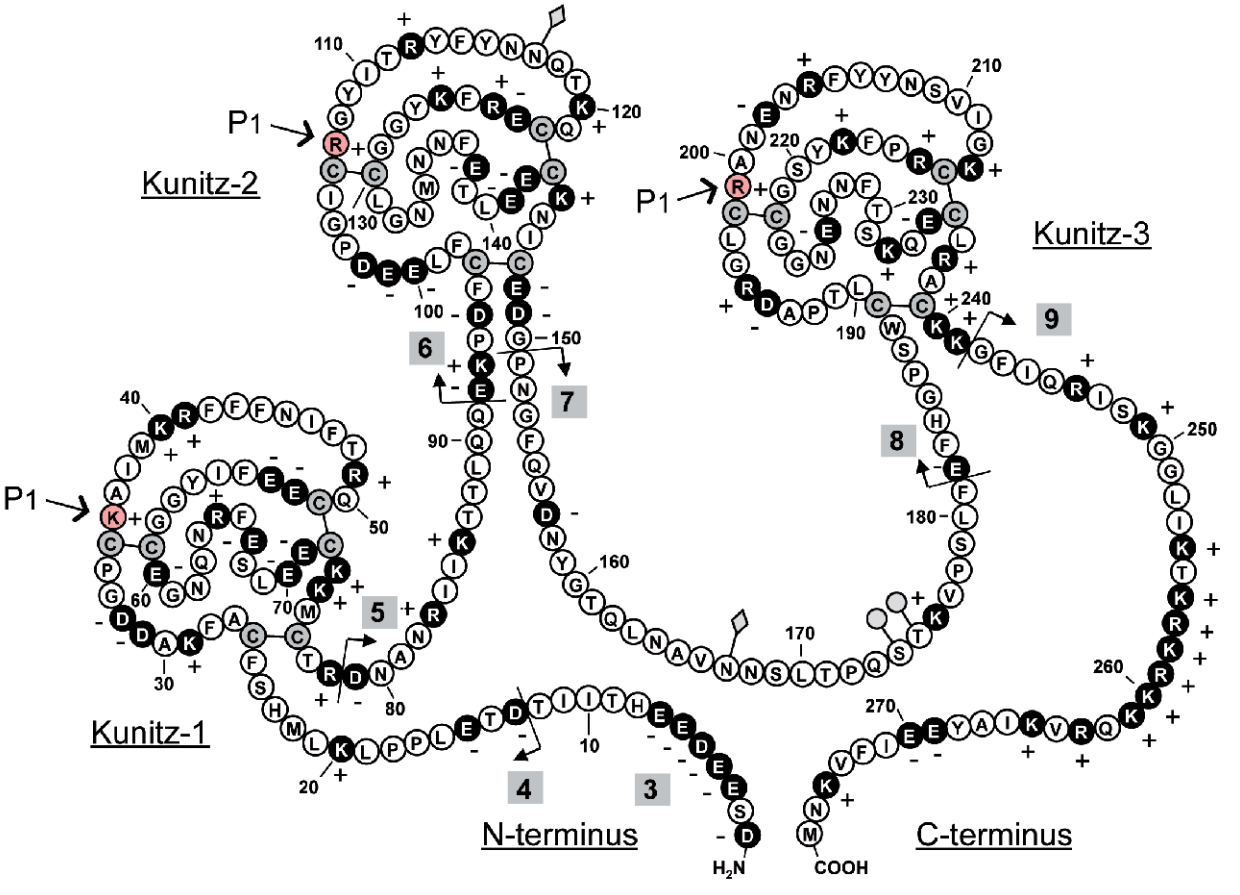


Figure 2. Primary structure of tissue factor pathway inhibitor (TFPI). TFPI is a 276-amino acid glycoprotein. Charged amino acids are indicated in black, P1 residues in red, cysteines in disulfide bonds in grey. Grey diamonds indicate N-linked glycosylation sites; grey circles indicate O-linked glycosylation sites. Backbone arrows and grey boxed numbers indicate the exon map. Adapted from Girard *et al.* Nature 1989, 338, 518-20.

and inhibits FVIIa, and that the second Kunitz-domain binds to and inhibits FXa.[4] The function of Kunitz-3 initially was less clear although it was reported that Kunitz-3 contained a heparin binding site[14] and that it was implicated in cell surface binding.[15] In addition, Kunitz-3 is involved in cross-disulfide linkages between various truncated forms of TFPI and low density lipoprotein.[16] More recently, however, it became apparent that Kunitz-3 is crucial for the TFPI-cofactor activity of protein S.[17,18] The basic C-terminal tail of TFPI was shown to interact with anionic membrane surfaces and heparin-like structures on the vessel wall.[14,19] The C-terminus of TFPI is required for optimal inhibition of FXa[4,20,21] and for the TFPI-cofactor activity of protein S[17] and therefore, full length (free) TFPI is now considered to be the only relevant anticoagulant TFPI form in the circulation.

TFPI: variants and distribution

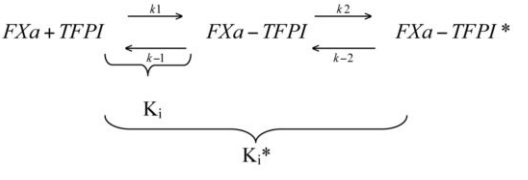
TFPI is produced and stored in endothelial cells, and on secretion, most of TFPI is bound to the endothelial cell surface through proteoglycans or GPI-linked proteins. In all, 80% of TFPI remains associated with the endothelium (Figure 1). The remainder of TFPI (20%) circulates in plasma at a concentration of approximately 2.5 nM.[22,23] The majority of circulating TFPI (70–80%) is truncated and bound to low-density lipoproteins through disulfide bonds with the Kunitz-3 domain.[16] Only 10% of plasma TFPI (2% of total TFPI: ~0.25 nM) circulates as free full length TFPI. (Figure 3). An alternatively spliced variant of TFPI (TFPIβ) has been identified, which lacks Kunitz-3 and the C-terminal domain but has a GPI-anchor by which it is directly bound to cell surfaces.[24,25] Administration of heparin releases the TFPI pool attached to endothelial cell surface proteoglycans, as well as intracellular stores of TFPI, resulting in a several-fold increase in plasma levels of full length TFPI.[26]

TFPI: mode of anticoagulant action

TFPI is a slow, tight binding inhibitor that regulates TF-activity through multiple mechanisms. TFPI inhibits TF/FVIIa via a two step feed-back mechanism, which involves formation of a bimolecular FXa/TFPI complex that subsequently interacts with TF/FVIIa, yielding an inactive quaternary complex and resulting in termination of TF/FVIIa-catalyzed FX activation.[4,27] In this negative feedback mechanism, the initial formation of a binary TFPI-FXa complex is a prerequisite for the final inhibition of TF/FVIIa by TFPI.[4] Alternatively, a more likely mechanism was proposed in which TFPI directly inhibits the trimolecular TF/FVIIa/FXa complex through primary Kunitz-2-FXa and secondary Kunitz-1-FVIIa interactions.[28]

The binding and inhibition of FXa by TFPI proceed through a two-step mechanism. First, a rapid encounter complex is being formed between FXa and Kunitz-2 of TFPI, in which the P1 residue Arg107 occupies the active site of FXa. Subsequently the encounter complex rearranges slowly into the tightly bound enzyme-inhibitor complex. During this process, the peptide bond between Arg107 and Gly108 is not hydrolyzed, making the final tight enzyme-inhibitor complex reversible.

During the first step of FXa inibition by TFPI, a small amount of loose complex between Kunitz-2 of TFPI and FXa is rapidly formed (*FXa-TFPI, equation a*), which results in quick inactivation of part of circulating FXa. During the second step, a slow rearrangement of this initial complex results in formation of the final tight FXa-TFPI*-complex[4] (*equation a*).



(*a*)

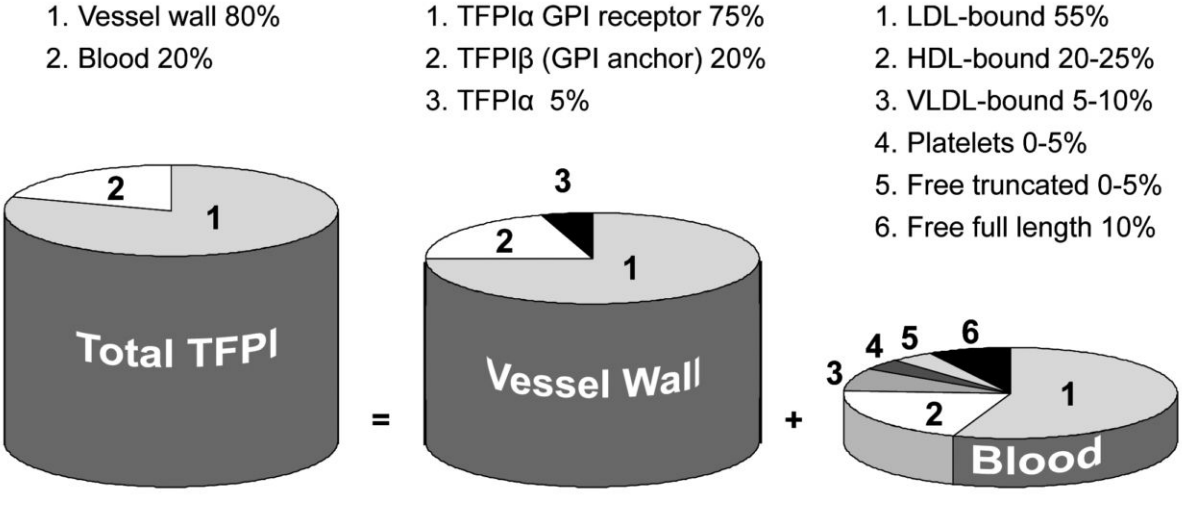


Figure 3. Distribution of TFPI variants and forms. From total TFPI, 80% is associated with endothelial cells and the vessel wall. Only 20% of TFPI circulates in plasma. From the circulating TFPI, 10% (2% of total TFPI) represents full length TFPI, the active anticoagulant form in plasma.

The dissociation constant of the first rapid equilibrium is represented by K_i ($K_i=k_{-1}/k_1$ = [FXa].[TFPI] / [FXa-TFPI]) and the overall equilibrium constant after the slow summarization is represented by K_i^* (K_i^* = [FXa].[TFPI] / {[FXa-TFPI] + [FXa-TFPI*]}). The Ki* for the final tight complex (0.05–0.07 nM) is several orders of magnitude lower than K_i for the initial encounter complex (5–15 nM).[17,29]

The fact that TFPI is a slow inhibitor of FXa has important implications for the down-regulation of the TF pathway by TFPI in plasma. For instance, it was observed that thrombin generation initiated by relatively high amounts of TF (14 pM) could not be effectively inhibited by TFPI. Only when TF concentrations went down to approximately 1 pM, efficient down-regulation of procoagulant response by TFPI could be observed.[30] This is not easy to understand if one realizes that at 14 pM of TF, the plasma concentration of free full length TFPI (0.25 nM) is still in more than 10-fold excess of TF and 5-fold over the reported Ki* for the final tight complex of FXa/TFPI of approximately 0.06 nM.[17,9] Under these conditions, efficient inhibition of FXa and thrombin generation by TFPI is to be expected. That this is not the case is caused by the fact that TFPI is a slow inhibitor of factor Xa.[31] In other words, only when FXa formation is slow and low, TFPI gets sufficient time to block the TF pathway before thrombin generation and clotting can occur. Therefore, at high TF concentrations, the rate of FX-activation exceeds a threshold that can effectively be managed by TFPI, and FXa can escape regulation.

So although kinetic parameters of final tight FXa/TFPI* complex formation are very favorable for FXa-inhibition, the slow rearrangement of the initial FXa/TFPI complex limits the efficacy of TFPI. In this respect, the rapid FXa/TFPI encounter complex gains importance as FXa is already inhibited in this loose complex. On the other hand, since the K_i of the initial inhibitory complex between TFPI and FXa (5–15 nM) is several times higher than the concentration of full length TFPI in plasma (~0.25 nM),[22] it was difficult to understand mechanistically how TFPI could be an effective inhibitor of TF-induced thrombin generation in plasma,[17,29] until it was uncovered that protein S acts as a cofactor for TFPI.

TFPI-cofactor activity of protein S

As described above, inhibition of TF-FVIIa by TFPI is a two-step process. In the first step, Kunitz-2 of TFPI binds to and inhibits FXa, while the second step involves binding of Kunitz-1 of TFPI to FVIIa. TFPI can either first form a bimolecular complex with FXa that subsequently acts on the bimolecular FVIIa/TF, or acts directly on tertiary FVIIa/TF/FXa to form the inactive quaternary complex.[4,28] In addition, the interaction of TFPI with FXa proceeds by a two-stage reaction, rapid formation of a loose complex followed by a slow rearrangement into a tight complex. More recently, it was observed that protein S reduces the Ki of the initial loose FXa-TFPI complex 10-fold from 5 nM to 0.5 nM, thereby enhancing the first step of the reaction and increasing the amount of FXa rapidly taken from the circulation by TFPI.[17] Initially, it was suggested that protein S accelerated the total feedback inhibition of the TF-mediated coagulation pathway, but alternatively it was proposed that TFPI stimulation by protein S only affects the inhibition of free FXa, and would be less important for the final inhibition of the TF-FVIIa-(FXa) complex.[17,32]

The identification of protein S as a cofactor for TFPI solved the long-standing question of how TFPI could be an important anticoagulant as kinetic experiments indicated that at its plasma concentration of 0.25 nM, full-length TFPI would be a poor inhibitor of thrombin generation in plasma.[33] By reducing the Ki of initial bimolecular FXa-TFPI complex formation from 5 nM to 0.5 nM, protein S brings the TFPI concentration necessary for effective FXa-inhibition well within range of the full-length TFPI concentration (0.25 nM) in plasma; in other words, protein S explains the physiological relevance of the plasma concentration of full length TFPI.

The current hypothesis is that TFPI can effectively inhibit the tertiary complex of FVIIa/TF/FXa, but when FXa dissociates from the extrinsic tenase on its way to participate in prothrombinase complex and thrombin formation, protein S is crucial for inhibition of free factor Xa by TFPI (Figure 4). When applying this hypothesis we should realize that some observations, such as the inability of TFPI and protein S to regulate FX-activation by the intrinsic route of coagulation, remain unexplained.[10]

TFPI-protein S complex

Somewhere in time during the protein S-catalyzed inhibition of FXa, a tertiary complex between FXa, TFPI, and protein S is likely to exist. Considering the fact that protein S is a cofactor for full length TFPI only, and that a truncated variant (1–161) of TFPI lacking Kunitz-3 and the C-terminus is not stimulated by protein S, the interaction between TFPI and protein S is mediated through the Kunitz-3/C-terminus of TFPI.[17] Later it was observed that Kunitz-3 of TFPI is responsible for the interaction with protein S.[18] As a result, almost half the plasma TFPI circulates in a complex with protein S.[34]

TFPI and thrombosis risk

The physiological relevance of TFPI was established by the uniform lethality of TFPI knock-out mice.[35] To date, no hereditary deficiencies of TFPI have been described in humans, but plasma TFPI levels show large inter-individual variations. Although TFPI in plasma represents only a fraction of all TFPI, several studies have shown that low levels of plasma TFPI (particularly free TFPI) are associated with approximately 2-fold increased risk of venous thromboembolism.[22,36-38] In 1999, it was reported that decreased levels of heparin-releasable TFPI were associated with venous thrombosis.[39] As heparin-releasable TFPI is the full length form of TFPI, this observation emphasizes the role of full length TFPI as an anticoagulant. In addition, it suggests an anticoagulant role of endothelial cell-bound TFPI, as lower availability of this pool leads to an increased risk for venous thrombosis.

