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

22nd Congress of the European Hematology Association
Madrid, Spain, June 22 - 25, 2017

EDUCATIONAL UPDATES IN HEMATOLOGY

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Words of Welcome

On behalf of the EHA Board and the Scientific Program Committee, we are pleased to present the Educational Updates in Hematology. This is a collection of short manuscripts providing an overview of recent developments in various fields covered in the Education Sessions.

The Education Program is the cornerstone of the congress and covers basic, translational and clinical research; it also highlights the state-of-the-art in the different fields of hematology. We are very thankful for the efforts of all the experts that took part and prepared this exciting educational program. In the beginning of each article, you can find the learning goals covering all three sections of the topic and every manuscript starts with take home messages. The goal of this short format is to enable the readers to get an overview of the most up-to-date progress in variety of topics in hematology. Deeper learning of a specific topic of interest can then be achieved by reviewing the specific webcasted presentation posted on the EHA Learning Center and by examination of the references that have been highlighted by the authors at the end of each short review. In addition, we hope that the presentation goals per speaker will allow you to easily assess if a particular session is of interest to you. The learning goals can be found in the Final Program book, EHA app, Online Program or EHA Learning Center.

For this congress we have adopted a holistic view of hematology. A view that integrates basic and clinical science, considers the hematopoietic cells within their microenvironment, and investigates the interaction between malignant cells and the defenses mechanism of the body. Accordingly, you will find exciting talks on the integration of novel genomics technologies into diagnosis and therapy and on the roles of manipulations of the immune system in therapies of hematopoietic malignancies. We have not forgotten the human perspective. Thus for the first time we have included a special education session on fertility preservation, a critical topic at this fortunate stage of increasing cure rate of hematological malignancies.

At the congress there is a very large variety of sessions describing the most recent advances in hematology. While these talks provide a vision as to how patients with hematological conditions might be treated in the future, the education sessions are designed to be practical and not lose sight of the needs of delegates involved in everyday patient care.

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

We trust that you will find the peer-reviewed articles a valuable source of information and reference. The Educational Updates in Hematology together with the webcasted presentations can be found online on the EHA Learning Center: learningcenter.ehaweb.org

On behalf of EHA, the Scientific Program Committee, and the congress organizers, I very much like to wish you a pleasant and informative congress!

Shai Izraeli

Chair Scientific Program Committee 22nd Congress

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We work towards a world without blood disorders by:

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EHA is the largest Europe-based organization that brings together all medical professionals, researchers and scientists with an active interest in hematology. We have more than 4,000 members from 100 countries and work with a vast network of national societies. Our annual congress is attended by more than 10,000 individuals with an interest in hematology who meet and learn together.

Supporting career development
To accelerate the careers of junior scientists, EHA provides research grants and training programs for basic, translational, and clinical researchers in the field of malignant and non-malignant hematology. EHA also honors the contribution of outstanding physicians and scientists for the advancement of hematology through awards.

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EHA is one of the largest international, independent providers of hematology education. A comprehensive and integral curriculum forms the basis of our Medical Education Program. Through this program, professionals acquire state-of-the-art knowledge by various means, such as an online learning platform, educational meetings and a European Hematology Exam.

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Table of Contents

Table of Contents

Table of Contents

MADRID
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Acquired and hereditary red cell anomalies

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Introduction

Two papers included here are investigating the relationship between iron overload and erythropoiesis from two different angles. The first, by Dr. Ginzburg, reviews how our knowledge in this area is advanced through studies in beta-thalassemic mice, focusing on novel findings on the role of apo-transferrin and transferrin receptor TfR1. The second, by Drs. Angelucci and Pilo, is focused on present practical implications of established knowledge, specifically how to competently combat iron overload and its detrimental effects in the context of hematopoietic stem-cell transplantation. A third paper, by Dr. Da Costa, is presenting new findings on pathogenetic pathways for Diamond-Blackfan anema.

1) The importance of mutual interaction between erythropoiesis and iron metabolism is well recognized. Erythroferrone signalling from the bone marrow, indicating erythropoietic demand, suppresses hepcidin release from the liver, with subsequent increase in iron absorption and recycling. While iron provision is essential for erythropoiesis, iron overload is thought to exacerbate ineffective erythropoiesis in beta thalassemia through the accumulation and toxicity of reactive oxygen species in the bone marrow.

The in-depth study of the underlying processes in beta-thalassemic mice has shown that our understanding is far from complete. Recent findings demonstrate that exogenous apo-transferrin in these mice ameliorates ineffective erythropoiesis and suppresses hepcidin release. The key mediator appears to be a reduction in effective TfR1 in erythroid precursors. The exact mechanism has not been elucidated, but implications for new therapeutic strategies can be derived.

2) Iron overload and cellular iron toxicity have been demonstrated to influence the outcome of hematopoietic stem cell transplantation. Sustained iron overload, subsequent labile plasma iron affecting labile cellular iron and, consequently, the formation of reactive oxygen species lead to cytotoxic injury of transplanted cells and hematopoietic niche in the recipient. Focus of iron chelation therapy therefore needs to be the reversal of acute and accumulated iron overload in the recipient before and after transplantation.

3) In Diamond-Blackfan anemia, mutations in ribosomal-protein genes, subsequent ribosome biogenesis impairment and erythroblastopenia are well established components of the pathogenetic process, but the exact nature of the link between them is still not fully understood. While the important involvement of p53 has been shown, p53-independent pathways have been identified and mutations in other genes found to underlie the condition. Recently, HSP70 has been identified as a key factor in its pathophysiology, being depressed in patients harbouring specific ribosomal mutations, while rectifying pathological processes when overexpressed *in vitro.*

Learning goals

1. ***To review the interplay of iron overload, ineffective erythropoiesis, hepcidin and erythroferrone in beta thalassemia including new findings obtained from the study of beta thalassemic mice.***
2. ***Discuss the suggested role of circulating apo-transferrin and transferrin receptor TfR1 in counteracting ineffective erythropoiesis and hepcidin repression, leading to potential novel therapeutic approaches.***
3. ***To review how our recently-improved understanding of iron toxicity informs strategies for iron chelation therapy before and after bone-marrow transplantation, but also to become aware of how impending diagnostic advances could require a modification of these strategies in the near future.***
4. ***Discuss recent findings on the pathophysiology of Diamond-Blackfan anemia.***

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Transferrin and TfR1 in co-regulation of erythropoiesis and iron metabolism

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Take-home messages

- Apo-transferrin decreases TfR1 expression and normalizes enucleation in β-thalassemic erythroid precursors.
- Decreased TfR1 expression causes iron restriction specifically in β-thalassemic erythroid precursors.
- Decreased TfR1 enables endogenous mechanism(s) to upregulate hepcidin in an iron- and erythroferrone-independent manner, re-enforcing sustained iron-restricted erythropoiesis in β-thalassemic mice.

Introduction

Hemoglobin synthesis during erythropoiesis is intrinsically dependent on iron. Iron delivery for erythropoiesis is exclusively dependent on the iron transporter transferrin and iron uptake by erythroid precursors requires transferrin-Fe binding to transferrin receptor 1 (TfR1).[1,2] Erythroid precursors regulate iron metabolism in part by secreting factors, such as the recently identified erythroferrone (ERFE),[3] which suppresses the hormone hepcidin, a key inhibitor of dietary iron absorption, recycling from senescent RBCs, and mobilization from iron stores.[4] As a physiological erythroid regulator of hepcidin, ERFE is increased in stress (i.e. phlebotomy) and ineffective erythropoiesis (i.e. β-thalassemia).[3] In spite of these recent discoveries, the regulatory interactions between erythropoiesis and iron metabolism remain incompletely understood.

Current state of the art

Exogenous iron-free apo-transferrin ameliorates ineffective erythropoiesis in β-thalassemic mice,[5] resulting in increased hepcidin. In addition, apo-transferrin-treated β-thalassemic mice exhibit normalized *Erfe* expression in sorted erythroid precursors and reversal of systemic iron overload,[6] suggesting that iron depletion improves ineffective erythropoiesis and thus increases hepcidin expression *despite* relative iron deficiency (**Figure 1**). This finding is counter-intuitive as iron deficiency suppresses hepcidin expression. We hypothesized that the beneficial effect of apo-transferrin in β-thalassemic mice is a consequence of *more than* frank iron restriction. Furthermore, we hypothesized that because ERFE expression is STAT5 dependent [3] and thus decreased with decreasing serum erythropoietin, *Erfe* expression in apo-transferrin-treated β-thalassemic mice is an indirect consequence of improved erythroid maturation.[5]

- First, we compared the results from apo-transferrin-treated β-thalassemic mice with the effects of other systemic iron depleting approaches. Apo-transferrin-treated β-thalassemic mice demonstrate results in contrast to the effect of dietary iron restriction, which results in decreased liver hepcidin expression[7] without improving hemoglobin in β-thalassemic mice[8] and is thus only partially beneficial in diseases of ineffective erythropoiesis. Furthermore, iron chelator therapy does not change erythropoiesis or hepcidin expression despite improved liver iron overload in β-thalassemic mice.[9] On the other hand, multiple means of increasing hepcidin expression to decrease iron overload in β-thalassemic mice result in both improved erythropoiesis and iron overload.[7,10-12] However, one of these approaches, using homozygous inactivation of *Tmprss6* leads to excessive hepcidin production, impaired dietary iron absorption, and microcytic anemia in mice.[13,14] Furthermore, Tmprss6-/- β-thalassemic mice exhibit increased erythropoietin,[10], suggesting that excessive hepcidin upregulation causes iron restriction[7] and prevents complete reversal of ineffective erythropoiesis. Taken together, *moderately* increased hepcidin results in iron sequestration, decreasing iron absorption and recycling, and resulting in restricted availability of iron for erythropoiesis. Thus, inducing iron-restriction specifically in the erythroid compartment may provide endogenous mechanism(s) for increasing hepcidin to maximize benefit for erythropoiesis and iron overload in β-thalassemia.
- Second, we hypothesized that apo-transferrin's effect is mediated via decreased TfR1 expression, resulting in disproportional decrease in erythroid precursor iron uptake. We thus evaluated TfR1 expression in β-thalassemic mice *in vivo* and *in vitro* with and without added apo-transferrin. Our cur-

rent findings demonstrate that β-thalassemic erythroid precursors overexpress TfR1, an effect which can be reversed by the administration of exogenous apo-transferrin. We anticipate that the increased number of β-thalassemic erythroid precursors limit iron availability per cell and, in conjunction with increased serum erythropoietin concentration, results in increased TfR1 expression *in vivo*. *In vitro* experiments demonstrate that apo-transferrin inhibits TfR1 and induce *Erfe* expression independent of erythropoietin- and iron-related signaling, decreases TfR1 partitioning to reticulocytes during enucleation, and enhances enucleation of defective β-thalassemic erythroid precursors. These findings strongly suggest that overexpressed TfR1 may play a regulatory role contributing to iron overload and anemia in β-thalassemic mice. Specifically, our data demonstrates that apo-transferrin functions via an effect on TfR1 trafficking, increasing enucleation as evidence of improved erythropoiesis and modulating iron metabolism by decreasing *Erfe* expression. Taken together, our findings suggest that novel regulatory pathways for transferrin and TfR1 are central to the crosstalk between erythropoiesis and iron metabolism.