It has been reported that protein S and TFPI covariate in normal individuals[40] and that plasma levels of full-

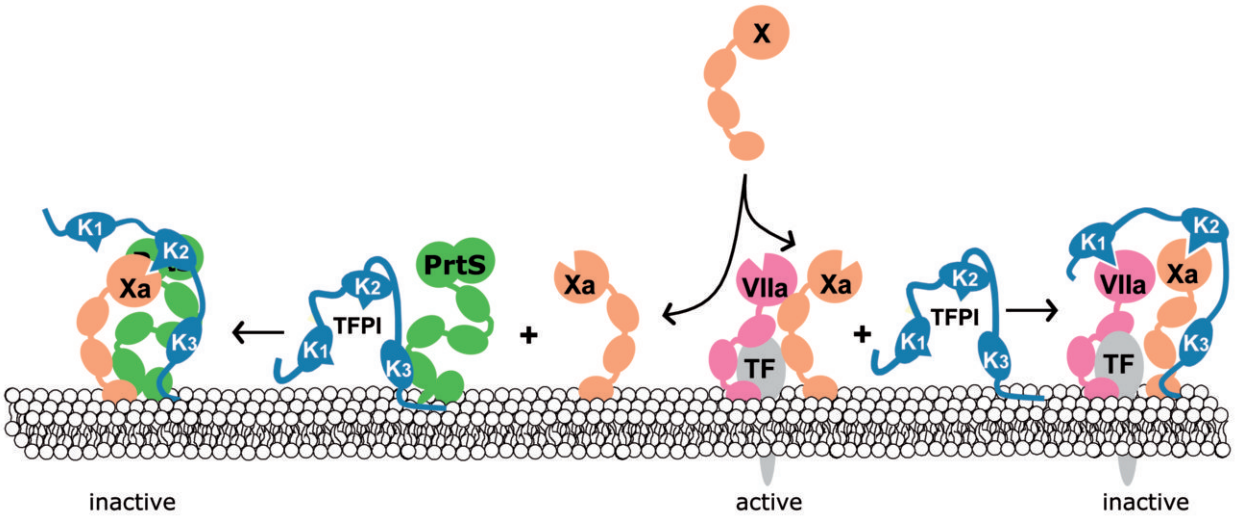


Figure 4. Regulation of coagulation by full length TFPI; state of the art. Factor X (X) is activated by tissue factor/factor VIIa (TF/VIIa). The tertiary complex of TF/VIIa/Xa reacts with TFPI to form an inactive quaternary TF/VIIa/Xa/TFPI complex. When Xa dissociates from the TF/VIIa complex to generate the prothrombinase complex, protein S acts as a cofactor to TFPI in the inactivation of Xa by stimulating Xa/TFPI complex formation.

length TFPI are low in protein S-deficient patients.[34] Since TFPI and protein S act in close collaboration to downregulate thrombin generation in plasma, combined low levels of both proteins may synergize in causing thrombosis. Both TFPI and protein S deficiencies are risk factors for development of venous thrombosis[41] and in this context, part of the thrombosis risk associated with protein S deficiency might actually be mediated by accompanying low levels of TFPI.[34]

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Recurrent venous thrombosis: a disorder of thrombin generation stimulus-response coupling?

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A B S T R A C T

One-quarter of patients who suffer an initial episode of venous thrombosis have no identifiable provocation. The risk of recurrent venous thrombosis in these patients is much greater than in patients who have an identifiable trigger. However, it is uncertain if patients with unprovoked thrombosis genuinely have suffered from a 'spontaneous' event or whether the episode was triggered by a factor that was not readily identifiable, that is, this was venous thrombosis due to a 'silent' provocation. Characterization of hypercoagulability may help to elucidate the relative contributions of an underlying prothrombotic state and silent provocation to venous thrombosis risk. In this paper, a model of recurrent venous thrombosis as a disorder of exaggerated thrombin generation due to abnormal stimulus-response coupling is proposed.

Introduction

Venous thrombosis (VT), also referred to as venous thromboembolism (VTE), describes deep vein thrombosis (DVT) with or without symptomatic pulmonary embolus (PE). Superficial vein thrombosis might also be considered within the spectrum of disease. After a first episode of VTE, patients are 40 times more likely to suffer a further event compared with previously unaffected individuals.[1] The post thrombotic syndrome is more likely to occur after recurrent ipsilateral DVT, and chronic thromboembolic pulmonary hypertension is more than ten times as likely after recurrent PE as after a first event.[2] Therefore, preventing recurrent VTE prevents fatal recurrence and reduces the burden of disease in survivors.

Treatment with an oral vitamin K antagonist (VKA), such as warfarin, or a direct thrombin or factor Xa inhibitor will prevent more than 95% of recurrent episodes of VTE.[3,4] However, VTE is only prevented for as long as the anticoagulant therapy is continued. Therefore, anticoagulation must be continued indefinitely in patients at high risk to prevent recurrence. The risk of anticoagulant therapy-related bleeding precludes routine continued treatment for all patients, and long-term (lifelong) treatment should ideally be given only to patients considered to have a risk of recurrent VTE that exceeds the risk of clinically significant bleeding associated with continued treatment.

Clinical risk factors and likelihood of recurrent venous thrombosis

Patients with cancer are known to be at high risk of recurrent VTE, and treatment of cancer-associated VTE is usually continued for as long as treatment with chemotherapy and/or radiotherapy is being given and for as long as cancer is considered to be present. Treatment with a low molecular weight heparin is considered superior to a vitamin K antagonist for these patients, at least for the first 6 months[5,6] and is now the preferred treatment.[7]

Patients with detectable antiphospholipid antibodies are often thought to be at continued risk of venous thrombosis and are considered for continued anticoagulation. However, what constitutes an abnormal test result that predicts a high risk of recurrence remains uncertain. Furthermore, whilst criteria for Antiphospholipid Syndrome (APS) have been defined for the purposes of clinical study recruitment and reporting, the diagnosis of APS in an individual in a routine clinical setting can be difficult, as can the decision regarding duration of anticoagulant therapy.

Patients with other persisting risk factors are also typically treated for as long as the risk factor persists. For example, a woman who suffers thrombosis in pregnancy is usually treated until at least 6 weeks after delivery. The risk of recurrence in the absence of a further pregnancy is low.

In a study of unselected patients with a first episode of VTE, 15% of patients had cancer-associated venous thrombosis, 6% were diagnosed with APS, and 1.5% were women with pregnancy-associated thrombosis.[8] The remaining patients were all treated for 6 months with oral anticoagulant therapy. Since then, randomized trials have indicated that continuing treatment for 6 months is no more beneficial than treating for only 3 months.[9,10] This has resulted in the recent recommendation that after a period of venous thrombosis, initial treatment with anticoagulant therapy should be for 3 months.[11]

Since the early 1990s, it was recognized in clinical trials and observational cohort studies that patients who suffered a provoked episode of venous thrombosis, for example, after surgery, were at lower risk of recurrence than patients whose first episode was unprovoked.[12–16] This relationship between the likelihood of recurrence and the clinical circumstances at the time of the first event was demonstrated prospectively in a study, which determined recurrence rates after a first episode of venous thrombosis in relation to clinical risk factors and thrombophilia testing.[8] It is now accepted that the relationship between clinical factors at the time of venous thrombosis and likelihood of recurrence is sufficiently strong and robust as to be used as the basis for determining which patients should be considered for continued anticoagulant therapy.[11] However, as VTE is only prevented for as long as the anticoagulant therapy is continued, this equates to potential lifelong anticoagulation after a first episode of venous thrombosis. The recommendation for consideration of continued (lifelong) anticoagulation after a first episode of unprovoked venous thrombosis is considered contentious by some experts, and further clinical investigation into refining individual risk, both of recurrent thrombosis and anticoagulant therapy-related bleeding is necessary.

A further consideration when determining duration of anticoagulation for a patient is the likely consequence of recurrence if it were to occur. The risk of fatal PE is two to four times more likely in patients with symptomatic PE as compared with patients with symptomatic DVT alone,[17,18] and chronic pulmonary hypertension is at least ten times more likely after recurrence.[2] Therefore, if recurrence is more likely to be PE than DVT then the consequences of recurrence are potentially greater in patients with a first event manifesting as symptomatic PE. Previous studies suggest that 75% of recurrences are PE in patients initially presenting with PE, compared with 20% in patients presenting with DVT.[18–20] A patient level meta-analysis of seven recent prospective studies showed that patients presenting with a first episode of PE are at the same risk of recurrent VTE as patients presenting with a first episode of DVT alone but they are three times more likely to suffer PE than DVT as a recurrence.[21]

Unprovoked venous thrombosis and risk of recurrence

Within the group of patients who have suffered an unprovoked episode of venous thrombosis, there is a heterogeneous mix of individual risk. This is appreciated by observation of the distribution of D-dimer levels in these patients,[22–25] with approximately 50% having a D-dimer level below a predefined threshold.[26] The definition of unprovoked VTE is clinical and is dependent on an absence of identifiable risk in temporal association with the episode of VTE. A number of strong and moderate clinical risk factors have been used to distinguish provoked and unprovoked VTE in clinical studies (Table 1). In the absence of these recognizable factors, it is possible that some cases of unprovoked VTE are misclassified as unprovoked. Our understanding of the totality of environmental factors and how these interact at a moment in time is still limited. It is possible that a proportion of unprovoked cases are actually provoked by an unknown combination of temporary environmental risks. Patients with a low D-dimer following a finite period of anticoagulation may be representative of this group of patients, that is, patients with venous thrombosis due to 'silent provocation'. However, there appears to be a continuous accrual of recurrent events over time even in patients with a low D-dimer after unprovoked VTE, albeit at a lower rate than that observed in patients with a high D-dimer. This suggests either that silent provocation is a recurring theme or that these patients are at increased risk of genuine unprovoked VTE despite a relatively low D-dimer. The issue that now has to be addressed in clinical studies is whether measurement of hypercoagulability can identify a group of patients that exists with venous thrombosis due to 'silent provocation' who are not at risk of recurrence in the future, or alternatively if measurement of hypercoagulability is quite simply a measure of the rate of recurrence over time (for all patients). Studies with prolonged follow-up without intervention would be required to answer this question. There is a proposed illustration of alternative outcomes of recurrent venous thrombosis in relation to hypercoagulability (Figure 1).

Table 1. Examples of risk factors (and exclusions) used in clinical studies to distinguish provoked from unprovoked VTE.

Unprovoked
No identifiable provoking factors
Provoked
Surgery within previous 6 weeks (possibly within previous 90 days)
Trauma including fracture
Plaster cast immobilisation
Oestrogen exposure (contraceptive or hormone replacement)
Immobilisation for 3 days or more (including hospitalisation)
Travel (more than 6 hours continuous travel within 1 week of onset of symptoms of VTE)
Exclusions
Cancer
Myeloproliferative disorder
Antiphospholipid syndrome
Pregnancy

Definition of hypercoagulability and the prothrombotic state

Global tests that measure the composite effect of variation in procoagulant and anticoagulant factors can be used to quantify 'coagulability' as a parameter. Hence it might be possible to define 'hypercoagulability'. Two approaches have been used in clinical studies so far: measurement of biomarkers and determination of the thrombin generating potential. Biomarkers of thrombin generation reflect thrombin generation that has taken place *in vivo*, and D-dimer measurement after completion of a finite period of anticoagulation has been shown to stratify patient risk.[23,26] Measurement of the thrombin generating potential quantifies the ability to generate thrombin *in vitro* (typically in a plasma sample)

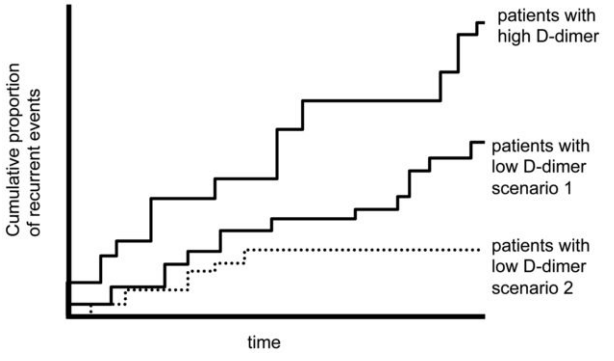

Figure 1. Illustration of alternative outcomes of recurrent venous thrombosis in relation to coagulability. Patients with elevated D-dimer have a cumulative risk of recurrence greater than those with a low D-dimer. For patients with a low D-dimer two survival curves are shown. The first (solid line, scenario 1) shows a continuous accrual of recurrent events and is in keeping with the hypothesis that measurement of coagulability (as measured by D-dimer in this example) is quite simply a measure of the rate of recurrence over time for all patients. The rate of recurrence is simply lower in patients with a low D-dimer but given sufficient time all patients will eventually suffer a recurrence. The second (broken line, scenario 2) shows a plateau implying that within the cohort of patients with a low D-dimer some are not at risk and do not suffer a recurrence. The distinction between patients with a low D-dimer who are and who are not at risk of recurrence might be determined prospectively by measurement of thrombin generating potentials or characterisation of the genetic architecture of an individual's thrombin generating potential.

in response to a pre-defined stimulus, usually a low concentration of tissue factor.[27] These complimentary approaches might be used to define hypercoagulabilty and the prothrombotic state as distinct entities.[28] Hypercoagulability might be defined as a predetermined 'exaggerated' response of thrombin generation to a stimulus. This might be identified by measurement of thrombin generating potentials or characterization of the genetic architecture of an individual's thrombin generating potential. The prothrombotic state might be defined as active (ongoing) exaggerated thrombin generation. This might be identified by measurement of D-dimer in the absence of an identifiable reason for increased thrombin generation, that is, in the absence of infection, pregnancy, recent surgery, and so on. This model of recurrent venous thrombosis is proposed as a disorder of exaggerated thrombin generation due to abnormal stimulus-response coupling (Figure 2).

It is not inconceivable that patients with extreme hypercoagulability are in a constant prothrombotic state as the normal environment in those individuals is a sufficient trigger for increased thrombin generation. Patients with a persistently elevated D-dimer after an episode of VTE may be representative of such patients who are at high risk of recurrence, either of unprovoked events or in response to 'silent' provoking factors.