- Lastly, we hypothesize that the beneficial effect of exogenous apo-transferrin on ineffective erythropoiesis is a consequence of reduced TfR1 expression or altered TfR1 trafficking in erythroid precursors. TfR1 is characteristically expressed on erythroid precursors, progressively decreasing during erythroid differentiation.[15] TfR1 is lost both during enucleation and reticulocyte maturation, the latter a consequence of proteolytic cleavage, leading to soluble TfR1 in circulation.[16] TfR1 expression itself is upregulated in iron deficiency[17] and by increased erythropoiesis,[18] but its function in erythropoiesis beyond its canonical involvement in cellular iron uptake

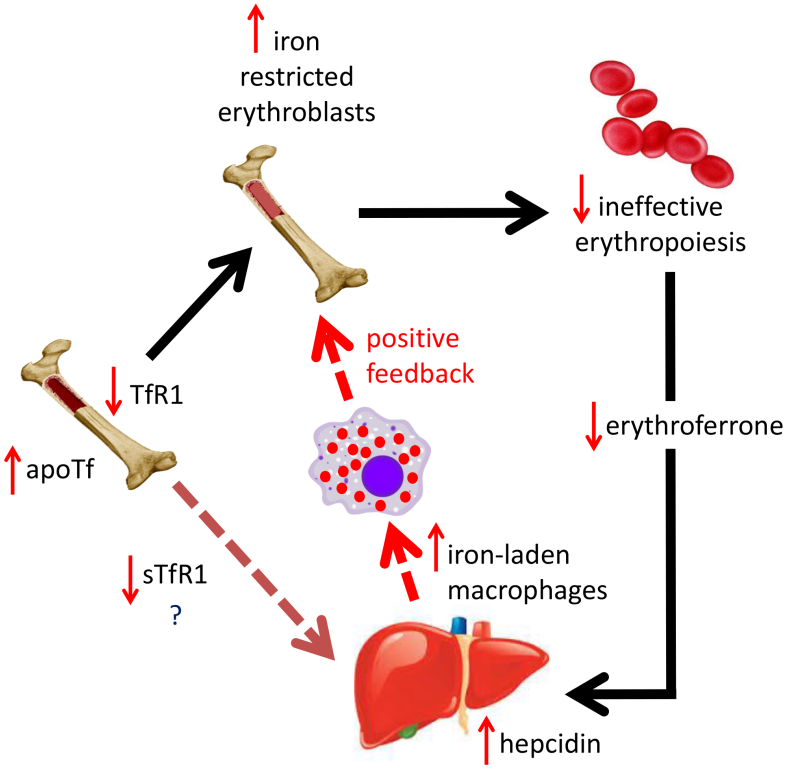

Figure 1. Working model of the effect of exogenous apo-transferrin and restricted TfR1 expression in β-thalassemia. The administration of exogenous apo-transferrin and restricted TfR1 expression both result in iron restriction disproportionately in erythroid precursors, in light of the highest concentration of TfR1 on these cells, and improve ineffective erythropoiesis in β-thalassemia. With increased hemoglobin, fewer erythroid precursors are required to maintain homeostasis, resulting in reversal of splenomegaly and a decrease of circulating erythroferrone and soluble TfR1 concentrations. As a consequence, hepcidin is de-repressed and re-enforces the sequestration of iron within macrophages and iron restricted erythropoiesis. We are currently exploring the role of soluble TfR1 in more directly regulating hepcidin expression. apoTf = apo-transferrin; TfR1 = transferrin receptor 1; sTfR1 = soluble transferrin receptor 1.

is incompletely understood, and hepcidin expression in TfR1 heterozygous mice (TfR1+/-) is surprisingly increased despite relative iron deficiency, iron-restricted erythropoiesis, and increased *Erfe* expression.[19] To evaluate further, we crossed TfR1+/- mice—themselves exhibiting iron-restricted erythropoiesis with increased hepcidin—with β-thalassemic mice. Resultant double-heterozygote mice demonstrate long-term improvement in ineffective erythropoiesis, hepcidin de-repression, and increased erythroid enucleation relative to β-thalassemic mice. Furthermore, hepcidin de-repression occurs in an iron- and erythroferrone-independent manner, suggesting that iron restriction within erythroblasts may de-repress hepcidin directly, resulting in a positive feedback to maintain iron restricted erythropoiesis. Taken together, the effects of apo-transferrin treatment and TfR1 haplo-insufficiency on β-thalassemic mice thus provide evidence of an additional mechanism for increasing hepcidin by *erythroid regulation*.

Future perspectives

Further studies are necessary to explore the potential use of exogenous apo-transferrin to reverse ineffective erythropoiesis in β-thalassemia and other diseases of concurrent anemia and iron overload. Our data present additional therapeutic targets in this pathway, and support our hypothesis that reversal of ineffective erythropoiesis and iron overload require parallel management in β-thalassemia. This data also raises numerous questions to explore. For instance, reticulocyte release of exosomal TfR1 is altered in apo-transferrin treated β-thalassemic mice. How is this effect related to improved erythropoiesis after exogenous apo-transferrin? What are the *regulatory* implications of altering TfR1 expression or localization? What is the role of exosomal or soluble TfR1 in regulating hepcidin expression in β-thalassemia? Are TfR1 antagonists—similar to the effect of exogenous apo-transferrin—potential therapeutic targets to simultaneously improve erythropoiesis and de-repress hepcidin? By exploring the impact on iron transport in circulation as well as erythroid precursor import and export, these results will extend our understanding of co-regulation of erythropoiesis and iron metabolism in iron overload anemias.

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First evaluation of hepcidin in TfR1+/- mice, suggesting a function of other erythroid regulators other than erythroferrone.

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Acquired and hereditary red cell anomalies - Section 2

Iron overload before, during and after hematopoietic stem cells transplantation

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Take-home messages

- Iron toxicity depends on the presence of free iron species [non transferrin bound iron (NTBI), labile plasma iron (LPI) and labile cellular iron (LCI)].
- Iron toxicity during hemopoietic stem cell transplantation (HSCT), can impair the bone marrow microenvironment, the quantity and quality of bone marrow mesenchymal stem cells, the ratio of immature hematopoietic cells and the clonogenic capacity of hemopoietic stem and progenitor cells.
- After successful hemopoietic stem cell transplantation, one should aim to achieve normal iron levels (i.e. normal transferrin saturation).

Introduction

Iron overload (IO) and consequent cellular iron toxicity are conditions often accompanying hemopoietic stem cell transplantation (HSCT) and have been associated with transplant outcome since the pioneering Pesaro experience in thalassemia[1,2]. Here will be discussed the implications of IO with HSCT and the mechanisms of iron toxicity, where our understanding has significantly evolved in the last few years[2].
In physiological situations, most iron in the plasma is bound to transferrin, a carrier protein that mediates cellular iron uptake. In the presence of IO, i.e., when transferrin saturation is >70%, plasma iron appears as non-transferrin bound iron (NTBI). A component of NTBI, called labile plasma iron (LPI), is potently redox-active and capable of permeating into cells, inducing cellular iron overload[3] and impacts the delicate equilibrium of labile cellular iron (LCI). The breakage of this balance catalyzes the formation of reactive oxygen species (ROS), which, with concomitant decrease in antioxidant enzymes, leads to cytotoxic cell injury (DNA damage, lipid peroxidation, protein modification and mitochondrial damage).
The mechanism underlying tissue iron toxicity has been recently summarized by the following equation[4]:

Tissue iron toxicity:
Σ tissue reactive iron x genetics x environmental factors x time.

Tissue iron toxicity depends on many factors in addition to the iron level per se[4]: the quantity of toxic iron related species, duration of exposure, individual's anti-oxidant genetics and environmental factors.

Current state of art

Iron overload before HSCT (before the start of conditioning)

In transfused patients, an effort should be made to continuously and regularly reduce the level of LCI and ROS in the years before transplant to prevent tissue damage because it is now clear that the critical point is duration of exposure to tissue reactive – toxic- iron forms[2,4]. This can be achieved with regular iron chelation. In any patient receiving significant transfusion therapy that may have an HSCT in the future, a decision on starting regular chelation is critical and should be undertaken as soon as possible.
There are limited data on the rationale for intensive pre-transplant chelation therapy unless sufficient time is available to correct IO and permit tissue repair.

Iron overload during HSCT (from the start of conditioning up to sustained engraftment)

Recent animal studies demonstrated that iron toxicity could impair the hematopoietic niche by damaging hematopoietic stem cells' (HSCs) self-renewal potential, proliferation, differentiation and the marrow microenvironment. This suggests that IO can impact the HSCT engraftment and outcome.