The thrombophilia paradox

Heritable thrombophilia describes an inherited tendency for venous thrombosis. So far, only deficiencies of antithrombin, protein C, and protein S due to mutations in the corresponding genes *SERPINC1*, *PROC*, *PROS*, and the two common mutations *F5*G1691A and *F2*G20210A have been shown to be unequivocally associated with venous thrombosis, as defined by at least a two-fold increased risk.[29] Deficiencies of antithrombin, protein C, and protein S might be considered 'high risk' thrombophilias compared with the 'low risk' *F5*G1691A and *F2*G20210A mutations.[30–33]

At a patient-group level, it has been demonstrated in prospective cohort studies of consecutive unselected patients that testing does not usefully predict likelihood of recurrence after a first episode of venous thrombosis.[8,34,35] This also holds true for the group of patients who suffer an unprovoked first episode of venous thrombosis. A review of the clinical utility of thrombophilia testing, published in 2008, concluded that testing for heritable thrombophilia serves a limited purpose and should not be performed on a routine basis.[36] An analysis of the Multiple Environmental and Genetic Assessment (MEGA) study showed that testing for inherited thrombophilia did not reduce recurrence of venous thrombosis.[37] Guidelines now recommend thrombophilia testing in a minority of patients with venous thrombosis.[38]

A paradox seemingly exists, namely that these five thrombophilias are associated with an increased risk of a first venous thrombosis but not, apparently, of a high risk of recurrence.[28] This is likely the result of limitations imposed by testing for only a minority of heritable thrombophilic defects and adopting a dichotomous testing strategy, whereby a defect is defined as present or absent rather than quantified in terms of risk. The complete genetic contribution to thrombosis risk in patients is not known. Multiple other genetic factors will be present, which may be associated with a low risk in isolation[39] but result in a significant risk when clustered in an individual[40] or present in addition to one of the five 'usual suspects'. Therefore, only a fraction of an individual's genetic framework is appreciated with a limited dichotomous testing strategy. Consequently, the material contribution of an individual's genetic framework is not accurately estimated by current thrombophilia testing strategies. These limitations are likely compounded in practice by test inaccuracy and imprecision, such that the intermediate phenotype of anticoagulant deficiency (defined by a low plasma level of antithrombin, protein C or S) is not fully concordant with the heritable genotype.[41] As rapid inexpensive genotyping becomes a reality in the next 5 years, the identification of the totality of low risk mutations combined with characterization of the structure-function consequences of high risk mutations may improve the estimate of genetically determined thrombosis risk in an individual. Such analysis may equate into a useful predictor of recurrent thrombosis risk, such that individual genomic analysis will be considered to have clinical utility. There is already proof of principle that multiple testing for common mutations (single nucleotide polymorphisms) quantifies the risk of recurrent VTE,[40] and the number of common gene variants shown to be possibly related to risk of VTE is increasing.[39,42] Ultimately, the potential application of genomic DNA analysis to individualized risk assessment remains to be determined as the interaction of complex

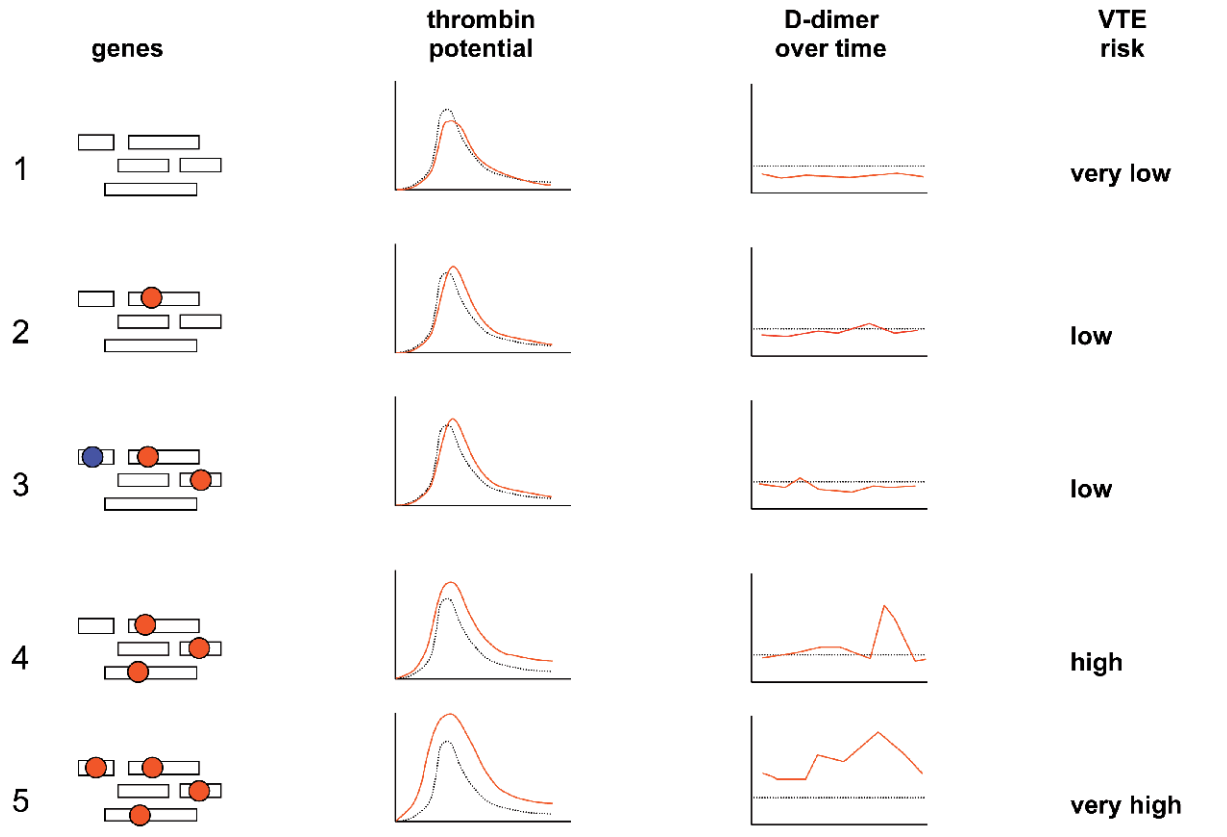

Figure 2. Proposed model of recurrent venous thrombosis as a disorder of exaggerated thrombin generation due to abnormal stimulus-response coupling. An exaggerated thrombin generation response would be genetically determined and might be identified by measurement of thrombin generating potentials or characterisation of the genetic architecture of an individual's thrombin generating potential. Some patients with extreme hypercoagulability are in a constant prothrombotic state as the normal environment in those individuals is a sufficient trigger for increased thrombin generation (individual 5). Others require a trigger to produce a prothrombotic state but depending on the degree of hypercoagulability a trigger may be slow slight as to not be readily identifiable; the 'silent trigger' (individual 4). In the model 5 individuals are presented: 1) normal with no thrombophilic mutations, 2) normal with minimal thrombophilic mutations, 3) normal with balanced thrombophilic and haemophilic mutations, 4) mild hypercoagulablity due to more thrombophilic mutations, 5) severe hypercoagulability due to most thrombophilic mutations. Thrombophilic mutations are shown in red and haemophilic mutations in blue. An average normal thrombin generation curve and the upper limit of normal (threshold) for D-dimer are shown as dashed lines.

combinations of gene variants with environmental factors may still prove to be relatively unpredictable at an individual level. On the other hand, if it is shown to have useful predictive power, it may also help our understanding of hypercoagulability and of how this translates into a prothrombotic state. In other words, does an individual's genotype result in a constant hypercoagulable state and hence constant relatively increased risk or venous thrombosis, or alternatively does it predispose to hypercoagulability in response to environmental stimuli and hence, only an increased risk of venous thrombosis on occasion. Current evidence in patients with type 1 antithrombin deficiency suggests the latter relationship.[43] In a study from Leiden, the annual incidence of venous thrombosis in antithrombin-deficient intervals who were not exposed to an environmental risk (for example, as in Table 1) was 0.3%. In patients who had surgery, the annual risk in the year that surgery was performed was 20%. Clearly, these individuals were at very high risk of provoked venous thrombosis. This suggests that measuring both thrombin generating capacity and D-dimer may be helpful in identifying patients at high risk of recurrence and whether recurrence is likely to be provoked (which would indicate the need for prophylaxis at times of identifiable risk, for example, patient 4 in Figure 2) or unprovoked (which would indicate a need for lifelong prophylaxis, for example, patient 5 in Figure 2).

Clinical utility of measures of hypercoagulability and the prothrombotic state

D-dimer is a marker of fibrin degradation formed by the sequential action of three enzymes: thrombin, factor XIIIa, and plasmin.[44] Therefore, increased thrombin generation is associated with increased D-dimer formation. In a series of studies from observation through to a patient management study (PROLONG), Palareti and colleagues showed that measurement of D-dimer levels following cessation of anticoagulant therapy predicts likelihood of recurrent thrombosis.[23–25] A meta-analysis of cohort studies indicates that the annualized risk of recurrence is 9% in patients with an elevated D-dimer

compared with 3.5% in patients with a low D-dimer after completion of a finite period of anticoagulation after a first venous thrombosis (relative risk 2.4, 95% CI 1.9 to 3.1).[26] In the PROLONG II study, D-dimer measurements were repeated at 2 monthly intervals for 1 year after an initial normal D-dimer following completion of initial therapy.[45] D-dimer was normal in 68% of patients 1 month after stopping treatment. Fourteen percent of patients developed an abnormal D-dimer 2 months after an initial normal result. The rate of VTE recurrence over a mean follow up of 10.6 months was 22.6% in these patients compared with 4.6% in patients whose D-dimer remained negative. This is an important finding that needs to be replicated in further studies. The predictive value of D-dimer may be influenced by interacting or confounded factors, such as sex and age. Furthermore, the predictive value of D-dimer measurement has typically been evaluated in patients with unprovoked venous thrombosis and is different in patients after provoked events.[46] A secondary analysis of the PROLONG study indicated that in patients with a normal D-dimer after completion of anticoagulant therapy after unprovoked venous thrombosis, recurrence rates were higher in males than females (7.4% vs. 4.3% patient-years) and in patients aged 65 years or more (8.4% vs. 3.6%). However, in a meta-analysis of cohort studies, only male sex had a significant effect on risk for recurrent VTE independent of D-dimer status; age, hormone therapy use at the time of the index event, body mass index, and timing of post-anticoagulation testing did not influence the predictive value of the D-dimer test result.[47]

Measurement of the thrombin generating potential is an alternative 'global testing' strategy that is possibly complimentary to measurement of D-dimer. This measurement has been shown in independent cohort studies to predict likelihood of recurrence with hazard ratios from 2.5 to 4.0.[48–50] Measurement of thrombin generation is technically difficult, and results are more influenced by pre-analytical variables than D-dimer measurements.[27,51–53] Thrombin generation assays measure the thrombin-time curve, which is the enzymatic work potential of thrombin. The Calibrated Automated Thrombogram ® (Thrombinoscope BV) and Technothrombin ® TGA (Technoclone) utilize a fluorogenic substrate, and the Endogenous Thrombin Potential Assay ® (Siemens healthcare Diagnostic Inc.) and Pefakit Thrombin Dynamics Test ® (Pentapharm) employ a chromogenic substrate. Activation of thrombin generation and interpretation of the thrombin-time curve varies between assays. Various parameters of the thrombin-time curve can be reported, including lag time, peak thrombin, time to peak, and the area under the curve (AUC, Endogenous thrombin Potential).

A recently developed alternative to these optical detection methods is continuous registration of the thrombin-time curve using an electrochemical biosensor.[54] Sensor strips with an amperometric substrate are electrically connected to a measuring unit. Thrombin cleaves the substrate producing an electric current. As electrochemical detection of thrombin activity is not affected by color or turbidity, the measurement can be performed on a whole blood sample. Clinical studies utilizing this technology have not yet been reported.

More studies are required to examine the clinical utility of measurement of D-dimer and thrombin generating potential and to determine:

- the performance characteristics of different assays;
- the value of quantitative (continuous variable) versus qualitative (dichotomized positive/negative or high/low) measurement;
- the influence of the clinical profile of the patient on the predictive value of the test result;
- the value of serial measurement, including measurements during and after completion of an initial period of anticoagulant therapy.

Conclusion

Prevention of recurrent venous thrombosis prevents fatal recurrence and reduces the burden of disease in survivors. Distinguishing patients at high and low risk of recurrence will permit continued anticoagulation in those patients in whom it is beneficial and avoid anticoagulant therapy-related bleeding in those who do not require continued treatment. In the last 10 years, measurement of 'coagulability' has been shown to be able to stratify patient risk and when used in conjunction with assessment of clinical risk factors, can increase the objectivity of clinical decision making. It is still not fully understood why some patients are at risk of recurrent unprovoked recurrent VTE. As well as validating measures of global coagulability for clinical use, ongoing studies may help to explain the mechanisms leading to thrombosis and clarify the relationship between thrombophilia, hypercoagulability, and the prothrombotic state.

As the 'silent' environmental trigger factors are currently unknown, it is not feasible to measure D-dimer at the time of an 'unknown risk event' to determine a patient's thrombin generating response. Therefore, all that can be suggested at present is measurement of D-dimer 'randomly', which in practice means repeated measurements. The PROLONG II study has paved the way for this approach. From a pragmatic clinical perspective, it may not be necessary to perform more than a few measurements to identify patients who readily 'slip into a prothrombotic state'.

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Thrombosis and pregnancy

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A B S T R A C T

Venous thromboembolism (VTE) is one of the most important causes of morbidity and mortality during pregnancy and in the peri- and postpartum period. The incidence of VTE is around 1 in 1000 pregnancies. Vitamin K antagonists have a teratogenic effect when given between the sixth and ninth week of gestation, but Heparin (unfractioned and low molecular weight heparin) can safely be used for treatment and prophylaxis during pregnancy. Acute VTE events are treated with therapeutic doses of low molecular weight heparin (LMWH), and treatment is continued until 6 weeks to 3 months after delivery. Lower doses of LMWH may be considered after 4–6 weeks on therapeutic doses. Prevention of VTE is recommended for women with a history of idiopathic VTE and suggested for those with antithrombin deficiency, homozygous factor V Leiden, and the lupus anticoagulant. Although no interventional trials on anticoagulation in pregnant women are available, treatment and prophylaxis of VTE in pregnancy can nowadays be regarded as highly effective and safe.

Epidemiology of pregnancy-associated thrombosis

VTE is an important and frequent complication of pregnancy, and pulmonary embolism (PE) is nowadays the most frequent cause of maternal mortality in the developed countries.[1] The incidence of VTE is around one episode per 1,000 deliveries,[2] and is thus much higher than in non-pregnant women of comparable age. About two-thirds of DVT occur antepartum; VTE may occur at any trimester and is more or less equally distributed throughout all three trimesters.[3] Approximately 50% of pregnancy-related episodes of PE occur in the first 6 weeks after delivery.[4] The incidence of VTE is higher in women with hereditary thrombophilia. In epidemiological studies from Scotland, the incidence of pregnancy-associated VTE was 1 in 437 in women with heterozygous factor V Leiden, 1 in 113 in those with Protein C deficiency, and 1 in 2.8 in women with antithrombin deficiency.[5] Similar or even slightly higher risks were found in a study from Norway. In women with heterozygous factor V Leiden, the risk was estimated to be 5.4 in 1000 pregnancies and was 9.4 in 1000 in those with heterozygous prothrombin G20210A variation.[6] During pregnancy, the risk of developing venous thromboembolism is about 4-fold in comparison with non-pregnancy, and the risk is considerably higher in the 6 week postpartum period. In a Norwegian study, significant risk factors for thrombosis during pregnancy were overweight (OR 7.7), especially with immobilization (OR 62), while smoking more than 10 cigarettes and pregnancy with twins were also risk factors with an OR above 2.0.[6] Risk factors for postnatal thrombosis were preeclampsia (OR above 3), gestational diabetes (OR 4.5), overweight (2.5), specifically with immobilization (OR 40), and postpartum bleeding (OR 4.0), specifically with surgical delivery (OR 12).[6]

Anticoagulants

Since controlled clinical trials are lacking, recommendations on the use of anticoagulants in pregnant women are largely based on data obtained from non-pregnant patients and from case series of pregnant patients.

Heparin, including low molecular weight heparin (LMWH), does not cross the placental barrier and is thus non-teratogenic, whereas vitamin K antagonists cross the placental barrier and may cause coumarin embryopathy, which is characterized by nose deformation and other deformations of the skeletal structure (stippled epiphysis).[7] Furthermore, bleeding might occur in the anticoagulated fetus, leading to severe and life-threatening clinical situations, such as cerebral bleeding. Chan and colleagues found that the use of vitamin K antagonists during pregnancy was associated with congenital fetal anomalies in 35 of 549 live births.[8] The dimension of the teratogenic risk remains controversial, ranging from 0% up to 29.6%.[7] This risk is only present when vitamin K antagonists are taken between the sixth and ninth week of gestation. Coumarins have also been associated with anomalies of the central nervous system after exposure to them during any trimester. Moreover, Vitamin K antagonists have been associated with abortion.[8,9]

Women receiving vitamin K antagonist therapy have to be clearly informed about the risks of oral anticoagulant therapy. The use of danaparoid treatment has been reviewed recently. Fifty-one pregnancies occurred in 49 patients, three fetal deaths were reported, but all of them were associated with maternal complications antedating danaparoid use.[10] Although not licensed, danaparoid seems to be an alternative in case of allergic reactions to LMWH. Reports on the successful use of fondaparinux in pregnant women,[11–13] have been published; however, potential adverse effects on the fetus cannot be excluded. Thrombolytic agents should only be used in life-threatening situations. There are still concerns about the use of vitamin K antagonists in nursing mothers, however, many experts in the field agree on the potential use of these agents in nursing mothers.[14] It is suggested to substitute vitamin K orally in the child (e.g., 1 mg of vitamin K once a week). The use of heparin including LMWH, danaparoid, and fondaparinux in the lactating woman is regarded as safe for the child.[7] There are no reports on the use of new anticoagulants, such as Dabigatran, Rivaroxaban, or Apixaban, during pregnancy. Due to the fact that these are small molecules, it can be anticipated that these substances cross the placental barrier and lead also to anticoagulation of the fetus.

Whereas thrombo-prophylaxis may not be justified on the basis of caesarean section alone, it is clearly recommended in women with one or more risk factors.[7] No adequately powered trials have been performed in women undergoing caesarean section.[15–18] Usually, based on experience from clinical trials in high-risk patients, thrombosis prophylaxis with LMWH may be recommended the way it is used in general surgery in high-risk patients. As there are also no data concerning the duration of prophylaxis, experts suggest a minimum duration of 5 days.

Acute VTE during pregnancy

When an acute event of VTE occurs in a pregnant woman, LMWH is the treatment of choice. The dose is adapted to the body weight of the woman.[19] It has been shown that during the course of pregnancy, the dosage of LMWH needed to reach a therapeutic anti-Xa level increases.[20–22] However, experts do not agree as to whether measurement of anti Xa levels is preferable in pregnant women with acute VTE or whether it is sufficient to adapt the dosage of LMWH to the weight of the pregnant woman. Since vitamin K antagonists are contraindicated during pregnancy due to their potential of causing embryopathy, secondary prophylaxis with LMWH is used in pregnant women. LMWH has a lower risk for development of osteoporosis and also for this reason, is the preferred option for these women.[23] Whether or not dose-adjustments are necessary during the course of pregnancy is still seen as controversial. Whereas some experts suggest increasing the dosage according to the increase of weight, others prefer adjustment of dosage of LMWH to the actual anti-Xa level. A target level of 0.6 to 1.2 units/mL is recommended when a twice-daily regimen is used and a slightly higher target if once-daily regimen is chosen. Measurement is performed approximately 4 hours after the administration of LMWH.

Yet, it still remains unclear whether the dose of LMWH can safely be reduced after the acute thromboembolic event after an initial phase of therapeutic anticoagulation. Several suggestions have been made, either the maintenance of therapeutic doses of anticoagulation throughout pregnancy or the reduction of LMWH to prophylactic or 50–75% of full dose LMWH after 4–6 weeks of full therapeutic anticoagulation. Such an approach may reduce the risks of anticoagulant-related bleeding and heparin-induced osteoporosis.

A dangerous complication of anticoagulation is bleeding during labor.[24] When induction of labor or elective caesarean section is planned, doses of LMWH are reduced 24 hours before the planned intervention and anticoagulation is reinstalled after delivery. In women planning to have a spontaneous delivery, the doses of heparin might be reduced to prophylactic levels after the 37th week of gestation. When the thromboembolic event has occurred shortly before delivery (e.g., within 6 weeks before delivery), the insertion of a caval filter has to be suggested. Filters can be removed after a therapeutic anticoagulation has been reinduced after delivery (3–10 days after delivery). Women with a very high risk for recurrent VTE could also be switched to therapeutic intravenous unfractioned heparin (UFH), which is then discontinued 4 to 6 hours prior to the expected time of delivery.

No studies clarifying the duration of anticoagulation after delivery are available. It is accepted by most experts that a minimum of 6 weeks of anticoagulation after delivery should be recommended, allowing for duration of up to 3–6 months in women, in whom VTE had occurred shortly before delivery or in those who still have an increased risk, for example, due to infection, immobilization, or other predisposing factors.

Prevention of VTE in pregnant women with a history of VTE

Women with a history of VTE are at increased risk for recurrence during the pregnancy period.[25] There are no sufficiently powered studies examining the incidence of VTE in pregnant women with and without heparin. From observational studies, the risk for recurrence can be deduced to be between 5–10 % during pregnancy.[26–28] In a prospective study designed to estimate the true incidence of recurrence in 125 women with prior VTE, Brill-Edwards.[26] found a risk of 2.4% of recurrence (95% confidence interval (CI) 0.2–6.9%). In a retrospective study of 159 women without thromboprophylaxis, the probability of developing antepartum VTE was 6.2% (95% CI, 0.2–6.9%), while that for postpartum VTE was 6.5% (95% CI, 3.5–11.9%).[27] The magnitude of the risk is similar throughout the whole period of pregnancy and thus is already increased during the first trimester. The risk of recurrence has been balanced against the risk of treatment, inconvenience, and costs. The risk of prophylaxis is heparin-induced thrombocytopenia and bleeding, and when higher (therapeutic) doses are administered, there is also a risk of developing osteoporosis and osteoporotic fractures, which has been

observed in prospective studies.[23,29–31] At the site of injection, allergic reactions and bleeding might occur, which causes itching, pain, and a small risk of heparin-induced skin necrosis. Since LMWH is administered during very long time periods (up to or even more than 9 months), a cost issue also has to be taken into account. An important task would be to identify women with a high risk of recurrence. Up to now, just antithrombin deficiency seems to be clearly associated with an increased risk of recurrence. There is ongoing discussion whether milder risk factors, such as factor V Leiden or the prothrombin variation in heterozygous from, can be regarded as risk factors for an increased risk for pregnancy-associated recurrence. There is a clear recommendation for prophylaxis after delivery in comparison with antepartum prophylaxis because of the shorter duration of required treatment (i.e., 6 weeks) and the higher average daily risk of VTE in the postpartum period. During prophylaxis with LMWH, the rate of primary and recurrent thrombosis is low. The risk of heparin-induced thrombocytopenia and osteoporosis is also very low.[23] There are no controlled studies available with regard to the dosage of LMWH and the avoidance of thrombosis. In retrospective and prospective cohort studies involving LMWH either in prophylactic.[27,32,33] or risk-adjusted doses of dalteparin,[34] antepartum recurrence rates were between 0% and 3%, postpartum the risk was up to 6.1%. Most of the women, in whom recurrence occurred despite LMW prophylaxis, were high-risk patients, often with thrombophilia, such as the lupus anticoagulant, antithrombin deficiency, or homozygous factor V Leiden.

Risk of pregnancy-associated VTE and prevention in women with thrombophilia

There are several risk factors for thromboembolism ("thrombophilia") causing a considerable increase of the risk of VTE.[35] The most important one is antithrombin deficiency type I.[36] Rates of VTE of 20–40% have been described in women with antithrombin deficiency, sometimes occurring very early during the course of pregnancy.[37] Women with homozygous factor V Leiden are also at a high risk for VTE during pregnancy,[38] and there are only very scarce data on patients with homozygous prothrombin variation. The risk for VTE in women with factor V Leiden in heterozygous form is clearly increased (HR 52);[39] in absolute terms, a risk of 1 in 430 women with hetereozygous F V Leiden has been described.[5] There is also an increased risk in women with protein C- and, to a lesser extent, in women with protein S deficiency.[5]

There is ongoing discussion as to which women should receive primary antepartum prophylaxis. The author of this manuscript suggests thrombosis prophylaxis in women with antithrombin deficiency and in those with homozygous factor V Leiden. Additionally, women that fulfill the criteria for an antiphospholipid syndrome would most probably benefit from thrombosis prophylaxis with LMWH including aspirin with a better outcome for the fetus.[37] In all other women, the recommendation is rather against thrombosis prophylaxis, but it seems mandatory to provide profound information for these women on the symptoms of VTE and, if symptoms compatible with VTE occur, to verify or exclude a thrombotic event immediately and objectively.

Venous Thromboembolism has become one of the most important complications of pregnancy. Although systematic clinical studies have been performed only rarely, the knowledge on prophylaxis and management of venous thromboembolism has increased considerably within the last decades. Specifically low molecular weight heparins play an important role, as in cohort studies, they have been shown to be safe for the woman and the fetus, and effective. Further advances might be reached by defining more clearly women at risk for venous thromboembolism and by defining more precisely the dosage of anticoagulants.

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Red blood cell alloimmunization: of mice, men, and women

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A B S T R A C T

Red blood cell (RBC) alloimmunization can be quite problematic from a medical, as well as a logistical viewpoint. Alloimmunized patients are at risk for hemolytic transfusion reactions, and highly alloimmunized patients are at risk of not having compatible RBC units available for transfusion. Although some factors influencing rates of RBC alloimmunization are obvious, others are less well understood. Animal models have begun to bridge the gap in the understanding of both donor and recipient factors influencing rates and degrees of RBC alloimmunization. Murine models have most frequently been utilized secondary to their known genetic backgrounds and the number of immunologic tools available for use. To date, four transgenic mouse models of RBC alloimmunization have been described: the Tg-FVB model (with presumed ubiquitous expression of the human Duffy^b antigen); the mHEL model (with ubiquitous expression of membrane bound hen egg lysozyme); the HOD model (with RBC specific expression of hen egg lysozyme, a portion of ovalbumin, and human Duffy^b); and the hGPA model (with RBC specific expression of the human glycophorin A antigen). This review focuses on the strengths of each model system, discussing what is known and unknown about RBC alloimmunization in mice, men, and women.

Introduction

Red blood cell (RBC) alloimmunization, or the formation of antibodies after exposure to non-self antigens, can be a clinically significant problem. Sequelae of RBC alloimmunization include timely and costly evaluations for antibody identification and for location of compatible blood for transfusion, acute or delayed hemolytic transfusion reactions, and hemolytic disease of the newborn.[1] Although some patients ("non-responders") fail to develop RBC alloantibodies despite exposure to large numbers of blood products, others ("responders") make multiple antibodies despite limited blood product exposure.[2] In some instances, a "responder" may develop so many anti-RBC alloantibodies that compatible RBCs for transfusion (aside from autologous RBCs) do not exist.

Outside of the very immunogenic Rh(D), alloimmunization to other RBC antigens occurs in approximately 3–10% of transfused individuals,[3–6] with some patient populations (including patients with sickle cell disease) having alloimmunization rates up to 40–50%.[7–9] The prevalence of alloimmunization may be even higher than previously appreciated, as a number of anti-RBC antibodies may disappear and then reappear at a later date ("evanescence").[10] The pathophysiology of this process is not well understood, but certain antibodies are more likely to evanesce than others, with antibody pairs (e.g., those developing at similar time points after transfusion) sharing a similar evanescence fate. Another consideration in interpretation of alloimmunization statistics is that agglutination based assays utilized in blood banks may fail to detect low levels of anti-RBC antibodies, should they be present. Thus, taking into account the large number of RBC units transfused annually (>15 million in the US alone),[11] the overall prevalence of RBC alloimmunization is quite high.

In order for a recipient to develop an anti-RBC alloantibody, several conditions must be met. First, there must be antigenic differences between donor and recipient. Given that there are hundreds of described RBC antigens,[12] there are typically a large number of antigenic differences between donor and recipient during each RBC transfusion. Next, the recipient must be able to recognize and present the foreign antigen. If the antigen in question cannot fit into the recipient's antigen presenting cell MHC pocket, then the steps to initiate alloantibody formation cannot occur. For example, it is thought that Rh(D) can be presented by an MHC in nearly all transfusion recipients,[13] whereas Fy^a appears to be preferentially presented by transfusion recipients with HLA DRB1*04 (0401 or 0403) or HLA DRB1*15.[14,15] Finally, it has been hypothesized that a danger signal[16] of sorts is necessary for an immune response in general to occur. Additional recipient factors that influence alloantibody formation in humans, however, are ill-defined.

Besides recipient factors, a number of donor and product specific factors may potentially influence alloantibody response. It has been proposed that length of RBC storage may influence recipient outcomes, such as infection, deep vein thrombosis, and gen-

eral morbidity and mortality.[17–21] Prospective studies evaluating RBC storage and rates of RBC alloimmunization have not been completed; however, a small retrospective study found no correlation between rates of anti-D formation and RBC storage length.[22] Furthermore, it may be hypothesized that factors intrinsic or extrinsic to the donor RBC could also influence the recipient immune system rate of recipient RBC clearance, potentially influencing alloantibody response. These factors may lead to an increased sensitivity to oxidant stress hemolysis or to hemolysis in general. Furthermore, certain manipulations within the blood bank, such as washing of RBCs, decreases post-transfusion RBC recovery. Thus, although not well studied, donor specific RBC factors and manipulations of RBC units prior to transfusion may ultimately influence recipient immune response to the transfused RBCs.

Animal models of RBC alloimmunization

Although much knowledge has been gained about RBC antigen structure/function and anti-RBC alloantibody prevalence in humans over the past few decades, in depth mechanistic studies of RBC alloimmunization are more logistically difficult to design and to complete in humans. Animal models, however, circumvent a number of these limitations. Transfusion medicine studies have been completed utilizing rabbits (with a well defined Hg RBC antigen system), dogs, sheep, and other animals. Although much has been learned by these models, some models are limited by xenoantigen concerns (e.g., sheep RBCs transfused into mice), and others are limited by the lack of availability of immunologic tools. Mice are useful for reductionist RBC alloimmunization studies, given their well-defined genetic backgrounds, the availability of analytic tools including T and B cell transgenic animals specific for certain antigens, the ease of manipulation, and the ability to study a large number of recipients. Furthermore, approximately 99% of human genes have a mouse homologue.[23,24]

There are approximately 10 mouse blood group systems (Ea-2)[24] but the polymorphisms are not well described. Thus, genetically engineered mice are particularly useful for studying the immunologic effects of transfused RBCs. To date, four unique transgenic mouse models of RBC alloimmunization have been described: 1) Tg-FVB (transgenic Duffy^b);[25] 2) mHEL (membrane bound hen egg lysozyme);[26,27] 3) HOD (hen egg lysozyme, ovalbumin, Duffy^b);[28] and 4) hGPA (human glycophorin A).[29] In addition to the models that came before them, these transgenic model systems have each made unique contributions to the understanding of RBC alloimmunization.

Tg-FVB mouse model

The Duffy blood group system in humans contains Duffy^a (Fy^a) and Duffy^b (Fy^b), with the Duffy protein being a receptor for Plasmodium vivax.[12] Duffy is also a receptor for chemokines, such as interleukin-8 (IL-8), MCP-1, and RANTES.[12,30–32] The majority of humans express the Duffy antigen not only on RBCs but also on endothelial and epithelial cells. Fy^a is a clinically significant antigen in humans, with some sickle cell centers prophylactically transfusing Fy^a negative RBCs to all patients, and others transfusing Fy^a negative RBCs to "responder" recipients.