Xiao Chai and coworkers[5] described, in a IO mouse model, how iron overload can increase ROS levels of HSCs progenitors, leading to defective ratio of immature hemopoietic cells and clonogenic capacity compared to a control group. In the same paper, in a mouse-transplant-model, flow cytometry analyses demonstrated that recipient mice of iron-overloaded donors had lower levels of myeloid, B and T- lymphocytic lineage engraftment compared to control transplants. Both effects were reversed after treating iron overloaded mice and transplant recipient mice with an iron chelator or a powerful anti-oxidant. The same group demonstrated that iron overload could impair the bone marrow microenvironment and the quantity and quality of mesenchymal progenitor's cells[6].
From a clinical point of view, apart from single case reports, little evidence is available. Visani and colleagues demonstrated that in cases of poor and delayed engraftment, iron chelation can help in stabilizing hemopoietic engraftment[7]. Studies are ongoing in patients undergoing allogeneic HSCT which addressing the issue of the positive effects of iron chelation on NTBI and LPI during conditioning and their prognostic value.[8,9]

Iron overload after transplantation (after sustained engraftment has been achieved)

After successful transplantation, patients are usually free from transfusion support. It should be examined whether in the absence of further blood–iron input, LCI levels can be maintained within the physiological range by existing iron homeostatic mechanisms that coordinately regulate uptake vs storage, so as to support iron utilization and minimize iron oxidation.
However even in this condition it is reasonable to think that the already acquired iron intracellular storage could continue to disrupt the delicate equilibrium of LCI and promote the generation of ROS by reacting with respiratory oxygen intermediates and thereby overriding the cellular antioxidant defenses leading to chemical damage to cell components and functions. From the clinical point of view, it has been demonstrated in transplanted thalassemia patients (transfusion free but still with acquired IO) that elevated transferrin saturation persists and liver disease progresses even in the absence of other comorbidities[10].
In this context, an iron toxic effect can be present even with a lower level of accumulation, and can result in cumulative tissue damage as demonstrated in the case of IO and hepatitis C infection in the development of liver fibrosis and cirrhosis[10].
Therefore, even because of the results of epidemiologic studies in thalassemia[11,12] and in the normal population[13] in the post-transplant setting (i.e., a patient cured from his/her disease) the target should be a normal iron levels, normal transferrin saturation and no evidence for toxic iron reactive species (NTBI, LPI and LCI).
Taken together, these findings implicate that iron chelation or phlebotomy[14] have a key role in the post-transplant setting. Table 1 reports the pros and cons for selecting iron chelation versus phlebotomy.

Future prospective

A growing body of evidence demonstrates how iron toxicity could impair the hematopoietic microenvironment niche by damaging hematopoietic stem cells' self-renewal potential, proliferation, and differentiation.
Standardization of the quantification methods for NTBI, LPI and ROS levels are emerging[15]. In the near future, availability and validation of these tools could contribute to a "precision medicine" clinical decision regarding IO before, during and after HSCT.

Table 1. Factors in favor or against the use of phlebotomy or deferasirox in long follow-up after successful hematopoietic stem cell transplantation.

	Phlebotomy	Chelation
Pros	Efficient	Efficient
	Safe	Safe
	Inexpensive	Immediate effect on NTBI/LPI
	Permits complete iron removal and normalizes iron body content	Hospital access not required
Cons	Requires sustained engraftment (not usable in the early post-HSCT period)	Expensive
	Immediate effect on NTBI/LPI still remains to be verified	Warning of renal toxicity in the case of concomitant use of cyclosporine
	Hospital access required	Possible increase in toxicity for low level of iron burden

Acquired and hereditary red cell anomalies - Section 2

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Availability and validation of methods to detect NTBI, LPI and ROS levels are mandatory for a "precision medicine" and adequate clinical decision.

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Heat Shock Protein S70 (HSP70), one of the key factors in Diamond-Blackfan anemia

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Take-home messages

- DBA is a congenital erythroblastopenia amongst the inherited bone marrow failure syndromes (IBMFS), highly heterogenous in phenotype and genotype.
- DBA is the first ribosomopathy described and p53 stabilization is involved in DBA erythroblastopenia.
- HSP70 is one of the major key factors involved in DBA pathophysiology.

Diamond-Blackfan anemia (DBA)[1, 2] has been the first ribosomopathy described[3,4] and belongs to the inherited bone marrow failure syndromes (IBMFS). DBA is characterized by a specific erythroid tropism and is associated with a lower risk of malignancies (5% of the DBA cases)[5,6] compared to Fanconi anemia, Shwachman-Diamond syndrome or dyskeratosis congenita. DBA is revealed early in infancy (median age of 2 months) with a moderate to severe, usually macrocytic aregenerative anemia and a normal platelet and white blood cell count in the vast majority of the DBA patients. The erythroblastopenia in an otherwise normal bone marrow is the main feature of the disease. The erythroid blockade has been stated between the BFU-e and the CFU-e stages.[7] DBA phenotype is however highly heterogeneous and in 50% of the DBA cases, various malformations mostly in the cephalic area and the extremities have been reported.[8,9] Steroid therapy should be initiated only after one year of age, in order to protect growth during the first year of life. More than 60% of DBA cases are steroid good responders. Corticoresistant or corticodependent more than 0.5 mg/kg/day (or even >0.3 mg/kg/day) DBA patients should enter into a regular transfusion program associated with an iron chelation after a certain amount of transfusions and based on ferritin level. However, so far bone marrow transplantation with an HLA identical intra-familial and non silent phenotype donor is the only curative treatment for DBA.[10] DBA genotype is also highly heterogeneous. A heterozygous mutation is found in more than 70% of the DBA affected patients in one of the 14 ribosomal protein genes, which have been shown to be involved in DBA, including *RPS19* (25%), *RPL5* (7%), *RPL11* (5%), *RPS24* (2,4%), *RPS26* (7%), *RPS10* (3%), *RPL35a* (3%), *RPS17* (1%), *RPS7* (<1%), *RPS28* (<1%), *RPS27a* (<1%), *RPL15*, *RPL9, RPL26*. Large deletions in these genes are reported.[11-13] The occurrence of mutations is sporadic, or *de novo*, in 55% of DBA affected patients, while in the familial cases, the inheritance is dominant. The RP gene mutation is responsible for a defect in rRNA maturation at different level depending on the RP gene.[3,14] The link between the mutation in an RP gene, the ribosome biogenesis impairment and the erythroblastopenia is still to be fully defined. However, several groups, including ours, identified p53 as one of the major proteins involved in the disease.[15,16] Indeed, it has been shown that rRNA maturation impairment leads to nucleolar or ribosomal stress which, in turn, leads to an increase in the expression level of various RP genes, with an RP binding to MDM2 (or HDM2). MDM2 is an E3 ubiquitin ligase, which binds p53 and directs it for proteasomal degradation. During the nucleolar stress, p53 is thus free and induces apoptosis and cell cycle arrest, responsible at least in part for the erythroblastopenia, the feature of the disease.[15,16] However, p53 independent pathways are now described[17] and non-RP genes have been identified in DBA, which may open new pathway(s) involved in DBA pathophysiology. Recently, two genes, namely *TSR2*[18] and *GATA1,*[19] which are not related to an RP gene have been identified in a few DBA patients. GATA1 is the major erythroid transcription factor. In GATA1 mutated DBA patients[19] and in RP mutated DBA patients,[20] the long form of GATA1 disappears, with only the short form remaining. The ribosomal defect in GATA1 mRNA translation in DBA results from this mRNA having a higher threshold for initiation of translation (highly structured 5'UTR are more poorly translated).[20] However, we recently identified another key factor that may explain the more severe DBA phenotype. Indeed, we were previously able to identify two DBA *in vitro* phenotypes resulting from the mutated RP

gene.[16] The respective DBA patients, affected by an *RPS19* mutation, exhibited a decrease in erythroid proliferation, but a normal erythroid differentiation and no apoptosis, while DBA patients carrying a mutation in RPL5 or RPL11 exhibited a dramatic decrease in erythroid proliferation, a delayed erythroid differentiation, and significant erythroid cell apoptosis.[16] In all these DBA patients, independently of the RP gene mutated, we found a cell cycle arrest in G_0/G1. Seeking to decipher the significance of these dual phenotypes, we were able to identify the protein involved in these discrepancies. Strikingly, the Heat Shock Protein S70 (HSP70) was expressed at a normal level in RPS19 mutated DBA patients and in shRNA-RPS19 infected $CD34^+$ from cord blood, while absent or largely decreased in RPL5 or RPL11 mutated DBA patients or in the shRNA-RPL5 or -RPL11 infected erythroid cells (manuscript in revision). The decrease in HSP70 is due to an enhanced proteasomal degradation of polyubiquitinylated HSP70. Overexpression of wild type HSP70 is able to restore the erythroid defect caused by DBA, in particular the erythroid proliferation and differentiation defect in the severe DBA phenotype, confirming the specific role of HSP70 in DBA pathophysiology. Furthermore, overexpression of wild-type HSP70 reduced p53 stabilization. In a parallel study, we were able to show a disequilibrium between the heme and globin synthesis. We observed a normal or slightly decreased total heme content, but an excess of free heme, in DBA affected patients in association with both, transcriptional and translational, defect in globin protein expression level. GATA1 targets, namely HRI, ALAS2 and globin genes, were indeed downregulated. Wild-type HSP70 overexpression was able to increase GATA1 and its targets, restoring globin chain expression levels. HSP70 should thus be considered as one of the key factors of DBA pathophysiology (Figure 1). New therapeutic developments involving the HSP70 nuclear re-localization and its GATA1 chaperon function may be considered as an innovative treatment for DBA patients, who faced until now in the vast majority of cases, only iterative transfusions or long-term steroid therapeutics options.