The Tg-FVB mouse model, developed at the New York Blood Center, was described by Chaudhuri *et al*. in 2004.[25] Two mice were initially developed: one with tissue but not RBC expression of Duffy^b ("Tg-FYB^{ES}" or "erythroid silent") and one with both tissue and RBC expression of Duffy^b ("Tg-FYB"). Tissue expression of Duffy^b was reported in brain, heart, liver, and spleen of both transgenic animals by RT-PCR. RBC alloimmunization studies utilizing the Tg-FYB mouse have been completed by Campbell-Lee *et al*.,[33] demonstrating the development of anti-Fy (Fy^3, Fy^6, and Fy^b) following transfusion of washed buffy coat-depleted RBCs from B6CBA-Tg-FYB donors into B6CBA-F1 recipients (Figure 1 shows a typical RBC alloimmunization study design). Additionally, biotin labeled Tg-FYB RBCs have decreased survival following transfusion into alloimmunized recipients compared to control recipients. The pre-

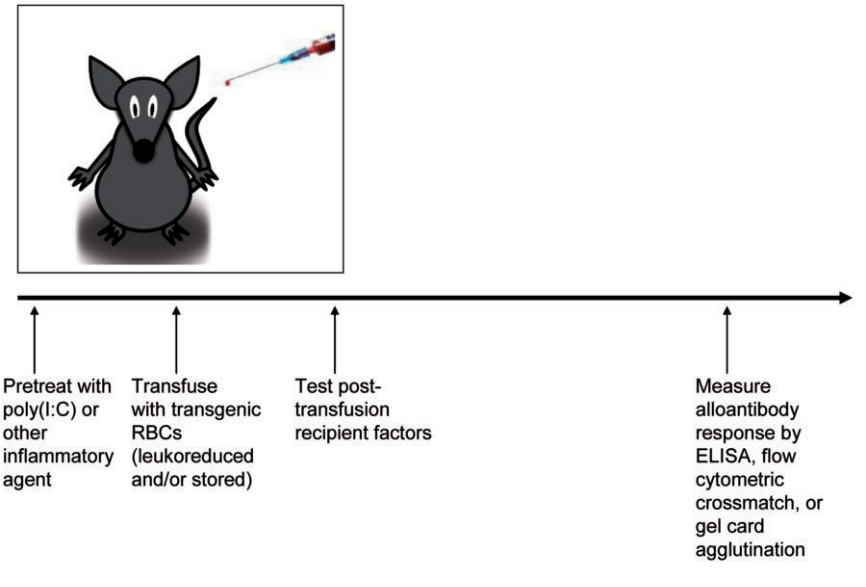

Figure 1. General overview of a murine alloimmunization experimental design.

sumed co-expression of Duffy^{b} on WBCs is a limitation of this system, given that the majority of human RBC antigens are not expressed on WBCs. Furthermore, the lack of immunologic tools, such as T and B cell transgenic animals, limit the number of in depth mechanistic studies that can be completed with this system. Nonetheless, this alloimmunization model has provided useful information on antibody responses to transfused RBCs containing a clinically relevant human RBC antigen.

mHEL mouse model

The mHEL (membrane bound hen egg lysozyme) mouse, created by Goodnow *et al*.[26,27] in 1988 using a class I promoter, expresses mHEL on RBCs, white blood cells, platelets, and a number of tissues. RBCs from the mHEL mouse circulate with a normal lifespan, with stable expression of the mHEL antigen.[34] Transfusion of RBCs from the mHEL mouse into wild type C57BL/6 x B10.BR F1 recipients that lack mHEL on their RBCs creates a model of immunization to transfused cells.[35] The lysozyme contained on mHEL RBCs is not entirely foreign to wild type transfusion recipients, who themselves express mouse lysozyme with limited homology to mHEL.

Transfusion of stringently leukoreduced RBCs from mHEL donors into wild type C57BL/6 x B10.BR F1 recipients leads to a low level alloantibody response in a subset of recipients.[35] This alloantibody response is presumably an anti-RBC response; however, as is the case with the Tg-FYB mouse, the potential role of residual mHEL expressing WBCs and/or platelets cannot be ignored. The weak recipient anti-HEL response can be detected by sensitive HEL specific ELISA but not by flow cytometric crossmatching, nor by traditional blood banking methodologies (including gel card agglutination).[35] However, this response can be significantly enhanced with the adoptive transfer of additional HEL specific CD4+ T cell[36] help prior to mHEL transfusion.[37]

The alloantibody response to transfused mHEL RBCs requires the presence of a spleen, with splenectomized mice failing to make anti-HEL following mHEL transfusion.[38] Furthermore, splenectomized mice also fail to make anti-HEL when transfusion occurs following the adoptive transfer of HEL specific CD4+ T cells. The lack of response to the mHEL antigen in splenectomized mice is not due to an inability of these animals to respond to the HEL antigen in general, as they make a robust antibody anti-HEL response to a non-RBC source of mHEL (mHEL-linked to murine cytomegalovirus (mHEL-MCMV)).[38] The generalizability of the requirement of a spleen in alloimmunization to other RBC antigens (and/or to human RBC alloimmunization) remains unknown. Furthermore, the presence or absence of a spleen upon initial antigen exposure may play a pivotal role in future responses to that particular RBC antigen.

Consistent with the theory that an inflammatory "danger signal" of sorts is necessary for an immune response,[16] recipient inflammatory status has been hypothesized to play a role in RBC alloimmunization. In fact, murine recipients treated with the double-stranded RNA poly (I:C) prior to mHEL transfusion have significantly higher anti-HEL response rates and antibody titers than control recipients.[35] Poly (I:C) is viral-like stimulus that agonizes toll-like receptor (TLR) 3, promotes the survival of helper CD4+ T cells, and activates NK cells and anti-viral responses. The exact mechanism(s) by which poly (I:C) enhance alloimmunization, however, remain unclear. Although the majority of transfused RBCs are cleared by macrophages in the spleen in the non-inflamed state, treatment with poly (I:C) increases RBC consumption by dendritic cells in the liver and spleen. Furthermore, poly (I:C) increases co-stimulatory molecule expression on antigen presenting cells, leading to increased proliferation and division of adoptively transferred HEL specific CD4+ T cells.[37]

As expected, recipient anti-HEL alloantibodies bind to circulating mHEL RBCs. However, instead of inducing hemolysis of mHEL RBCs, bound anti-HEL antibodies lead to loss of the HEL antigen ("antigen-loss").[34] Thus, mHEL RBCs remain in circulation, even after being bound with anti-HEL alloantibodies. However, these RBCs cannot be identified using polyclonal or monoclonal anti-HEL in the days following transfusion, indicating an alteration in or loss of the HEL antigen. Antigen loss in the mHEL system requires the Fc gamma receptor III (FcγRIII) (presumably on a phagocytic cell), and also requires anti-HEL antibodies simultaneously recognizing two distinct epitopes (presumably with cross-linking of the target antigen).[39,40] This antigen loss phenomenon, also known as "depressed antigen," "antigen suppression," and "weakened antigenicity," is not unique to the mHEL system, having been described more than 20 times in humans in the Kell, Rh, Kidd, Gerbich, LW, and Cromer blood group systems.[41]

In sum, the transgenic mHEL mouse and companion HEL-specific T cell transgenic mice have provided unique insights into RBC alloimmunization. However, shortcomings include the expression of mHEL not only on transfused RBCs, but also on contaminating WBCs and platelets. Furthermore, although there is some homology between murine lysozyme and mHEL, recipients completely lack mHEL on their RBCs. This situation is unlike that observed most often in the human setting, where recipients and donors may express antithetic RBC antigens that differ by a single amino acid.

HOD mouse model

The transgenic HOD mouse was created by Zimring *et al*. in 2009.[28] This animal, generated using a B-globin promoter[42,43] and made on an FVB (H-2^{q}) background, has RBC specific expression of hen egg lysozyme, a portion (N-terminus) of ovalbumin, and the multipass human Duffy^{b} antigen. This animal lacks expression of HOD by flow cytometry on WBCs and platelets, thus resembling most authentic human RBC antigens, which are not expressed on non-RBCs. Furthermore, the inclusion of OVA in the construct allows for the application of a large number of immunologic tools, including CD4+ T cell transgenic mice (OTII, whose CD4+ T cell recognize a portion of OVA presented in I-a^{b})[44] and CD8+ T cell transgenic mice (OTI, whose CD8+ T cell recognizes a portion of OVA).[43]

The majority of mice transfused once with leukoreduced HOD RBCs make an anti-HOD response, with anti-HEL being the primary antibody response.[45] This response is more robust than the response to transfused leukoreduced mHEL RBCs, potentially due to extra CD4+ T cell help afforded by the inclusion of OVA in

the HOD construct. Furthermore, the robust response is detectable not only by sensitive HEL-specific ELISA, but also by flow cytometric crossmatching with HOD RBCs.

Similar to what has been described in the mHEL system, recipient pre-treatment with poly (I:C) significantly enhances alloimmunization to transfused leukoreduced HOD RBCs.[46] These data suggest that RBC expression of the antigen is sufficient for poly (I:C) to enhance alloantibody response. In addition to being enhanced in the presence of recipient inflammation with poly(I:C), the recipient response to transfused HOD RBCs is significantly enhanced by the presence of additional CD4+ T cell help (with either HEL (3A9) or OVA (OTII) specific CD4+ T cells). Finally, similar to what has been observed in the mHEL system, the response to transfused HOD RBCs is nearly completely blunted in the absence of a spleen.

However, unlike what has been observed in the mHEL system, LPS treatment of recipients prior to HOD transfusion does not inhibit anti-HOD alloimmunization.[46] In fact, treatment of recipients with LPS at multiple different dosages leads to enhanced alloantibody response to transfused HOD RBCs, in the presence or absence of additional CD4+ T cell help. This enhancement of alloimmunization magnitude, which is significantly lower than that seen in the presence of poly (I:C), remains statistically significant and reproducible. Ongoing experiments are attempting to define better the relationship between types of inflammation and RBC alloimmunization responses, with a working hypothesis that antigen expression on contaminating WBCs may be involved in the decreased response to transfused mHEL RBCs in the presence of LPS.

It is known that a number of microbes (e.g., Hemophilus influenza, Bacteroides, Vibrio Cholera) share amino acid sequences with RBC antigens.[47] Patients may be infected at some point prior to transfusion and thus in theory, may be primed to make a response to transfused RBCs with shared antigen sequences. In fact, mice infected prior to transfusion with polyoma virus containing an OVA CD4+ T cell epitope make more alloantibodies (anti-HEL) compared with mice infected with virus lacking this epitope or uninfected mice.[47] Although these data suggest that patients may indeed be primed to respond to RBC antigens through prior microbial exposure containing a shared CD4+ T cell epitope, current limitations of clinical blood banking methodologies (which focus on humoral and not cellular responses) would fail to predict such a response.

Human RBCs are rarely collected and transfused in the same day. Thus, a murine model of RBC storage was developed by Gilson *et al*.,[48] in part to compare immune responses of fresh and stored RBCs. HOD RBCs, collected in CPDA-1, leukoreduced, and stored for 14 days prior to transfusion, result in high levels of anti-HOD alloantibodies compared with freshly collected and transfused RBCs.[45] Additionally, high levels of the pro-inflammatory cytokines interleukin-6 (IL-6), KC (CXCL/1) and monocyte chemoattractant protein-1 (MCP-1) are seen in recipients following the transfusion of stored but not fresh HOD RBCs.[49] This enhanced response is not due to bacterial contamination of the units, nor is it due to altered HOD antigen expression over time, as gram stain/cultures are negative and flow cytometric evaluation shows similar levels of HEL, OVA, and Fy^3 expression on stored RBCs and fresh RBCs.[45] Furthermore, this effect is dose dependent, with two units of stored RBCs being significantly more immunogenic than one unit of stored RBCs and inducing higher levels of cytokines as well.[49] It must be mentioned that HOD RBCs, like most human RBCs, contain the human $Duffy^b$ antigen, which has been shown to bind interleukin-8, MCP-1, and RANTES.[12] The role of the Duffy antigen in any of the described findings within the HOD model system is unclear; however, its presence is consistent with protein biology of human RBCs.

HOD RBCs (on an FVB background) store more poorly than RBCs from mice on a C57BL/6 background, with higher degrees of hemolysis during storage and inferior 24 hour post-transfusion recovery rates.[45] The contribution of the rapid post-transfusion clearance to alloimmunogenicity is currently under investigation. Recent studies show transfusion of HOD RBCs treated with a chemical known to cause oxidant stress damage and rapid post-transfusion clearance (phenylhydrazine) results in high levels of recipient pro-inflammatory cytokines and high levels of alloimmunization.[50] Furthermore, transfusion of HOD RBCs treated in a non-oxidant stress manner (with heat treatment at 50° for 30 minutes) also results in high levels of recipient pro-inflammatory cytokines and high levels of anti-HOD alloantibodies.[50] As seen in the storage experiments, expression of HEL, OVA, and Fy^3 appear largely unaltered in phenylhydrazine or heat treated HOD RBCs. Thus, rapid clearance of damaged RBCs appears to be a risk factor for alloimmunization to the HOD antigen in mice, which raises the question of whether human donor RBCs with rapid post-transfusion clearance may be more immunogenic than RBCs with better post-transfusion recoveries.

In summary, the HOD model is the first model of RBC alloimmunization with RBC specific expression of a model antigen that allows for the use of a number of immunologic tools (including HEL and OVA specific CD4+ T cells, OVA specific CD8+ T cells, and HEL specific B cells). Once this model is fully backcrossed onto a C57BL/6 background, a number of additional immunologic tools and transgenic animals will be available. In addition to RBC alloimmunization studies, the HOD mouse is useful for studies of antigen loss, cellular immunity, and autoimmunity, the details of which are beyond the scope of this manuscript. Limitations of the HOD system include the antigen not being an authentic human blood group antigen and being present on donor RBCs yet entirely absent on recipient RBCs.

hGPA mouse model

The hGPA (human glycophorin A) mouse, described in 2001 by Auffray *et al*.,[29] was generated in FVB founders using a bacterial artificial chromosome clone containing the promoter region and entire genomic hGPA gene. Transgenic hGPA mice have RBC specific expression of human glycophorin A, with decreased expression of mouse glycophorin A on RBCs but stable expression of band 3.[29] Yu *et al*. have shown that multi-

ple transfusions of buffy coat depleted hGPA RBCs fail to lead to recipient anti-hGPA responses.[51] However, transfusion of buffy coat depleted hGPA RBCs mixed with unmethylated bacterial CpG dinucleotides (CpG ODN) (followed by weekly transfusions of buffy coat depleted hGPA RBCs in the absence of an agonist) leads to presumed anti-hGPA alloimmunization in some recipients. Similar to what has been observed in Tg-FYB alloimmunized recipients transfused with Tg-FVB RBCs, hGPA RBCs have a reduced circulatory half life in hGPA immunized recipients.

Yu *et al*. have also reported that depletion of regulatory T cells with anti-CD25 enhances anti-hGPA alloantibody responses, introducing the idea that recipient regulatory T cells play a role in RBC alloimmunization.[51] Subsequently, Bao *et al*. have studied regulatory T cell status in hGPA alloimmunized "responder" and "non-responder" recipient mice, finding reduced *in vitro* and *in vivo* Treg-suppressive activity in responders.[52] Furthermore, similar to the observation of "antibody pair" formation in humans,[10] they have reported that murine "responders" are more likely than "non-responders" to develop additional alloantibodies to a second immunogen (in this case Tg-FYB).

Besides being a useful model of RBC alloimmunization, the hGPA model has been useful to study the fate of incompatible RBCs.[53] Many monoclonal anti-hGPA antibodies are available, and passive immunization with IgG1 anti-hGPA monoclonal antibodies induces rapid clearance of incompatible transfused hGPA RBCs.[54] Furthermore, this rapid clearance is accompanied by a pro-inflammatory recipient cytokine storm.[55] Mechanistic studies investigating the role of the spleen, complement and activating FcγRs in this incompatible RBC clearance, are ongoing.

Limitations of the hGPA model, present in any mouse model utilizing donors that differ genetically from recipients, include difficulties in distinguishing anti-hGPA responses from anti-MHC responses. MHC-I is present on multiple cell types in mice, including RBCs, and also present on WBCs and platelets that may contaminate RBC preparations. Thus, careful controls are necessary to ensure observed alloantibody responses are in fact against transgenic RBC antigens and not against MHC-I. Accepting this limitation, a significant strength of the hGPA model includes hGPA being an authentic human blood group antigen. Furthermore, studies generated in the hGPA system have served as a springboard for ongoing translational experiments investigating the role of regulatory T cells in human alloantibody responses.