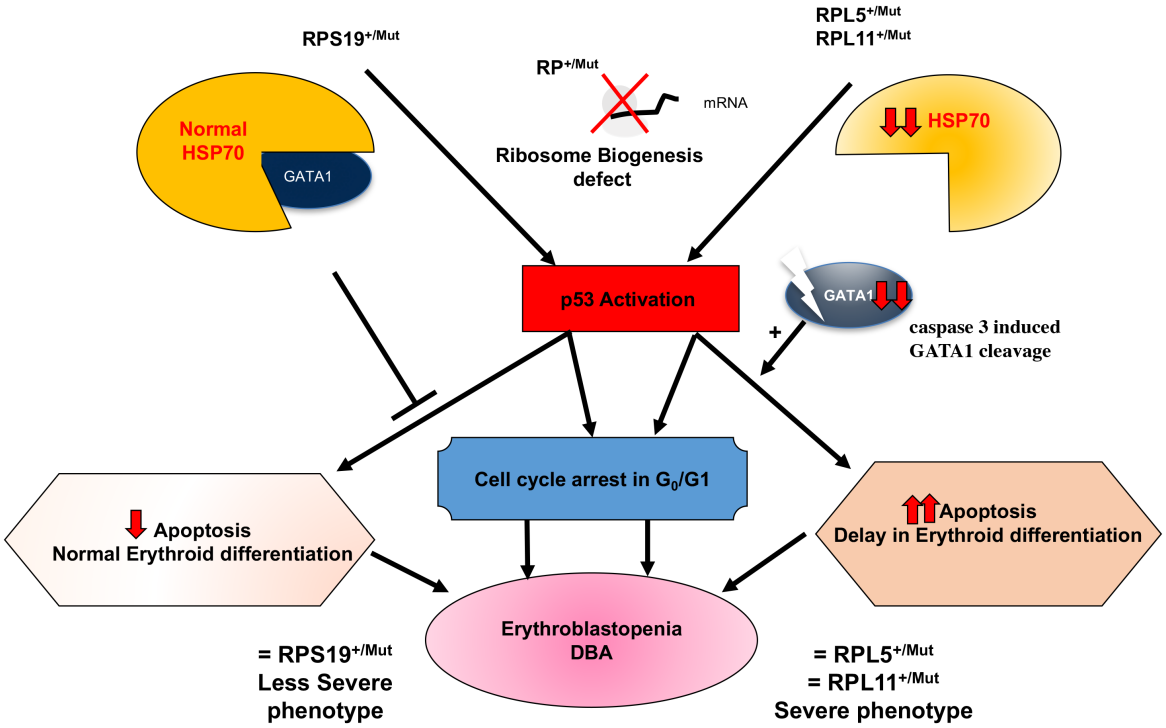

Figure 1. HSP70 as a major key factor in DBA pathophysiology.

Acquired and hereditary red cell anomalies - Section 3

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Acute lymphoblastic leukemia
The worst and the best

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Introduction

In this session, three experts will discuss current important topics that gain a lot of interest in the word of acute lymphoblastic leukemia. First, overall survival for childhood acute lymphoblastic leukemia (ALL) has increased from zero to 90% over the last 6 decades by intensifying therapy for all of them. It becomes important to identify the cases with a survival chance of close to 100% and to study whether therapy can be reduced in these. Second, several immunotherapeutic modalities for treatment of ALL have emerged in the last decade. Most promising are different types of antibodies and most recently the use of CAR-T cells genetically engineered to attack CD19 and other B-cell antigens. Infusion of CAR-T cells results in very high rates of molecular remissions. Not only the successes but also the limitations and treatment failures will be discussed. Third, although the number of new drugs approved for its use in ALL is limited, there is a large pipeline of drugs and immunotherapeutic strategies that are highly promising. These are mainly based on new knowledge of molecular-genetic abnormalities such as the bcr-abl like subtype. The status of these new therapeutic modalities will be discussed.

Learning goals

1. ***To learn in which ALL patients therapy can be reduced and how this can be done.***
2. ***To learn about the successes and also the limitations and failures of CAR-T cells in ALL.***
3. ***To learn about the new therapeutic strategies largely based on molecular genetic abnormalities in ALL.***

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Balancing efficacy and toxicity in the treatment of childhood acute lymphoblastic leukemia

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Take-home messages

- Reductions in relapse risk after first line treatment of children with acute lymphoblastic leukemia observed with intensified treatment strategies in the 1970s to 1990s have unmasked the morbidity and mortality associated with intensive therapy and revealed late treatment-related side effects on long term follow-up.
- Advances in understanding of the biological complexity of childhood acute lymphoblastic leukemia and development of sensitive methods for detecting Minimal Residual Disease (MRD) have improved molecular profiling and risk stratification models.
- Risk adapted intensification and de-escalation of treatment, drugs against specific molecular targets and immune based treatment approaches will be the basis for design of future protocols to improve efficacy while minimizing toxicity.

Introduction

The full curative potential of intensive chemotherapy in childhood acute lymphoblastic leukemia (ALL) is handicapped by treatment associated mortality and morbidity. Despite improvements in supportive care, intensive therapy carries a significant risk of mortality (4 -6 %) and morbidity (30 - 60% serious adverse event rate), especially when viewed against a low (<10%) relapse risk in recent trials[1] (Table 1). Additionally, patients remain at risk of late neurocognitive side effects and secondary cancers. It's important, therefore, to identify groups of patients who remain at high risk of relapse to direct further intensification of treatment towards them, while trying to de-escalate treatment for the remainder who achieve high rates of event-free survival with 'standard' therapy.

Current state of the art

Risk stratification

Although treatment stratification based on clinical and cytogenetic criteria have been in use for many years, risk groups identified by these variables are relatively non-specific. For example, a high-risk group with a 5 year EFS of around 50% defined by age, gender and presenting WCC identifies only 20% of patients destined for relapse, with the majority of relapses still arising out of the remaining, apparently, low risk patients.[2,3] Assessment of minimal residual disease (MRD) at post-remission time points offers a very sensitive and specific means of distinguishing between patients who will and will not relapse. Hence, most current treatment protocols use a risk stratification approach incorporating MRD assessment at one (end of induction) or two time points (and end consolidation).[4] However, although undetectable MRD at end of induction identifies a group at very low risk of relapse, a high risk group defined solely on the basis of MRD does not capture a majority of relapses[5] as these occur within the MRD intermediate risk group whose outcome can be further stratified by molecular profiling.[6,7]

De-escalation of treatment

Durable remissions of ALL were reported in roughly 50% of patients treated on St Jude total therapy V in the 1960s. Subsequently, the Berlin-Frankfurt-Munster (BFM) group showed that the event free survival could be improved to 70% by intensified induction and consolidation therapy, later confirmed by the UK MRC group in a randomised trial.[8,9] Although the BFM strategy gained wide acceptance internationally, almost without exception the original model required modification because its toxicity did not allow delivery as in Germany. Two recent trials by the UK (UKALL 2003)(1) and Dutch (DCOG 10) groups[10] have demonstrated that modest de-escalation of treatment is feasible for a MRD defined low risk group, although a contemporary European study (AIEOP-

Acute lymphoblastic leukemia - The worst and the best - Section 1

BFM 2000) found a slight increase in relapse risk associated with a reduced intensity delayed intensification course.[11] Since DS-ALL has an inferior survival due in large part to a high treatment related mortality (TRM), many groups reduce the intensity of treatment for this group of patients.[12, 13]

CNS directed therapy- is cranial radiotherapy essential?

Having been standard practice for prevention of central nervous system (CNS) relapse in older treatment protocols for children with ALL, pre-emptive cranial radiotherapy (CRT) has increasingly been replaced by other treatment strategies due to its associated high risk of late neurocognitive sequaelae, endocrinopathy and secondary cancers. A systematic review and meta-analysis of 47 randomized trials of CNS-directed therapy conducted between the 1970s and 1990s showed that CRT can generally be replaced by intrathecal therapy.[14] This observation has been confirmed in single group studies[15] and in a more recent meta-analysis of T-lineage ALL only.[16] Another recent meta-analysis demonstrated that CRT is of no benefit in prevention of relapse after contemporary first line therapy except for a small sub-group of patients with overt CNS disease at diagnosis for whom CRT reduced isolated CNS relapse, but did not affect overall survival which was poor, with or without CRT.[17]

Limiting exposure to toxic drugs

UK and US COG groups limit exposure to anthracyclines in induction to NCI high risk patients only (age >10 years or WCC >50x10^9/L) to reduce the depth and duration of marrow failure, severity of mucositis and risk of late cardiotoxicity. In view of excess infection related induction mortality in Down syndrome patients, in the UK, even NCI HR patients start 3 drug induction without anthracycline which is added at day 15 for those with a slow early response (day 15 M3 marrow). Although thioguanine is more effective than mercaptopurine at preventing CNS relapses, its association with an increased risk of death in remission and veno-occlusive disease (VOD) of the liver[18] precludes its use for the maintenance phase of treatment. The risk of osteonecrosis might be reduced by using an alternate week schedule of dexamethasone during delayed intensification,[19] but appears not to be higher in patients who receive steroid pulses in maintenance.[20]

Limiting the proportion of patients receiving Hemopoietic Stem Cell Transplant (HSCT)

The proportion of patients transplanted in first remission varies by study group from <5% to 15%. Some groups have reported a benefit of matched related donor HSCT compared

Table 1. Outcomes with contemporary childhood ALL protocols.

Trial	Group	Region	Years	Subgroup (n)	EFS (yrs)	OS (yrs)
Several	COGUS, Canada, Australia, New Zealand		2000-05	All patients (6994)	N/A	91.3% (5-yr)
				B-ALL (5845)	N/A	92.0% (5-yr)
				T-ALL (457)	N/A	81.5% (5-yr)
Total XV (age 1-18)	SJCRH	US	2000-07	All patients (498)	85.6% (5-yr)	93.5% (5-yr)
				B-ALL (422)	86.9% (5-yr)	94.6% (5-yr)
				T-ALL (76)	78.4% (5-yr)	87.6% (5-yr)
00-01(age 1-18	DFCI	US, Canada	2000-04	All patients (492)	80.0% (5-yr)	91.0% (5-yr)
				B-ALL (443)	82.0% (5-yr)	N/A
				T-ALL (49)	69.0% (5-yr)	N/A
AIEOP-BFM 2000 (age 1-18)	BFM	Western Europe	2000-06	All patients	N/A	N/A
				B-ALL (4016)	80.4% (7-yr)	91.8% (7-yr)
				T-ALL (464)	75.9% (7-yr)	80.7% (7-yr)
ALL-10 (age 1-18)	DCOG	Netherlands	1997-2004	All patients (865)	87% (5-yr)	92% (5-yr)
				B-ALL (661)	88% (5-yr)	93.3%(5-yr)
				T-ALL (116)	80% (5-yr)	88%(5-yr)
UKALL 2003 (age 1-25)	MRC/NCRI	UK	2003-11	All patients (3126)	87.3% (5-yr)	91.6%
				B-ALL (2733)	88% (5-yr)	92.3%
				T-ALL (386)	82% (5-yr)	86.4%

*AIEOP-BFM: Association of Italian Pediatric Oncology and Berlin Frankfurt-Munster; COG: Children's Oncology Group; SJCRH: St. Jude Children's Research Hospital; DFCI: Dana Farber Cancer Institute Consortium; MRC/NCRI: Medical Research Council/National Cancer Research Institute; *infants <1-year-old excluded.*

with chemotherapy in high risk sub-groups,[21] but a transplant related mortality (TRM) of 5-20% associated with unrelated and mismatched donor transplant limits the benefit of HSCT. Although TRM has improved with the incorporation of standardized donor matching and conditioning therapy,[22] it remains a significant concern as does acute and late HSCT-related toxicity especially that associated with total body irradiation (TBI) based conditioning. An on-going randomized international study (FORUM) is testing whether radiation free conditioning is associated with reduced toxicity without compromising efficacy. Most groups have also narrowed the indications for CR1 HSCT with a focus on MRD response based criteria rather than solely clinical or genetic features.

Future perspectives

As cure rates improve, greater attention should focus on reducing treatment related deaths which make up an increasing proportion of treatment failures. Identification of groups at high risk of toxicity (e.g., Down syndrome) and pharmacogenomic analysis will guide targeted supportive care and individualized drug dosing to reduce toxic deaths. There is evidence that gene expression signatures of leukemic blasts can predict in-vitro and *in vivo* chemosensitivity and treatment in future could be customized to a patient's pharmacogenomic and leukemia genotype. Translation of recent advances in understanding of the molecular biology of ALL and its influence on phenotype and clinical outcome will help define specific sub-groups that might benefit from such an approach. Targeted and immune based treatment could replace elements of conventional chemotherapy regimens responsible for some of the major toxicities, thereby reducing toxicity whilst retaining overall efficacy of treatment. These include tyrosine kinase inhibitors for Philadelphia chromosome negative ABL class fusions, antibodies such as blinatumomab and cellular therapy with autologous and universal chimeric antigen T cells (CART). The efficacy and toxicity of these interventions as single agents or in combination with chemotherapy will need to be tested in controlled clinical trials with long term follow-up.

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Acute lymphoblastic leukemia - The worst and the best - Section 2

Immunotherapy for acute lymphoblastic leukemia: From biology to the clinic and back

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Take-home messages

- CD19-targeted immunotherapy has been highly successful in the treatment of relapsed and refractory ALL and can generate durable remissions.
- A substantial number of patients will relapse due to either leukemic resistance (antigen loss) or immunotherapeutic failure.
- CD22 is another validated ALL target creating opportunities for multispecific targeting with the potential to reduce antigen-loss relapses.

There has been a recent explosion of successful immune-based therapeutic options in oncology, including the approval of immunotherapeutic agents for treatment of multiple types of cancers, such that immunotherapy can now be considered a part of standard cancer treatment.[1] This review will summarize the status of immunotherapeutic approaches for the treatment acute lymphoblastic leukemia. Dramatic therapeutic success has been achieved with agents that block or inhibit negative regulators of the immune response (immune checkpoint inhibitors).[2] Initially demonstrated with anti-CTLA4 in melanoma, this approach has now extended to other immune regulatory pathways such as PD1/PDL1 and to other types of cancer. Unfortunately, the preliminary experience with these agents in pediatric cancers and for many hematologic malignancies has been disappointing. The reasons for this have not been entirely elucidated but a prevailing thought is that pediatric cancers have less neoantigens to which pre-existing immune responses can be 'unleashed' through checkpoint inhibition[3-5] due to the low nonsynonymous mutation rate in pediatric cancer compared to adult epithelial malignancies.[6]

One strategy to overcome a lack of natural T cell immunity is to 'redirect' the specificity of immune cells towards tumor-expressed targets using agents that bridge immune cell receptors and malignant cells. A bispecific immune engager that binds CD3 on T cells and CD19 on B cell malignancies has been successful in both adult and pediatric B lineage acute lymphoblastic leukemia (ALL) and has been approved for the treatment of relapsed and refractory patients.[7] Other multi-specific immune engagers are currently being tested in clinical trials against a number of other hematologic malignancies such as acute myeloid leukemia (AML), as well as engagers of other immune effector cells such as NK cells.

A second redirection strategy is to genetically engineer immune effector cells to artificially express a receptor that recognizes a tumor antigen. Chimeric antigen receptors (CARs) contain a domain that binds a cell surface antigen (typically a via an antibody-derived sequence) combined in the same construct with T cell receptor (TCR) signaling machinery (Figure 1A).[8] Second generation CARs contain both a TCR signaling sequence (typically CD3zeta) and a co-stimulatory signaling domain (for example, derived from CD28 or 41BB). CARs require cell surface expressed targets but have the advantage over TCRs of not being restricted by the major histocompatibility complex such that they can be used in all patients. Both TCR- and CAR-based gene-modified T cell therapies are being tested in the clinic.[9,10]

Genetically engineered immune effector cells have now been generated successfully at clinical grade and in sufficient numbers for infusion as patient specific products across multiple clinical trials. The most dramatic example of success with this approach has been the use of T cells engineered to express a CD19-targeted CAR. Remissions were achieved in 60-80% of patients with relapsed or refractory B lineage ALL in multiple phase I clinical trials.[11] One lesson learned from this early experience with CARs is that the dose required for remission is remarkably small (typically $<10^8$ cells). This allows for a relatively short production time but response requires the ability for the T cells to expand dramatically after infusion. Based on the success with the CD19 CAR in phase I trials, phase II trials are underway. Thus far, preliminary data has demonstrated comparable response rates in phase II trials to initial phase I studies and trials incorporating CD19 CAR T cells early in ALL therapy based on standard risk assessment are being planned.

Longer term follow-up of patients treated with CD19 CAR suggests that relapse may occur in 1/3 to 1/2 of patients

achieving remission (Figure 1B). One pattern of relapse seems to result from lack of CAR persistence. Although optimal length of CAR T cell persistence has yet to be defined, some centers consider 3-6 months to be a reasonable target. For patients with sub-optimal persistence, strategies to modulate T cell behavior and immune biology would be predicted to improve durability of remission. Studies are underway to look for markers of inferior T cell quality both in the product and in patients after infusion. Approaches to improve persistence such as cytokine administration or boost vaccines are also being explored.

The second pattern of relapse occurring following remissions induced by CD19 CAR and CD19 bispecific immune engagers is loss the targeted CD19 antigen as an example of immune escape. Interestingly, many of these relapses may not involve loss of the full CD19 protein but rather an alternative post-transcriptional splicing event such that the RNA transcript no longer contains the exon encoding the targeted epitope.[12] Another mechanism of immune escape that was initially identified in a murine model involved lineage switch of a pre-B cell ALL to a myeloid phenotype occurring in the presence of persistent CD19 CAR.[13] This mechanism of resistance has also been seen in patients with MLL-rearranged ALL following CD19-targeted immunotherapy, suggesting recapitulation of leukemia biology under lineage-targeted immune pressure.[14,15]

CD22 has been well validated as an alternative B cell restricted ALL target based on high response rates using a CD22-targeted immunotoxin conjugate (Inotuzumab Ozogamycin).[16] Success with a second CAR targeting CD22 has recently been reported with a 75% remission induction, including patients with CD19 antigen loss after CD19-targeted therapy. This experience is proof of principal of success with CARs targeting other antigens besides CD19 and that patients relapsing with resistant leukemia after immunotherapy can be salvaged with an alternative immunotherapy. Although durable remission is possible after the CD22 CAR, relapse due changes in CD22 expression occurred. Interestingly, rather than complete loss of CD22, most patients relapsed with leukemia that had reduced density of CD22 expression.