Future mouse models of RBC alloimmunization

Future directions in murine alloimmunization studies include the development of additional model systems with advantages not held by existing models. This includes the development of a model with donors and recipients expressing an RBC specific antigen that differ by a single amino acid, akin to human antithetic blood group antigens (e.g., E/e). Additionally, an Rh(D) transgenic animal, though technically challenging to develop, would also be quite useful. To increase the analytical power of such models, antigen specific CD4+ and CD8+ transgenic T cell companion mice would ideally be constructed in parallel, potentially along with tetramer reagents and B cell transgenic mice. Besides being useful in studying antibody responses and antibody coated RBC clearance mechanisms, the development of these additional mouse models could potentially shed light on the mechanisms of action of prophylactic anti-D (e.g., RhoGam) and could also lay the groundwork for a mouse model of hemolytic disease of the newborn.

Conclusions

In summary, much has been learned about RBC alloimmunization over the past few decades, and mouse models have proven useful as a supplement to existing knowledge about human RBC antigens and anti-RBC antibodies. Being cognizant of inherent limitations of model systems in general and of mouse models in particular, the Tg-FVB, mHEL, HOD, and hGPA mouse models of RBC alloimmunization have each provided insight into the immune sequelae of transfusion. These include a better understanding of both donor and recipient factors influencing rates of RBC alloimmunization, mechanisms of anti-RBC antibody formation, and clearance patterns of antibody coated RBCs. Furthermore, these model systems have generated discrete hypotheses and questions that are testable in humans (Table 1). For example, ongoing human studies are investigating the impact of regulatory T cells on RBC alloimmunization response rates in patients with sickle cell disease. Additionally, human clinical trials investigating the relationship between recipient serum cytokine profiles or gene polymorphisms and responder/non-responder status have been proposed.[56] Lastly, the effects of stored human RBCs are currently being examined in multiple prospective randomized clinical trials.[57–60]

Table 1. Clinically relevant questions raised by murine models of RBC alloimmunization.

1)	Does the inflammatory status of the human recipient play a role in their immune response to transfused RBCs?
2)	Do recipient gene polymorphisms influence RBC responder/non-responder status?
3)	How do recipient regulatory T cells impact rates and degree of RBC alloimmunization?
4)	Are older, stored human RBCs more immunogenic than freshly collected and transfused RBCs?
5)	Are leukoreduced human RBCs less immunogenic than non-leukoreduced human RBCs, and if so what is the mechanism behind this?
6)	Is a spleen required for RBC alloimmunization in humans? (realizing the presence of the spleen on initial exposure to the antigen may be critical)
7)	Can humans be primed for RBC alloantibody responses by prior exposure to pathogens containing CD4+ T cell epitopes shared with RBC antigens?
8)	How common is antigen loss in humans, and how often is it not recognized due to lack of awareness?
9)	How are incompatible transfused RBCs cleared in humans, and how can sequelae of incompatible transfusions be minimized?

Goals of future murine studies include a refined understanding of questions generated in both human and murine systems, as well as an ongoing generation of novel hypotheses to be tested in humans. The ultimate goals of both murine and human RBC alloimmunization studies include the development of rational strategies to minimize rates of alloimmunization formation, and the development of strategies to minimize complications in patients with existing anti-RBC alloantibodies. These goals can best be accomplished using the combined strengths of human and murine systems, in a bench to bedside and back or bedside to bench and back model.

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Relevance of RH variants in transfusion of sickle cell patients

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A B S T R A C T

Transfusion remains the main treatment of sickle cell disease (SCD) patients. Red cell allo immunization is frequent because of the antigen disparities between patients of African descent and donors of European ancestry. Allo immunization is associated with severe hemolytic transfusion reaction, auto antibody formation, and difficulties in the management of transfusion compatibility. Beside common antigens, a number of different RH variant antigens found in individuals of African descent can be involved in allo immunization. If some variants, such as Hr^S negative antigens, are known to prone significant allo antibodies and DHTR, it is not clear whether all the described variants represent a clinical risk for SCD patients.

The knowledge of the clinical relevance of RH variants is a real issue. An abundance of molecular tools are developed to detect variants, but they do not distinguish those likely to prone immunization from those that are unlikely to prone immunization and DHTR. A strategy of prevention, which generally requires rare red blood cells, cannot be implemented without this fundamental information. In this review, we discuss the relevance of the RH variants in SCD based on the published data and on our experience in transfusion of SCD patients.

Introduction

Blood transfusion is a cornerstone of the management of sickle cell disease (SCD).[1] The goals of blood transfusion are to increase oxygen distribution to tissues, and/or to replace rigid sickle-shaped red blood cells (RBCs) by deformable RBCs. However, blood transfusion in patients affected with SCD is associated with a high rate of delayed hemolytic transfusion reactions (DHTR). One major cause of these reactions is the development of allo-antibodies to RBCs promoted by the high polymorphism of blood group antigens between the patients of African ancestry and the donors primarily of European descent.[2] In these situations, the rate of allo-immunization is about 10 to 45%.[3-5] It is likely that in Africa, where there is homogeneity between donors and patients, the rate of allo-immunization is lower.[6] The common antigens expressed in donors and causing allo-immunization in patients are well known. They are C and E in the RH blood group. Few individuals of African descent express C and E when they are D positive. In Caucasians, the presence of D antigen is always accompanied by the presence of either C or E antigen. In the other blood groups, the disparities are mainly for Fy^a, Jk^b, and S antigens. In every country with a European background, there is a shortage of matching units. Limited antigen matching for E, C, and Kell has become the standard of care.[7] Matching for the other antigens is generally extended when allo-antibodies are developed. This approach reduces the rate of allo-immunization considering that patients who form antibodies show an increased risk for additional allo-antibodies upon further transfusion exposure.[8] It remains that this approach does not take into account the numerous RH variants that are encountered in these patients of African ancestry, representing potentially an additional risk for allo-immunization and DHTR. They are encoded by altered alleles at the RH locus. The RH variants may prone allo-immunization when the antigen is incomplete (or partial) and when the carrier is exposed to the complete antigen through transfusion or pregnancy.

It is important to mention that it is not clear which RH variants induce allo-immunization when the carrier of the variant is exposed to the normal antigens. It also remains unclear which allo-antibodies to RH variants are clinically significant. This knowledge is a major issue to determine which variants have to be considered in the prevention of allo-immunization and DHTR. The RH variants have been widely described. There is a consensus to recognize DNA analysis as the best tool to detect and characterize RH variants.[9-11] In the last years, industrials have developed many tools to detect variants. They are qualified as "high throughput genotyping" because of the numerous detected alleles in one assay.[12,13] With those commercial tools, all biologists can type variants for SCD patients. Therefore, they are supposed to assist the clinician to make an informed decision

regarding selection of units. However, they remain frequently puzzled because they do not really know the clinical significance of the variant they have found. After a long period of describing new variant alleles, the next goal is to determine which variants represent a risk for the SCD patients frequently exposed to foreign antigens, to adapt DNA-based typing to the clinical situation,[14] and to implement prevention only when necessary.

The RH blood group and the variants

The RH blood group is composed of two highly homozygous genes, the *RHD* and the *RHCE* genes.

The *RHD* gene produces the D antigen, and the *RHCE* gene produces a polypeptide carrying two antigens: C or c and E or e.[15] In Caucasians, the main haplotype is *DCe*, in Afro-Americans and Afro-Caribbeans, the main haplotype is *Dce*.[16] The result of this distribution is a high frequency of SCD patients with the D+C-E-c+e+ phenotype. Only 20% of patients express the C antigen.[5] Within the five main antigens (D, C, E, c, e), schematically, two types of variants are described, and they are encoded by point mutations, multiple missense mutations or hybrid alleles.[17] First, those named partial, because they lack some immunogenic epitopes, as shown by allo-immunization of the carriers against missing epitopes when exposed to the complete antigen through transfusion or pregnancy. Second, are the weak antigens. Individuals carrying weak antigens do not get immunized when exposed to the normal antigen. Therefore, a carrier of a partial antigen should receive RBCs which do not express the antigen to prevent allo-immunization. When a new variant is discovered because the carrier has developed an antibody, the variant is classified as partial on an immunological point of view. When a new variant is described, because of a weak reactivity, it is much more difficult to decide the category.[18] There is a consensus to classify the variant based on the predicted localization of the substitution. The Rhesus index determining the antigen density can help also to decide.[19] Schematically, when localized at the outer surface of the membrane, the variant is considered partial, when localized in the intra membrane or in the intra cellular domain, the variant is considered only weak. But it has been shown that carriers of some variant categorized as weak did get immunized. It is the case for the weak D type 11, type 15, and also for the weak D type 4.2, which is found in SCD patients.[20,21] Then, as stated by the Bristol team, the weakD/partial D dichotomy is artificial.[18] Therefore, the risk of allo-immunization in a "variant" situation is fundamental to know to manage safely and efficiently the transfusion of the carrier. There is already a register for anti-D immunization (http://www.uni-ulm.de/~wflegel/RH/), but there are no data regarding the clinical significance of the antibodies. There is no register for RHCE variants prone to immunization.

The RH variants in SCD patients and the associated risk of allo immunization

Sickle cell disease patients are highly polymorphic at the RH locus compared with individuals of European ancestry. In the *RHD* gene, the first difference is the molecular basis of D-negative individuals. As compared with D-negative European individuals who display mainly a deletion of the *RHD* gene,[22] D-negative individuals of African ancestry exhibit a silent gene produced either by a 37 base pair insertion that lead to a premature stop codon or a hybrid *RHD-CE-D* gene characterized by the production of a partial C antigen but no D.[23,24] These differences do not bring any specific risk for SCD patients, as serologically there is no difference between D negative individuals from European or African ancestry. This background has to be considered only when determination of D is based on DNA-based typing methods. The variants that have to be taken into account are those prone to allo-immunization. They are the partial variants of the five main antigens (D, C, E, c, e), the variants characterized by absence of expression a high frequency antigen, mainly produced by abnormalities within the *RHCE* gene, and variants characterized by the expression of a new antigen (low incidence antigens). Management of prevention of allo-immunization can be extremely difficult in certain situations because of the shortage of identical blood supply.

The partial D variants (Figure 1)

In Europeans, 1 to 2% carries *RHD* variant alleles, which can produce weak D with no risk of allo-immunization or partial D. In most of the cases, a D antigen with a weak expression is frequently due to the weak D type 1, 2, and 3 alleles, which are not prone to anti-D immunization.[21] There are some major differences within D variants among individuals of African ancestry: firstly, the frequency of D variants is probably much higher; secondly, a D antigen with a weak expression is frequently a partial antigen prone to anti-D immunization; thirdly, altered *RHCE* alleles are generally associated with altered *RHD* alleles. We show the results of the investigation of weak D samples from Afro-Caribbeans that have been referred in our laboratory in 2010 (Table 1A). These results can be extrapolated to our SCD population in term of the incidence of the variants that are detected because of a decreased expression. In the weak D type 4 cluster, many variants have been documented to prone anti-D in the carriers.[21] In this category, the *DAR* allele (*weak D type 4.2.2*) is inherited with *ceAR* allele, and in some cases, with other similar alleles (*ceEK* or *ceBI*). In the DIVa cluster, the *DIII* and *DIII type 5* alleles can appear normal or depressed, and are frequently inherited with *RHce* variant alleles, mainly the ce^S allele.[25] The *DAU* allele cluster produces also weak D antigens, which are partial for some of them.[26]

It is well known that D antigen is the most immunogenic antigen, but in case of partial D, the rate of allo-immunization cannot be compared with the rate in D-negative patients receiving D-positive units, as only one part of the epitopes of the complete antigen is missing. Some variant remained controversial regarding the allo-immunization risk. It is the case for the *weak D type 4.0*, as well as for the *DAU-0*, which are not supposed to prone anti-D allo-immunization.[26,27] The *DAU-0* allele is very frequent in some populations of Africa, reaching almost 20% in Mali.[28] The antigen and Rhesus index of the DAU-0 phenotype are about normal, rendering it indistinguishable from the normal antigen-D-positive

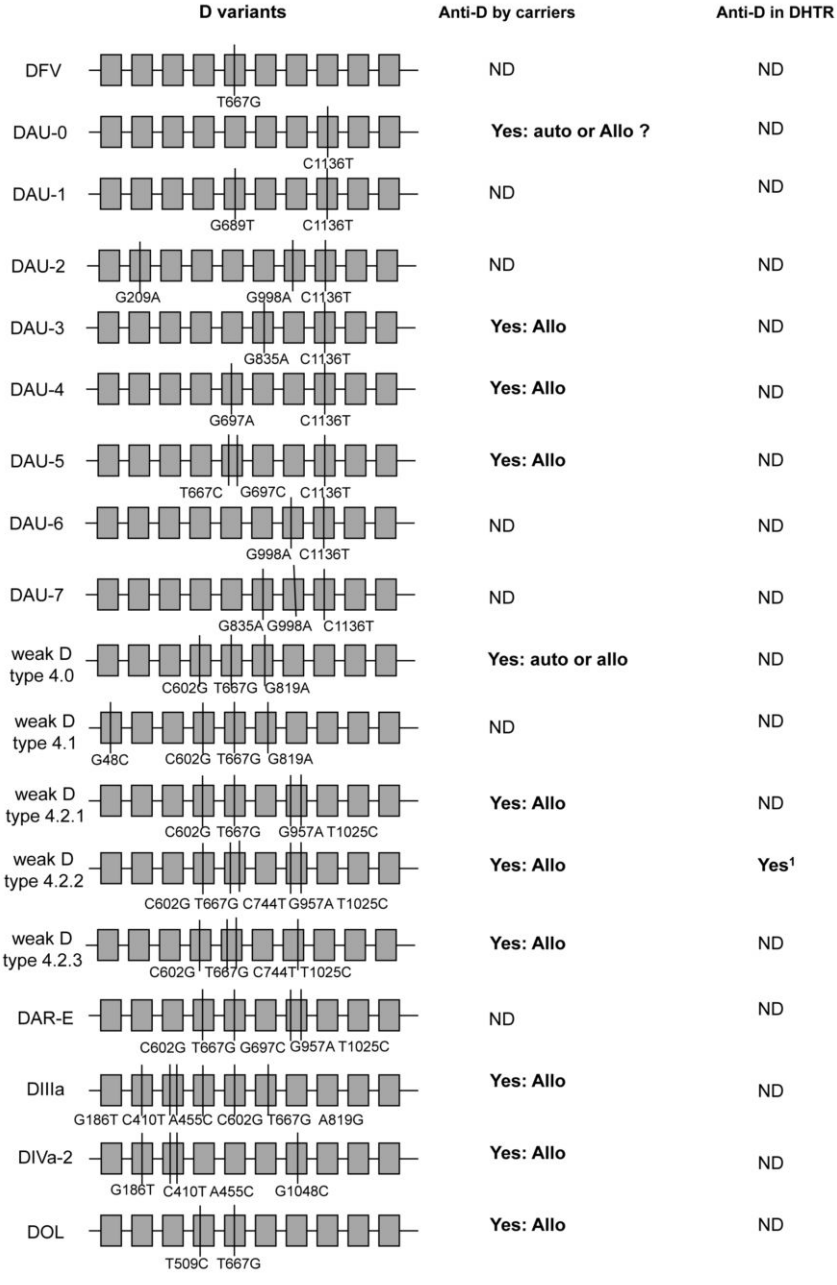

Figure 1. The *RHD* alleles encoding D variants in individuals of African descent are represented. The possibility of occurrence of antibodies in carriers is indicated and is based on published reports, this report, and the Rhesus Immunization Registry (for anti-D only) (http://www.uni-ulm.de/~wflegel/RH/).
ND: not documented.
[1] The antibody was found among other antibodies.

phenotype.[26] In 2010, we investigated 17 SCD patients with anti-D. Among them, one patient carried *weak D type 4.1*, and two patients carried *DAU-0*. One patient carried a new *RHD* allele with an 814 G>C mutation in exon 6, encoding a val281leu substitution on the RhD polypeptide. We found also anti-D producers without any abnormalities (48%) in the *RHD* gene (Table 1B). In SCD, the inflammation is a specific feature of the disease. It is known in mice that inflammation favors the presentation of foreign peptides by professional cells. Therefore, the inflammation status can make SCD patients more suitable to immunization when they are exposed to missing epitopes of variants with low risk of immunization compared with patients in a steady state clinical situation.[29] For other partials, there is no doubt regarding the risk of anti-D allo immunization. In a recent study, among 39 patients with *DIIIa*, 18 had allo-anti-D.[25] The *DAR* carriers are also prone to anti-D allo immunization.[30,31] The anti-D immunization risk in DAR has been estimated to be lower than 1% per D+ transfusion in SCD. This risk is likely to be lower for the other D variants.[31] Considering that there is a risk of allo immunization for many D variants, their detection prior to immunization could be performed on a routine basis in SCD patients as a part of the initial typing, at the same level as the complete phenotype. DNA analysis is the most adequate technology because serology does not detect all D variants. However, considering that all D variant patients transfused with D-positive units will not develop anti-D, the question whether these patients should receive D-negative units as a way to prevent anti D immunization is a real issue because of serious consequences. The first consequence would be to shorten the supply of D-negative RBCs, already extensively used for

Table 1A. RH genotype of individuals of African descent with weak D antigen.

Weak D type 4.0 or 4.1	89
Weak D type 4.2.2 or 4.2.3	100
DIII type 5	1
DVIa2	1
DLO	1
DAU-2	5
DAU-5	4
DAU-4	3
DAU-4 + DHK	1
Total	205

Table 1B. RH genotype of SCD patients who developed antibodies.

	Patients with anti-D	Patients with anti-e like
Normal genes	8	6
Weak D type 4	1	
Weak D 4.2.3	1	
DIVa-2	1	
DAU-0	2	
DAU-3	2	
DAU-5	1	
RHD: 841G>C	1	
ce(667)		1
ce^{s}(48)		1
ce^{s}(697)		1
ce^{s} HMZ		1

SCD patients to prevent anti-C and anti-E immunization. We showed that 26% of the units transfused to the D+C-E-c+e patients were D-C-E-c+e+.[5] The second consequence would be to increase the exposition of the patients to the common antigens (Fya, Jkb, S) expressed by the D-negative RBCs that are mainly from Caucasians donors. These antigens could represent a higher risk of allo-immunization and DHTR, compared with the risk encountered by the RHD variant status of the patient. Epidemiologic data with a statistical approach are needed to balance these different risks and demonstrate the real impact of D variants in term of allo-immunization, but also in DHTR.

The partial RHCE variants (Figure 2)

Within the *RHCE* variants in individuals of African descent, partial antigens are frequently encountered, which is not the case in the European population, where no altered antigen has been documented with antibodies. As compared with *RHD* variants in SCD patients, *RHCE* variants present additional complications. Firstly, for many variants, it is still controversial whether they are prone to immunization. The serological data that would confirm the "allo" character are difficult to obtain because of the multi transfused and multi allo-immunized state of the patients, but also because the occurrence of auto antibodies with the e or Ce "like" specificity is common in SCD. Therefore, it is likely that, considering the frequency of *RHCE* variants, auto-antibodies can be produced concomitantly with the expression of a variant, but not prone by this variant. Secondly, *RHCE* variants, when they are at the homozygous state, are very difficult to manage in term of prevention of allo-immunization and DHTR. Taking the case of a partial e allele (*ceMO* allele) at the homozygous state (D+C-E-c+e+), prevention of allo-immunization will require transfusion with RBCs with a similar molecular background, as e-negative RBCs are always E-positive (e and E antigens have an antithetical relationship). The supply is extremely rare and cannot be compared with the D-negative supply required for the partial D carriers.

Some *RHCE* alleles are well known to produce variants prone to immunization. The *ceMO*, and *ce^{s}(340)* alleles produced partial e.[32] The *ceAR* allele represents the main molecular background of the Hr^{s} negative phenotype. The Hr^{s} negative phenotype can be also encoded by other alleles, such as *ceBI* and *ceEK*. The difference between negative Hr^{s} phenotype and partial e is that the abnormality is not restricted to the partial e situation; the associated antibody recognizes the entire Rhce polypeptide. The complication of these variants is that serologically, antibodies produced by carriers of the different type of e variants and Hr^{s} negative variants may cross react.

Then, the safest way to transfuse those patients when they developed antibodies is to give RBCs with the same molecular background. This is especially true for the *ceAR* situation, which has been documented to induce DHTR.[32] The additional complication with *ceAR* is the association to the DAR variant prone to anti-D immunization. Recently, it has been shown that *ceAR* in a C+c+ SCD patient was also a risk for anti-c immunization.[33]

Other *RHCE*ce* alleles encoding partial antigens have been described in individuals of African descent. The *RHCE*ceCF* encodes both partial c and partial e, the *RHCE*ce 48C, 733G* with nucleotide 941C in exon 7 encodes partial e.[34,35] The *RHCE*ce^{s}(340)* allele with a JAL+ phenotype, already known to encode partial e, has been shown also to produce an altered expression of the c antigen allowing the patient to make an allo anti-c.[36] The occurrence of anti-e in e-positive SCD patients is not uncommon. In our cohort of 1065 e-positive patients, 4% produce anti-e like antibodies. This result has to be compared with the 1% occurrence of anti-D in D-positive SCD patients in the same cohort. When looking to the 10 referred samples of SCD patients for anti-e investigation in 2010, only 4 showed abnormalities in the *RHCE* gene. We could not get (except for one) any serological proof of the link between the abnormality and occurrence of the antibody (Table 2).

Regarding the C antigen, only two haplotypes have been shown to encode partial C in C+c+ patients: the *(C)ce^{s}* and the *RN*. In a retrospective study, we showed that among a cohort of C-positive SCD patients with history of transfusions, 30% were partial C, and within these partial C individuals, 30% developed anti-C, mainly those with the *(C)ce^{s}* background. Based on this result, we have decided to genotype all C+ SCD patients in order to know their C status, and to transfuse C partial individuals with C-negative RBCs, as the anti-

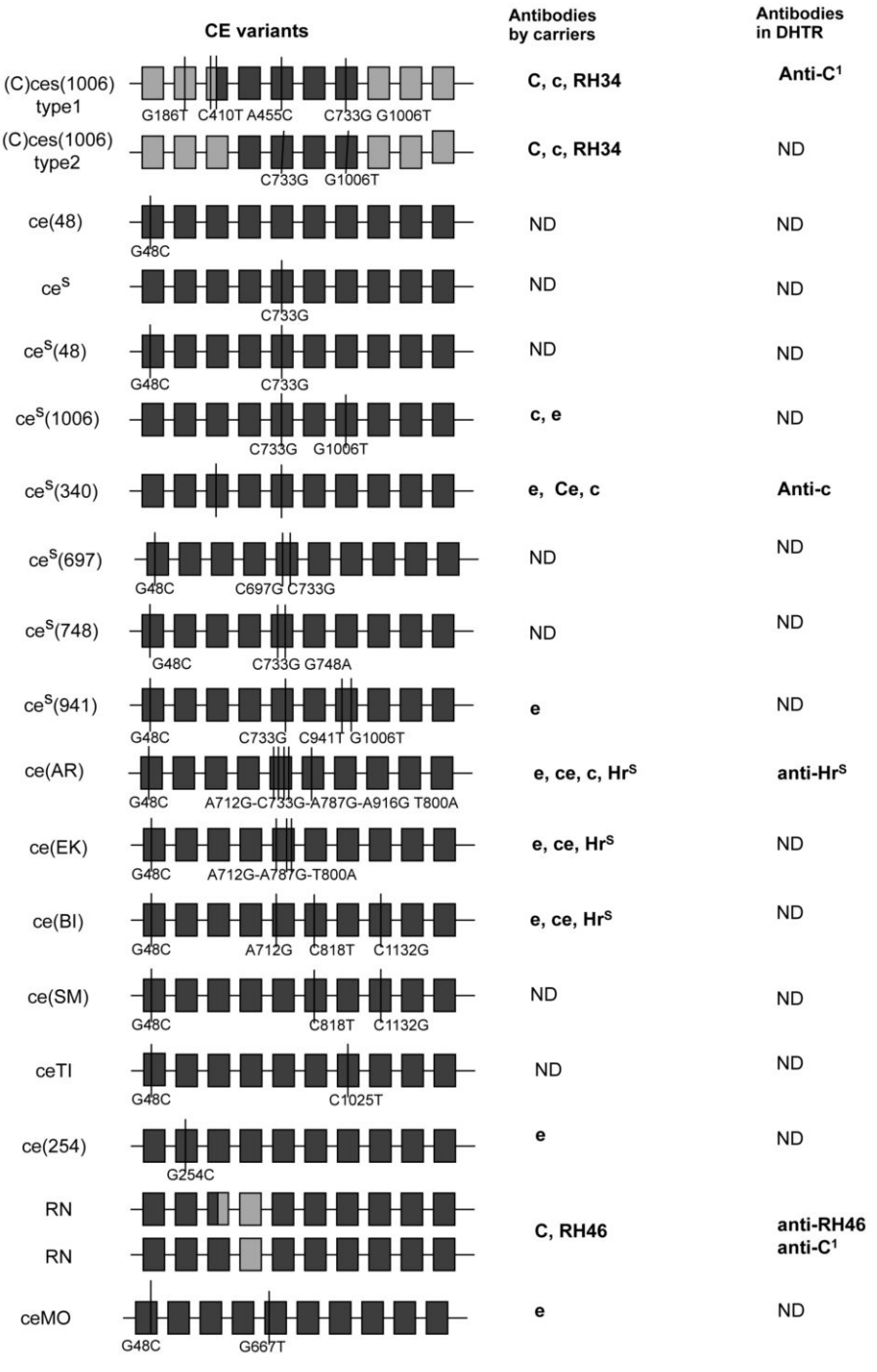

Figure 2. The *RHCE* alleles encoding CE variants in individuals of African descent are represented. The antibodies prone by the variant and their clinical significance are indicated when documented in published reports.
[1] The anti-C was found after DHTR. Then the real involvement cannot be proved, as the antibody was not eluted from the transfused RBCs.

body has been involved in DHTR.[37] The *(C)ce*^{S} has been associated with a partial c in one case, in which an anti-c was isolated from a mixture of antibodies.[38] In all reported cases, the "allo" feature may be questioned when many serological manipulations have been performed to isolate the antibody. Of greater concern are the rare phenotypes encoded by *(C)ce*^{S} and the *RN* when they are present at the homozygous state. The *RN/RN* background encodes RH: 32,-46 and the *(C)ce*^{S}*/(C)ce*^{S} background encodes the RH:-34. Both these phenotypes, plus the RH:-18 phenotype and the homozygous partial e are real issues in the management of transfusion in SCD patients. They are known to prone alloimmunization, and supply is very rare. Therefore, alternative treatments, such as bone marrow graft, should be always discussed, balancing the risk of DHTR and the risk of the graft procedure.

The low incidence RH antigens

Substitutions on the Rh polypetides can encode antigens with missing epitopes as described in the partial situation, but can also cause creation of new epitopes. These new epitopes can induce production of allo-antibodies in a carrier who does not present the abnormali-

ty. These new epitopes are called "low incidence antigens" relatively to their low incidence in the Caucasian reference population. In the Black population, some of them are frequently expressed, such as the RH20 (VS) antigen, encoded by the *RHCE*ce^{S}* allele. Considering that this antigen is expressed in about 40% of individuals of African descent, a VS-negative SCD patient receiving blood from a donor with the same ethnic background can be exposed and get immunized to VS. Whether these antigens are immunogenic and clinically significant is also a real issue. Data are lacking on the subject. Antibodies against VS are not detected by the screening test because in most cases, RBC tests do not express VS. Then, it is quite difficult to evaluate the occurrence of immunization in case of mismatch. A prospective study on occurrence of immunization against VS with determination by DNA analysis of VS in both donors and recipients would be required to evaluate the risk, and decide whether a prevention strategy is necessary. The other described low incidence antigens in the RH blood group are linked to low incidence variants. Therefore, it is not likely that exposition of SCD patients occur frequently. It remains that a case of DHTR has been reported in a SCD patient who received RBCs from a partial DIVa donor who expressed the associated Go^{a} low frequency antigen.[39] In cases of DHTR without detectable antibodies, the possibility of the involvement of antibodies against a low incidence antigen have always to be questioned, but is difficult to demonstrate. Many low incidence antigens are described in Blacks: the RH32 encoded by the *RN* haplotype, the DAK encoded by *DIIIa, DOL*, and *RN*.[40,41]

Clinical significance of the antibodies linked to RH variants in SCD (Figures 1 and 2)

For all the RH variants, the main question in case of occurrence of an antibody is finally its clinical significance. In the published cases, few "variant"' situations have caused transfusion fatalities. They have been mainly encountered in patients lacking the Hr^{S} and RH46 high incidence antigen. We described two fatal cases with the *DAR-ceAR* haplotype at the homozygous state in SCD patients. They produced both anti-RH18 and anti-D.[32] Those cases have pointed out the need to store RH:-18 RBCs in the frozen rare bank. Other fatal cases with anti-Hr^{S} and anti-RH46 were described in the eighties.[42] Clinical significance has been shown for an anti-c developed by an African American woman with *ce^{S}(340)* at the homozygous state.[36] For the other variants, except the anti-Goa case, there is no published data on the occurrence of DHTR with demonstration of the responsibility of an antibody prone by a partial in the carrier or prone by another low incidence antigen in the donor. In some published cases of DHTR, some antibodies prone by variants are developed in a mixture of antibodies and are sometimes detected following the DHTR.[5,37,43,44] Within eight cases of DHTR in SCD children, we found one partial C (encoded by a *RN)* patient who experienced DHTR with production of anti-C at distance of the episode[45] Data are dramatically lacking regarding the involvement of variants in DHTR. One reason could be the rarity of the situation. It is specially the case for the anti-e like antibodies produced by e variant carriers. In some severe cases of DHTR in SCD, many patients are being identified who have developed red cell auto-antibodies. It is possible that in some cases, the auto antibodies have been mislabeled because the laboratory could not perform molecular analysis to type for variants. It is important that the biologist keep in mind that an auto-anti-e or a pan agglutinin could be a real allo antibody linked to variants carried by the patient. Therefore, the reasons for the absence of data regarding occurrence of DHTR in RH variant patients can be the absence of careful laboratory evaluation in case of DHTR, but can also be a low clinical relevance of variants. Considering the second reason, what would be the point to detect variants and to take into account the associated antibodies if only few of them reveal a clinical relevance? This question is a real issue as many costly tools are developed to type RH variants, and management of transfusion in a variant carrier can be very tricky.

However, if not directly involved in DHTR, variants could eventually promote allo-immunization to clinically significant antibodies (Fy, Jk, S) or to auto-antibodies that can compromise their clinical care. Some authors defend the hypothesis that occurrence of one antibody is a risk factor for the occurrence of many more antibodies.[8] Zimring *et al*. proposed an interesting hypothesis in which non exofacial polymorphisms (NEPs) contribute to the immunogenicity of blood group antigens, and provide a mechanism by which transfusion can lead to anti-RBC auto-antibodies.[46] Many of the RH variants encountered in individuals of African descent have NEPs.