Although recent experience with immunotherapy for hematologic malignancies has been promising, there are a number of

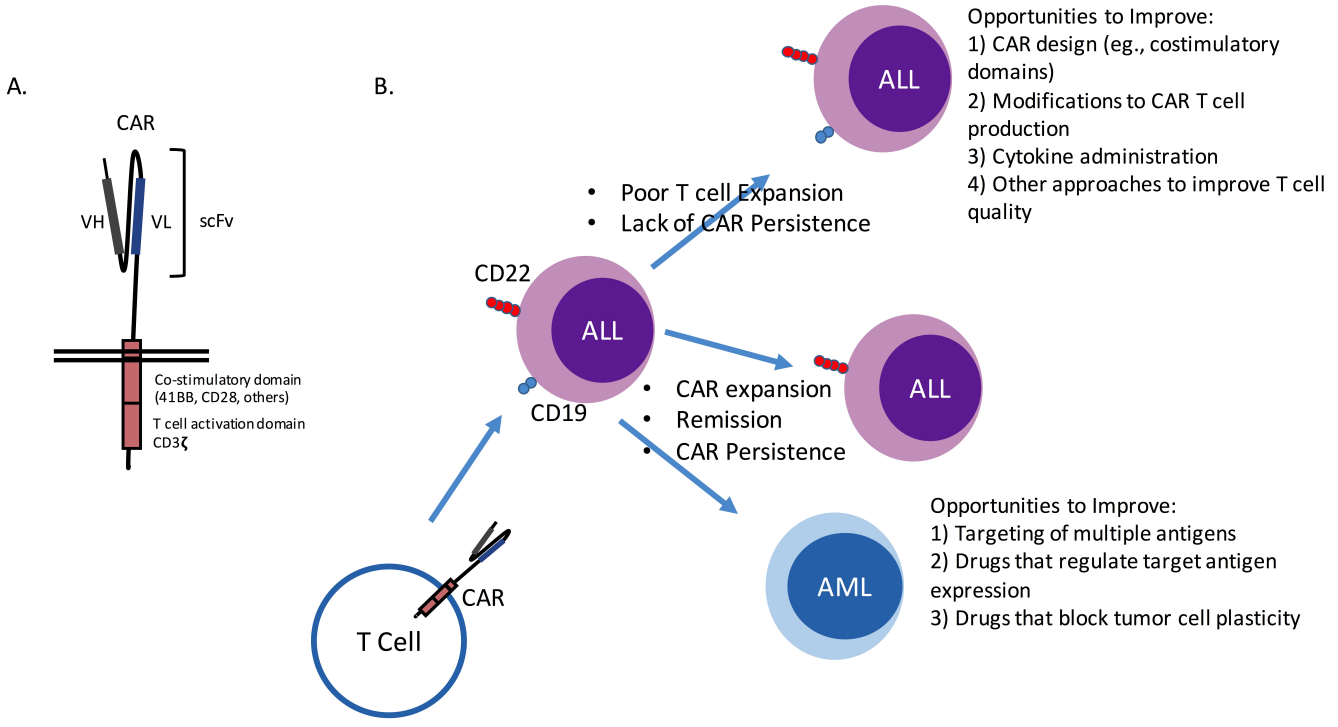

Figure 1. A) Schematic of a chimeric antigen receptor. B) Mechanisms of relapse after CAR therapy for ALL.

challenges. Multi-specific immune engagers and genetically modified T cells cause cytokine release syndrome a toxicity resulting in substantial morbidity and mortality can occur. In addition, significant neurotoxicity has been seen in some trials although the severity seems to be less in children than in adults. A better understanding of the pathophysiology of both CRS and neurotoxicity will be required to more safely administer these agents. For B lineage ALL, addressing antigen loss relapse will be important to improve durability of remissions. With recent identification of an active CAR targeting CD22 one approach would be to target 2 antigens simultaneously.[17,18] A CD19xCD22 bispecific CAR will enter clinical trials in the near future. Finally, although trials with CARs targeting AML are underway, it remains to be seen whether the success of CAR T cells in ALL can be reproduced in other hematologic malignancies.

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Acute lymphoblastic leukemia - The worst and the best - Section 3

Novel approaches with recently licensed drugs or recently studied in relapsed acute lymphoblastic leukemia

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Take-home messages

- Although acute lymphoblastic leukemia is highly curable with conventional chemotherapy (ALL), novel therapeutic approaches are still needed to improve outcomes for high-risk or relapsed ALL, especially in adults.
- Immunotherapeutic approaches have significantly improved the outcome of R/R ALL patients and are currently tested in early phases of the disease.
- Targeted therapy combined with conventional chemotherapy and/or immunotherapy can provide promising results in some specific subtypes of ALL.

Most drugs used in standard regimens for acute lymphoblastic leukemia (ALL) were developed more than 30 years ago and have contributed to the success of treatment, especially in children. Since that time, several new drugs have been developed and incorporated into ALL treatment. However, a small number of drugs have been approved by the regulatory agencies since the year 2000, including imatinib (2001), clofarabine (2004), nelarabine (2005), dasatinib (2006), liposomal vincristine sulfate (2012), ponatinib (2012) and blinatumomab (2014). In spite of this, novel therapeutic approaches are still needed to improve outcomes for high-risk or relapsed ALL, especially in adults. These include purine nucleoside analogs, mammalian target of rapamycin (mTOR) inhibitors, proteasome inhibitors, histone deacetylase (HDAC) inhibitors, hypomethylating agents, Bruton's tyrosine kinase (BTK) inhibitors, Janus kinase-signal transducer and activator of transcription (JAK-STAT) inhibitors, anti-programmed cell death protein (anti-PD-1) antibodies, mitogen-activated protein kinase (MEK) inhibitors, CXCR4 antagonists, poly (ADP-ribose) polymerase (PARP) inhibitors, and FMS-like tyrosine kinase 3 (FLT3) inhibitors, among others.[1] The immunotherapeutic approaches with monoclonal antibodies, antibody drug conjugates and bispecific antibodies have shown very promising results in relapsed or refractory ALL patients.[2] Apart of these drugs, therapeutic strategies harnessing T lymphocytes or NK cells have been developed, being the CAR T cells associated to an impressive short-term efficacy.[3] On the other hand, progress in the genetic characterization of ALL in both children and adults has allowed the recognition of specific subtypes with specific altered pathways targetable with specific drugs. Philadelphia chromosome-positive ALL was the first example of an ALL subtype in which the combination of tyrosine kinase (TKI) inhibitors with standard chemotherapy resulted in a significant improvement in the patients' survival. Similarly, the patients with the BCR-ABL like ALL subtype are currently enrolled in clinical trials with TKI inhibitors (e.g., dasatinib) or JAK inhibitors (e.g., ruxolitinib) combined with chemotherapy, depending on their mutational profile.[4] Progresses in the knowledge of the biology of MLL-rearranged ALL have led to evaluate the activity of hypomethylating drugs (eg, azacitidine), DOT1L inhibitors (e.g., EPZ5676), FLT3 inhibitors, MEK inhibitors or BCL-2 inhibitors, in combination with chemotherapy.[5]

Some of the novel approaches with recently licensed or recently studied drugs in relapsed or in newly diagnosed ALL have been associated with significant improvement in survival in phase 2 or phase 3 clinical trials. For mature B ALL, there is no doubt that the combination of rituximab with specific chemotherapy constitutes the treatment of choice.[6] Similarly, significant improvements in event-free survival have been observed with the incorporation of rituximab to the standard chemotherapy schedule in B-cell precursor (BCP) ALL with CD20 expression.[7] In patients with relapsed or refractory (R/R) BCP ALL two phase 3 studies with inotuzumab[8] ozogamycin and blinatumomab[9], respectively, have demonstrated significant improvement in the complete remission (CR) rate and the overall survival (OS) compared with standard of care (SOC) chemotherapy and these therapies should be currently considered as a bridge to hematopoietic stem transplantation (HSCT) in these patients. A global phase 2 study with blinatumomab in patients with minimal residual disease (MRD) positive ALL has shown 80% rate of molecu-

Acute lymphoblastic leukemia - The worst and the best - Section 3

Table 1. Antileukemic drugs in current clinical trials in acute lymphoblastic leukemia.

Class agent	Target	Indication
Purine nucleoside analogue		
Clofarabine	Ribonucleotide reductase; DNA Polymerase; mitochondria	All ALL
Nelarabine	Ribonucleotide reductase; DNA synthesis	T-ALL
Vinca alkaloid		
Vincristine sulfate liposome	Tubulin	All ALL
Kinase inhibitors		
ABL1 kinase inhibitors (dasatinib, ponatinib)	ABL1 kinase; PDGFR-B	BCR-ABL+ ALL
Aurora kinase inhibitors (alisertib)	Aurora A kinase	BCR-ABL-like ALL
Janus kinase inhibitors (JAK)	JAK	BCR-ABL+ ALL
Ruxolitinib, tofacitinib, other		JAK-mutated ALL
BCR-ABL-like ALL		
T-ALL		
Tyrosine kinase inhibitors	FMS-like tyrosine kinase 3 (FLT3)	MLL-ALL
Lestaurtinib, midostaurin, sorafenib, quizartinib, tandutinib, sunitinib		Hyperdiploid ALL
Other molecular or signaling inhibitors		
Proteasome inhibitors (bortezomib)	Ubiquitin pathway	All ALL
mTOR inhibitors (sirolimus, everolimus)	mTOR	All ALL
FT inhibitors (tipifarnib, lonafarnib)	Ras, lamin A	All ALL
γ secretase inhibitors	γ Secretase	T-ALL
Angiogenesis inhibitors (bevacizumab)	VEGF	All ALL
Apoptosis inducers (obatoclax, oblimersen)	Bcl-2	All ALL
CXCR4 antagonists	CXCL12(SDF1)/CXCR4 axis	All ALL
B-cell receptor inhibitors	Ibrutinib, idelalisib, other BTK inhibitors	B-precursor ALL
Epigenetic therapy		
DNA methyltransferase inhibitor (Azacitidine, decitabine)	DNA methyltransferase	All ALL
Histone methyltransferase inhibitor (EPZ-5676)	DOT1L	*MLL*-ALL
HDAC inhibitor (Vorinostat, panobinostat)	Histone deacethylase	All ALL
Immune therapy		
Monoclonal antibody		
Blinatumomab	*CD19 (engages CD3)*	CD19+ ALL
Coltuximab ravtansine	*CD19*	CD19+ ALL
Denintuzumab mafodotin	*CD19*	CD19+ ALL
DT2219ARL	*CD19 and CD22*	CD19/CD22+ ALL
Rituximab	*CD20*	CD20+ ALL
Epratuzumab,	*CD22*	CD22+ ALL
Moxetumomab pasudotox	*CD22*	CD22+ ALL
Inotuzumab ozogamycin	*CD22*	CD22+ ALL
Alemtuzumab	*CD52*	CD52+ ALL
Brentuximab vedotin	*CD30*	T-ALL
Cellular therapy		
NK cells	KIR ligand	
T cells with CD19 chimeric Ag receptor	*CD19*	CD19+ ALL
T cells with CD22 chimeric Ag receptor	*CD22*	CD22+ ALL
T cells with CD123 chimeric Ag receptor	*CD123*	CD19 negative relapses

lar response, being translated into improved OS, independent of the subsequent HSCT realization.[10] Another phase 2 study with blinatumomab in R/R Ph+ ALL has shown a CR rate of 36%, independent of the ABL mutation status.[11] Blinatumomab and inotuzumab are currently being investigated in newly diagnosed patients with BCP ALL, integrated with the chemotherapy schedule, especially during consolidation. In this sense, two ongoing phase 2 studies with inotuzumab combined with attenuated chemotherapy have shown promising results in adults and elderly patients with R/R or with newly diagnosed BCP ALL, respectively.[12,13]

Among the non-immunochemotherapeutic drugs, the combination of ponatinib with chemotherapy has shown very promising short-term results in patients with newly diagnosed Ph+ ALL,[14] being superior to those obtained with imatinib in historical comparisons.[15] If these results are confirmed in other ongoing trials this combination could be the treatment of choice for these patients. Other non-TKI approaches (e.g., allosteric inhibitors) are actively investigated. Current trials are evaluating the combination of immunotherapeutic drugs (e.g., blinatumomab) with TKI inhibitors in an attempt to treat Philadelphia chromosome-positive ALL with a non-chemotherapeutic approach.

Some approved drugs and drugs under development are actively investigated in phase 2 and phase 3 clinical trials. In this sense, randomized studies compare first-line chemotherapy with standard vincristine versus vincristine sulfate liposome in newly diagnosed Ph-negative ALL patients.[16] Clofarabine is currently being tested as part of the conditioning regimens for allogeneic HSCT in ALL patients.[17] Proteasome inhibitors are actively investigated in patients with R/R ALL (Table 1).

The therapeutic armamentarium with new drugs for T-ALL is limited. Nelarabine has been safely integrated into intensive chemotherapy regimens in newly diagnosed T-cell ALL in children and in adults, although more information is needed to know whether the addition of nelarabine improves their outcome.[18] Targeting NOTCH (gamma-secretase inhibitors), the IL7R-JAK1/3-STAT5 axis (ruxolitinib, tofacitinib), the PI3K/Akt/mTOR axis (PI3K or mTOR inhibitors), the NUP214-ABL1 rearrangement (dasatinib) or BCL2 (BCL2 inhibitors, ETP ALL) are promising areas of research.[19,20]

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Acute lymphoblastic leukemia - The worst and the best - Section 3

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Acute myeloid leukemia

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Introduction

Acute myeloid leukemia (AML) is a clonal disorder arising from hematopoietic progenitors developing in the myeloid pathway characterized by deregulated differentiation and maturation programs. Deciphering of the molecular heterogeneity by rapidly evolving genomic technology has resulted in a multitude of new mutations and gene expression signatures resulting in unique genomic make up of every individual AML. Prognostication is now also based on these new aberrancies. Treatment of AML relies for over 40 years on a combination of cytarabine and an anthracycline aiming to achieve a complete remission(CR) while post remission therapy (either chemotherapy or stem cell transplantation in its various forms) aims to maintain it by eradication of residual disease. Prognosis especially in the elderly population remains very poor, in the younger age segment a gradually improvement have been achieved in the previous decennia resulting in an OS between 40-50%. Nevertheless, new treatment strategies are urgently warranted to improve outcome and to decrease toxicity. A large number of new drugs targeting leukemic drivers or a multitude of deregulated pathways are awaiting clinical application. In this educational session on AML we focus on a selection out of these topics.

Learning goals

1. ***Get knowledge of the growing importance of molecular genomics to inform patient care with regard to improved disease classification and risk prediction, MRD monitoring and guiding targeted therapeutic approaches.***
2. ***To learn that many new therapies emerge that target cell surface markers, mutated genes, deregulated pathways or immune response that will improve the outcome of the standard of care for AML (3+7).***
3. ***To understand that FLT3 inhibition is likely to be incorporated into the management of FLT3-mutated AML.***

EUROPEAN HEMATOLOGY ASSOCIATION

Acute myeloid leukemia - Section 1

Molecular diagnostics in acute myeloid leukemia

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Take-home messages

- There is a growing need to implement novel next-generation-sequencing (NGS) based gene panel diagnostic tools to rapidly capture inter- and intra-individual disease heterogeneity.
- Future technological developments will enable genome-wide comprehensive genomic, epigenomic and transcriptomic characterization of the disease (at single cell level), but for now these approaches are reserved for research questions.
- Molecular genomics have started to inform patient care with regard to improved disease classification and risk prediction (knowledge databases), MRD monitoring and guiding targeted therapeutic approaches.

Introduction

For many years, genomic aberrations have been known to play an important role in the pathogenesis of acute myeloid leukemia (AML) and have become well established diagnostic and prognostic markers.[1,2] Since the turn of the century, advances in microarray and next-generation sequencing (NGS)-based "omics" technologies have contributed to an exponential knowledge growth of the molecular aberrations underlying AML,[3,4] but only recently molecular diagnostics have begun to translate into improved disease classification and clinical care.[5,6] In this article, I will provide a brief overview of the heterogeneous genomic landscape of AML and its impact on molecular diagnostics, including recent advances in genomics-based AML classification and patient care.

Current state of the art

Genomic landscape

Following first comprehensive studies using high-throughput microarray technologies, AML was also the first tumor genome to be completely sequenced using the novel NGS technology.[7] Subsequent studies led to the identification of novel recurrent somatic mutations of biologic, prognostic, and therapeutic relevance, and they identified AML as complex and dynamic disease characterized by a high inter- and intra-individual heterogeneity. Genome-wide profiling of 200 *de novo* AML cases within the 'The Cancer Genome Atlas (TCGA)' project revealed an average of 13 coding mutations [single nucleotide variations (SNVs), and insertions/deletions (indels)] per adult AML as well as a median of one somatic copy-number variant (e.g., trisomies or monosomies) and an average of less than one gene-fusion event.[3] While the recurrently mutated genes included known candidates (such as *NPM1, FLT3, CEBPA, DNMT3A, IDH1*, and *IDH2*) as well as genes just recently implicated in leukemogenesis (including *EZH2, U2AF1, SMC1A*, and *SMC3*), the mutational patterns were non-random of co-occurrence and mutual exclusivity. Especially *NPM1*, *CEPBA*, and *RUNX1* mutations were mutually exclusive of transcription factor fusions, thereby indicating that these aberrations might be leukemia-initiating events similar to the fusion genes.

Clonal evolution

Analysis of the variant allele frequency (VAF) demonstrated that over half of the TCGA cases exhibited at least one subclone in addition to a founding leukemia clone (the clone showing the highest VAF values).[3] Together with other studies, these data support a clonal evolution concept in which epigenetic regulator mutations (e.g. *DNMT3A, TET2*, and *ASXL1* mutations) or splicing factor gene mutations (e.g. *SF3B1,* and *SRSF2* mutations) occur as early founder events in pre-leukemic progenitor cells prior to transforming leukemogenic events (e.g. *NPM1* or signaling molecule mutations). In accordance, recurrent mutations in epigenetic regulators and splicing factor genes can be found in the blood of mainly elderly patients,[8,9] and the term 'clonal hematopoiesis of indeterminate potential' (CHIP) was proposed to describe the presence of leukemia-associated somatic mutations in blood or bone marrow in the absence of conventional diagnostic criteria for a hematologic malignancy.[10] While the transformation rate of CHIP into a hematologic malignancy is 0.5-1% per year, in the future the role of persisting CHIP following leukemia treatment will have to be better understood by monitoring of minimal residual disease (MRD) for both pre-leukemic and leukemic markers.

Molecular diagnostics

Today, conventional cytogenetic analysis remains mandatory for the AML workup, although molecular testing by reverse transcriptase–polymerase chain reaction (RT-PCR) for recurring rearrangements can be useful if cytogenetic analysis fails and in the future whole genome sequencing approaches might fill in. Molecular genetic diag-

nostics, as recommended by the European LeukemiaNet (ELN),[6] should comprise at least screening for (i) disease defining mutations in *NPM1*, *CEBPA*, and *RUNX1* genes; (ii) prognostic and targetable mutations in *FLT3*, both tyrosine kinase domain mutations (at codons D835 and I836) mutations and internal tandem duplications [ITDs] (including data on the mutant–to–wild-type allelic ratio); and (iii) mutations in *TP53* and *ASXL1* that have consistently been associated with poor prognosis (Table 1). While it is time consuming and cost ineffective to capture these aberrations by conventional sequencing strategies, the list of molecular markers informing clinical practice is growing and testing will have to be replaced by gene panel diagnostics. Currently, there are already a number of commercial and custom designed gene panels available,[11] but it will be crucial to invest in flexible platforms and to develop diagnostic tools that can simultaneously test for both gene mutations and gene rearrangements.[12,13]

Genomic classification

Leukemia-associated chromosomal translocations and inversions opened the avenue towards the genetic AML classification reflected in the currently updated World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia,[5] however, during recent years NGS studies have also been informing disease classification.[3,4,14] Beyond currently defined classes (such as the balanced rearrangements, AML with mutated *NPM1*, or biallelic mutated *CEBPA*), three more heterogeneous classes emerged, i.e. '*AML with mutated chromatin, RNA-splicing genes, or both*', '*AML with TP53 mutations, chromosomal aneuploidy, or both*', and '*AML with IDH2^{R172} mutation*' (without other class-defining lesions). Using this classification scheme, at least 80% of AML could ambiguously be categorized in a single group based upon the underlying genetic abnormalities.[4]

Genomics informed patient care

Recent advanced proved also that novel genetic information can be successfully applied to inform clinical practice. For example, a large knowledge bank of matched genomic-clinical AML data could be devised to accurately predict likelihoods of remission, relapse and mortality with findings being validated on independent TCGA data.[15] Future models based on increased patient numbers will allow to further reduce the error rate of such personalized treatment predictions, and European initiatives like HARMONY (Healthcare Alliance for Resourceful Medicines Offensive against Neoplasms in HematologY) are currently capturing, integrating, and harmonizing patient data from large AML cohorts to gain valuable novel insights (https://www.ehaweb.org/news/eha-news/article/125). Similarly, genomic knowledge does now also facilitate follow-up monitoring of MRD, and highly sensitive digital PCR as well as targeted ultra-deep NGS approaches are valuable novel tools adding to quantitative