Other intriguing questions about RH variants and SCD transfusion

In case of investigation of anti-e like antibody in SCD transfused patients, in our experience and in experiences of others, it happens that DNA analysis shows the presence of an altered allele, frequently the *ce^{S}* allele or the *(C)ce^{S}* haplotype, and in trans a normal *ce* or *Ce* allele.[9,47] From an immunology standpoint, there is no reason for the patient to produce an anti-e alloantibody. In other words, the anti-e like antibody cannot be considered as an allo antibody in a case of heterozygous abnormality compensated by a normal haplotype in trans. One case has been recently described in which the result of DNA analysis has contributed to hematopoietic stem cell transplantation.[48] The patient produced anti-e like antibody but carried only one *(C)ce^{S}*. The authors concluded to an allo antibody despite a normal *Dce* haplotype *in trans*, based on serological absorption studies. It remains that this antibody did not induce DHTR in the child, and disappeared during the bone marrow graft procedure. This case points out the real need to implement, not only a register for allo-immunization but also a register for clinically significant antibodies. In this time of development of DNA analysis, it became a real issue to define the features of a significant antibody in SCD patients, especially those linked to RH variants.

The second tricky situation is when the patient is found with a heterozygous composite background, the two different variants alleles encoding a known partial

antigen. Cases can be encountered during the investigation of a weak antigen. As exampled, it is frequent to find the presence of both *ceMO* and *ceAR*, or both *(C)ce*^{s} and *ceAR*.

We have no response to the question whether these heterozygous backgrounds in patients will prone allo immunization with a clinically significant antibody. Whether the expressed epitopes encoded by one allele may compensate the missing epitopes of the antigen encoded by the other variant (and vice versa) is not known. One reason is probably that transfused patients in this situation are rare. There is no data showing DHTR in SCD with these types of backgrounds. Since we do not know, and based also on our experience with SCD transfusion, we do not share the idea that a molecular compatibility should be performed for patients with variants that have not been implicated in a DHTR. This excess of precaution will introduce another high risk for SCD patients: the absence of transfusion when transfusion is the only option.

Conclusions

The relevance of variants in SCD has been demonstrated for a few cases of molecular backgrounds. In all other cases, even though the production of antibodies has been associated with the presence of the variant in the patient, there is no data on the clinical relevance of these antibodies. A register on DHTR with involvement of variants should be implemented. The point that could be eventually relevant considering these variants is the possibility that allo immunization to variants could prone allo immunization to other significant antibodies, frequently encountered in SCD DHTR, or to significant auto antibodies. In case of such a demonstration, it could be advice in some cases to consider variants in the choice of the units. Studies are needed to explore these hypotheses. In this prospect, molecular biologists, immunologists, and serologists have to work together. With the development of costly procedures to type variants, it becomes a real issue to determine the exact relevance of variants in the transfusion of SCD patients.

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Immunologic responders to red blood cell transfusions

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A B S T R A C T

Alloimmunization to RBC antigens is a significant risk factor for future transfusions and pregnancies. Analysis of patient databases strongly suggests that there is a distinct group of potential immunologic responders to RBC transfusions. This group, comprising 13–20% of the general patient population, is most likely genetically determined and is not related to disease. However, even potential immunologic responders do not respond to most transfusions. Though it is unclear what factors determine which RBC transfusions will induce an immune response, animal models suggest that inflammatory signals may contribute to responsiveness to individual transfusions. When a patient responds to one antibody, he or she is more likely to make additional antibodies, but development of those additional antibodies is a stochastic process. Youngest patients have the broadest repertoire of antibody responses. The anti-RBC antibody repertoire gradually declines with age.

Risks associated with anti-RBC antibodies

Allogeneic red blood cell (RBC) transfusions expose patients to dozens to hundreds of foreign antigens that are exposed on erythrocyte membranes. Remarkably, RBC transfusions do not usually result in a detectable immune response and transfused RBCs usually have a normal lifespan. Occasionally, however, an antibody response to one or more antigens occurs. Such an immune response can be dangerous for the patient, risking delayed hemolytic transfusion reactions, and/or hemolytic disease of the fetus and newborn.

The severity of a delayed hemolytic transfusion reaction depends in large part on whether the reaction is a primary immune response or whether it is a secondary immune response. If the response is a primary immune response, then the patient can develop a relatively mild delayed hemolytic transfusion reaction. However, secondary amanestic responses can be relatively rapid, and strong responses can cause severe delayed hemolytic transfusion reactions that in rare cases, can be fatal.

Approaches to prevent risks associated anti-RBC antibodies

Blood banks have procedures to avoid severe delayed hemolytic transfusion reactions associated with secondary immune responses. These procedures are generally aimed at detecting and identifying RBC alloantibodies prior to RBC transfusions and avoiding transfusing RBC units expressing the corresponding antigens.

Although current blood bank approaches prevent the most potential serious delayed hemolytic transfusion reactions, they do not prevent all such reactions. One deficiency in the current system concerns the fact that antibody screens are usually only performed prior to the anticipated transfusions, not in the weeks following RBC transfusions. Tests performed several weeks after a transfusion would be the most sensitive in detecting transfusion induced antibodies.[1] Instead, however, standard practice usually involves waiting until a transfusion itns anticipated to perform an antibody screen. Unfortunately, this may not detect prior immune responses because anti-RBC antibody titers often wane over time, often to undetectable levels after several years.[2] Although antibodies can wane over time, memory lymphocytes can persist throughout an individual's life. These memory lymphocytes can mount a rapid, vigorous response, resulting in a severe delayed hemolytic transfusion reaction even when no detectable antibody was present prior to a transfusion.

In addition to failing to prevent severe delayed hemolytic transfusion reactions, protocols primarily aimed at avoiding secondary immune responses to transfused erythrocytes ignore the risks posed by some antibodies to women and girls with child bearing potential. Some IgG antibodies can be transported across the placenta and react with fetal erythrocytes, resulting in hemolysis. The resulting clinical syndrome, hemolytic disease of the fetus and newborn, can be severe but rarely occurs during the first pregnancy of a non-transfused woman.

Recognizing the risks posed by primary immune responses to erythrocyte antigens, most modern transfusion services have adopted protocols to reduce the incidence of primary immune responses. Most transfusion services have minimized transfusion of

the antigens most likely to induce immune responses in susceptible individuals. Specifically, Rh(D)- patients generally receive Rh(D)- RBC units. Additionally, some centers transfuse K-negative RBC for females of child bearing potential and some match the K antigen and other select antigens for patients with hemoglobinopathies, such as sickle cell disease. Traditionally, such efforts have been difficult and costly, in large part because of the expenses incurred in performing extended erythrocyte phenotypes of patients and donor units.

Recent advances in molecular techniques are reducing expenses associated with determining extended erythrocyte phenotypes. As the costs decrease, RBC unit inventories will likely limit the ability to provide extended matched RBC units for patients.[3] Even if extended phenotypes are determined for all RBC units in the inventory, differences in the extended RBC phenotypes between patients and RBC units will preclude provision of perfectly matched RBC units for all patients. However, if extended matched units are provided for only a subset of patients who receive higher priority, then there is a higher likelihood that most of those patients can receive well-matched RBC units.

Analyzing immune responders

One approach for prioritizing extended matched RBC units would be to identify patients most at risk of immunologically responding to RBC transfusions. Such a question attempts to identify variability in the human immune response. Such a fundamental question follows a long history, in which blood transfusions have been used to explore the human immune response, both in animals and humans. Indeed, blood incompatibilities were some of the first indications of the presence of an immune response to foreign substances.

One approach to understand the human immune response to red blood cells is to analyze large patient data repositories to identify patterns of antibody formation. To understand the risk of antibody development and the risk of developing multiple antibodies, we studied the relationship between the number of transfusions and the number of patients who have made at least one anti-RBC antibody. The data weakly suggest that approximately 20% of patients make at least one antibody once they have received at least 20 RBC transfusions.[4] However, the data do not fit a line or curve very well, and the relationship between transfusion count and antibody development appears very weak, suggesting that while most transfusions do not induce antibodies, many transfusions that do induce antibodies induce more than one.

Another approach is to analyze the number of antibodies that develop in people who have made at least one antibody. Within this group, we hypothesized that development of each antibody is independent of development of other antibodies. This hypothesis assumes that all antibodies have an equal chance of developing. While this is not precisely true, the relative chances of developing different non-ABO, non-Rh(D) antibodies lie on a continuum so that the chance of developing an antibody is not that different from developing the next antibody. Hence, this hypothesis may be true for the overall population within the approximations inherent to retrospective data analysis.

Mathematically stated, this hypothesis states that the probability of developing (N+1) antibodies is some probability, p, of the chance of developing N antibodies for all values of N. This would mean that if M patients develop N antibodies, then pM patients would develop (N+1) antibodies.

We fitted the exponential decay curve predicted by this hypothesis to the data at two study hospitals. One was a tertiary care adult hospital from which data was obtained for a 15-year period and the other was a pediatric hospital. The adult and pediatric hospitals transfuse approximately 35,000 and 11,000 RBC units to approximately 6,625 and 2,750 patients annually. At a tertiary care adult hospital, the data fit the decay curve well, with a p value of approximately 0.3, meaning that approximately 30% of the patients who make N antibodies make N+1 antibodies (Figure 1). The same findings were made for a pediatric hospital and when analyzing the data previously reported for a large general hospital.[5] In all cases, antibodies appeared to develop independently of each other with a value of p of approximately 0.3. The only deviations to the curves were those that suggested that there may be a very small number of super-responders who make an exceptionally high number of antibodies. However, so few people appeared to be super-responders that the results were not statistically significant.

These results can be used to estimate the percentage of people who are potential responders. If 30% of the numbers of people who make N antibodies make N+1 antibodies, then 30% of the number of people who make zero antibodies should make one antibody, assuming there is nothing exceptional in making the first antibody. However, data from our adult hospital and from other reports suggest that 4–5% of people make at least one red blood cell antibody following transfusion or RBCs.[5] This suggests that 13% of people are potential responders, since if 13% of the patients make at least one antibody 30% of the time, then 4–5% of all patients would make an antibody with each transfusion.

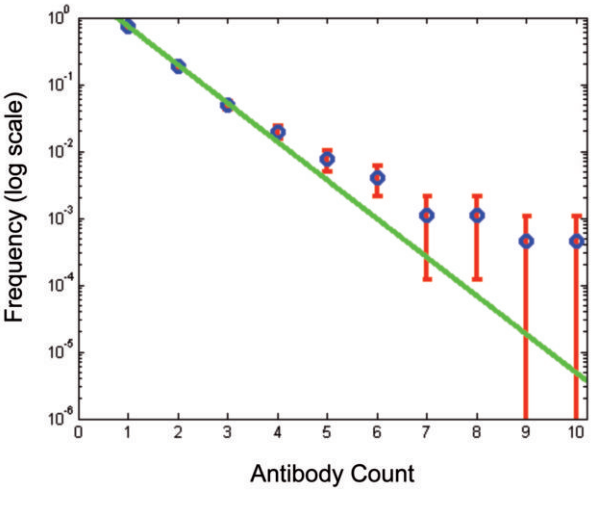

Figure 1. Antibody count frequencies of transfused alloimmunized patients at adult study hospital.

We also analyzed patient databases to determine the percentage of previously sensitized patients who made additional antibodies following transfusion. These individuals had proven themselves capable of responding to RBC transfusions. Our data found that 19% percent of these individuals made additional antibodies following an additional exposure. Similarly, Schonewille *et al*. have found that 21.7% of previously immunized hematooncology patients formed additional anti-RBC antibodies following a mean of seven transfused RBC units.[6] This data provides an independent approach to calculate the size of the potential responder population. If potential responders actually make a response 19% of the time and the entire population makes a response approximately 5% of the time, then potential responders should constitute approximately 20% of the population.

Taken together, this analysis suggests that there is distinct population of immunologic responders who are at a substantially increased risk of making anti-RBC antibodies. However, the size of the responder population is somewhat different depending on the assumptions inherent in the calculation. If we assume that the chance of making the first antibody is the same as the chance of making subsequent antibodies, then the potential responder population is approximately 13% of the general population. If, however, we assume that the *history* of making an antibody from prior transfusions does not impact the chance of making additional antibodies, then the responder population would be approximately 20% of the general patient population.

Biological considerations favor the second assumption, namely that the development of an-anti RBC antibody does not increase the risk of development of antibodies in subsequent transfusions. In general, subsequent transfusions lack the antigen recognized by the first antibody and hence should not stimulate the immune response during subsequent transfusions. In contrast, it is conceivable that it is particularly difficult to make the first antibody but that there is a lower barrier to making additional antibodies. To make the first antibody, the active immune response needs to overcome inhibitory signals made by T regulatory cells and inhibitory cytokines.[7] Once this barrier is overcome, it may be relatively easy for subsequent antibodies to develop.

Taken together, the data from our and other studies suggest that there is a larger barrier to developing the first antibody than additional antibodies. Since only 19% instead of 30% of responders make antibodies during a transfusion, then there is a 37% lower chance of developing one antibody than additional antibodies. Further exploration of this effect may lead to ways to reduce the chance of antibody development from RBC transfusions.

Effects of patient factors on immunologic responsiveness

Regardless of the obstacles in developing the first antibody, subsequent antibodies appear in a random process, indicating that the development of each antibody does not impact development of a second antibody. Although there are always many foreign RBC antigens in each transfusion, some have suggested that more antibodies might develop when there are more immunogenic foreign antigens present in the donor units. This, however, does not appear to be the case. African-Americans are generally exposed to more foreign RBC antigens than Whites during transfusions since most of the RBC units in our study hospitals are donated by Caucasians. However, African-Americans have the same value of p, meaning that they have the same random chance of developing additional antibodies as Whites.

Animal studies suggest that inflammation may be associated with development of an anti-RBC antibodies.[8] We have performed our analysis on defined patient populations and have found no influence of most disease states on the development of RBC antibodies. Specifically, patients with diabetes, atherosclerosis, orthopedic diagnoses, or hematologic malignancies do not have significant differences in the frequency of antibody development beyond the first antibody. While this does not totally exclude the possibility of an impact of an inflammatory state impacting the likelihood of developing anti-RBC antibodies, it suggests that development of multiple antibodies is a random process not significantly influenced by inflammation in humans. Indeed, there is variability in development of anti-RBC antibodies even in inbred strains of mice, supporting the idea that some stochastic process impacts antibody development.

The only diagnosis that impacts the rate of development of multiple antibodies is pregnancy. Although pregnant women have alternate modes of exposure to foreign RBCs, transfusion is a major factor in stimulating antibodies to non RhD antigens.[9] Pregnant women, like other patients make additional antibodies beyond the first antibody in a random stochastic pattern, but they make each additional antibody at about two-thirds the rate of other patients. The reason for this disparity is unknown but several possibilities may contribute to this phenomenon. The primary routes of exposure to foreign RBC antigens are from small fetal-maternal hemorrhages during pregnancy, labor, and delivery, or procedures. The immune system may process this differently from the relatively large intravenous infusions of RBC units that occur in other patients. Also, pregnancy itself confers immune suppression that one could hypothesize reduces the chances of immunologically responding to foreign RBC antigens. Finally, mothers are exposed to relatively few foreign RBC antigens on fetal cells because some antigens are poorly expressed on fetal cells and because of the close genetic relationship between the fetus and the mother.

In addition to being stable among disease diagnoses, the rate of antibody development among patients who make at least one antibody is stable among all age groups studied, ranging from patients less than 10 years old to patients in their 8th decade of life. Although the rate of antibody development remains constant through this broad age range, the range of antibodies appears to narrow as patients age. The immune systems of the youngest patients respond to a diverse range of RBC antigens. As patients' age, the diversity of their immune response to RBC antigens declines.

Taken together, these results suggest strategies to identify patients at greatest risk of developing anti-RBC antigens. Patients who have made prior anti-RBC antibodies are at increased risk of making new antibodies and in comparison with older patients, younger patients are at increased risk of making antigens to weakly immunogenic antigens. Currently, there is no way to identify potential responders prior to their first response to RBC antigens. Future studies aimed at discovering markers of potential immune responders would be extremely valuable.

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