Table 1. 2017 European LeukemiaNet (ELN) risk stratification by genetics.[a]

Risk Category[b]	Genetic Lesion
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITDlow(c) Biallelic mutated *CEBPA*
Intermediate	Mutated NPM1 and *FLT3*-ITD$^{high(c)}$ Wild type NPM1 without *FLT3*-ITD or with *FLT3*-ITD$^{low(c)}$ (w/o adverse-risk gene mutations) t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*[d] Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, *MECOM(EVI1)* -5 or del(5q); -7; -17/abn(17p) Complex karyotype,[e] monosomal karyotype[f] Wild type *NPM1* and *FLT3*-ITD$^{high(c)}$ Mutated *RUNX1*[g] Mutated *ASXL1*[g] Mutated *TP53*[h]

Adapted from: Dohner et al. Blood 2017;129:424-47; with permission. Frequencies, response rates and outcome measures should be reported by risk category, and, if sufficient numbers are available, by specific genetic lesions indicated; [b]prognostic impact of a marker is treatment-dependent and may change with new therapies; [c]low, low allelic ratio (<0.5); high, high allelic ratio (≥0.5); semi-quantitative assessment of FLT3-ITD allelic ratio (using DNA fragment analysis) is determined as ratio of the area under the curve (AUC) 'FLT3-ITD' divided by AUC 'FLT3-wild type'; recent studies indicate that AML with NPM1 mutation and FLT3-ITD low allelic ratio may also have a more favorable prognosis and patients should not routinely be assigned to allogeneic hematopoietic-cell transplantation; [d]the presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations; [e]three or more unrelated chromosome abnormalities in the absence of one of the WHO-designated recurring translocations or inversions, i.e., t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3); AML with BCR-ABL1; [f]defined by the presence of one single monosomy (excluding loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core-binding factor AML); [g]these markers should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes; [h]TP53 mutations are significantly associated with AML with complex and monosomal karyotype.

reverse-transcriptase polymerase chain reaction (qRT-PCR) and multiparameter flow cytometry (MFC) methods. The NGS-based identification of molecular markers in almost 100% of diagnostic AML cases provides a prerequisite for comprehensive and individualized MRD assessment to identify patients at high relapse risk at early time points and to detect persistent pre-leukemic hematopoiesis.[16,17] Finally, genomics knowledge will allow us to better guide the use of novel drugs such as protein kinase inhibitors, epigenetic modulators, immune checkpoint inhibitors and cellular immunotherapies.[2,6] However, selective inhibition may only address distinct leukemia subclones. Thus, future molecularly targeted treatment designs will have to take clonal relationships into account and treatment strategies should be adjusted based on longitudinal clonal monitoring.

Future perspectives

Given a growing list of disease-relevant genes in AML, NGS-based gene panel diagnostics have started to enter our daily clinical routine. Today, rapid technical NGS advances allow for more accurate MRD assessment and start to offer the possibility to capture leukemia heterogeneity at the single cell level.[18,19] In addition, future developments will ultimately allow genome-wide unbiased tests at high quality based on which individualized treatment approaches can be further advanced.

Acknowledgements

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Acute myeloid leukemia - Section 2

Targeting mutated FLT3 in acute myeloid leukemia

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Take-home messages

- FLT3-mutated AML evolves during therapy, with FLT3-addicted clones emerging at relapse.
- FLT3 inhibition is likely to be incorporated into the management of FLT3-mutated AML in the very near future, but the roles of selective versus non-selective inhibitors remains to be determined.

Introduction

Acute myeloid leukemia (AML) develops from mutations arising within the genomes of hematopoietic stem/progenitor cells. Our current understanding of this process is that founding mutations confer a proliferative advantage to stem cell clones, and the progressive occurrence of cooperating mutations leads to eventual transformation and overt clinical disease.[1] FLT3, which codes for a receptor tyrosine kinase, is one of the most commonly mutated genes in AML,[2] and because these mutations are associated with constitutive activation of that receptor, inhibiting FLT3 has been a goal of numerous clinical studies for several years now. This short review will summarize the current state of this field, and will offer some perspectives on the future of targeting FLT3 in AML.

Current state of the art

There are two types of FLT3 mutations. The first type, occurring in ~23% of newly-diagnosed AML cases, consists of internal tandem duplications (ITDs), in which duplicated sequence (3-200+ base pairs) is inserted in tandem, and in-frame, into the region coding for the juxtamembrane domain (and sometimes extending into the kinase domain).[3,4] The resulting additional amino acid sequence disrupts the auto-inhibitory function of this domain and leads to constitutive receptor activation. It is important to note, however, that FLT3 receptors with an ITD mutation are still dependent on the cognate ligand, FLT3 ligand (FL), for full activation, and that the addition of FL to FLT3-ITD AML blasts *in vitro* has proliferative and anti-apoptotic effects.[5] The second type of FLT3 mutations occur in 7% of patients at diagnosis and consists of point mutations in the tyrosine kinase domain (TKD).[3] While FLT3-TKD mutations also cause constitutive activation, the signaling of FLT3-TKD receptors is less aberrant as compared to FLT3-ITD receptors. FLT3-TKD mutations probably have a mild negative effect on prognosis (although this remains somewhat controversial), while FLT3-ITD mutations confer a clear negative impact on survival. AML patients with FLT3-ITD mutations typically present with pronounced leukocytosis, and while cytarabine-based induction therapy will often result in remission, these patients relapse more frequently, and relapse earlier, than AML patients with wild type FLT3.

FLT3 mutations were first identified in 1996, and their prognostic impact was established after 2002, following the publication of a series of retrospective studies.[3] At that time, the only small molecule tyrosine kinase inhibitors (TKIs) available were compounds that had been developed for other molecular targets. Midostaurin was first introduced as an inhibitor of protein kinase C (PKC) almost 30 years ago,[6] while lestaurtinib had been initially studied as an inhibitor of TrkA.[7] In reality, both compounds are highly non-selective, inhibiting many other kinases (and likely other ATP-binding enzymes) as well. Neither drug was particularly effective as monotherapy for FLT3-mutated AML.[8,9] Trials of lestaurtinib combined with chemotherapy for newly-diagnosed or relapsed patients yielded no benefit.[10,11] On the other hand, the recent results of CALGB 10603 ("RATIFY") indicate that midostaurin administered immediately after a standard 3 + 7 induction improves response rates and overall survival for newly-diagnosed patients with either FLT3-ITD or FLT3-TKD mutations.[12] Sorafenib, a drug initially developed as a RAF kinase and VEGF-R inhibitor, can induce compete responses as monotherapy in relapsed FLT3-ITD AML,[13] and has shown remarkable activity in the post-allogeneic transplant setting or in combination with azacitidine.[14,15] While all of these studies were proceeding, quizartinib emerged as the first TKI specifically designed to target FLT3,[16] and it displayed a high level of activity as a single agent in the relapsed setting.[17] Patients

responding to quizartinib quickly developed resistance-conferring FLT3-TKD mutations,[18] and so the next-generation FLT3 TKIs, crenolanib and gilteritinib (both of which were also effective against TKD mutations) were introduced.[19,20]

The pre-clinical and clinical studies of these drugs have provided us with considerable insight into the biology of FLT3-ITD AML. When we merge these insights with the data provided from next generation sequencing studies of AML mutations, we have a clearer understanding of the problem and how best to approach it. At diagnosis, like all AML, FLT3-ITD AML is polyclonal, and the majority of the clones are not dependent on mutant FLT3 signaling for survival. Next generation sequencing (NGS) studies on AML samples indicate that several sub-clones are present at diagnosis,[21] and *in vitro* studies of FLT3-ITD AML blasts suggest that only a subset of these clones are dependent on mutant FLT3 signaling for survival.[22] At relapse, the disease is more oligoclonal, and the blasts display far greater sensitivity *in vitro* to FLT3 inhibition, highlighting the need for potent selective inhibitors such as quizartinib or gilteritinib in this setting. The underlying mechanism for this selection is not known, but it is conceivable that FL plays a role. Plasma levels of FL increase exponentially during repeated rounds of aplasia-inducing chemotherapy,[23] and it is possible that this constant 'bath' of the very cytokine which the blasts depend on for survival selects for the outgrowth of FLT3-addicted clones.

Future perspectives

There are 9 completed or actively accruing phase 3/pivotal trials of FLT3 TKIs for the treatment of FLT3-mutated AML (Table 1). CALGB10603 met its primary endpoint of improved survival, and so FLT3 inhibition with a first generation inhibitor may well be a part of standard AML therapy in the near future. Assuming eventual approval of the later-generation drugs, how should we incorporate FLT3 inhibition into the current treatment paradigms? In keeping with the conventional approach used in BCR-ABL-driven acute leukemia, it seems likely that FLT3 inhibitors will be used early and continuously into induction, consolidation, and maintenance therapy (with or without allogeneic transplant) for FLT3-mutated AML. The uncertainty lies in the decision as to which drug to use, and when to use it. Data from CALGB 10603 suggests that midostaurin following induction chemotherapy produces more frequent and deeper responses, but its benefits in the maintenance setting seem much less clear, and it probably has no role in relapse.[12] In relapsed FLT3-ITD patients, potent selective like quizartinib and gilteritinib produce gratifyingly rapid responses, but these responses are incomplete and often short-lived. Interestingly, the combination of FLT3 inhibitors with azacitidine may be the most effective approach in the salvage setting,[15,24] although combinations with conventional intensive chemotherapy regimens is currently being studied. Finally, allogeneic transplant has emerged as a preferred consolidation for FLT3-ITD AML, and FLT3 inhibition appears to synergize with the allogeneic effect. Post-transplant maintenance with a selective, well-tolerated FLT3 TKI is a logical approach, and the benefit of this will be determined by an international trial just getting underway (BMT-CTN 1506).

Table 1. Phase 3 trials of FLT3 inhibitors for the treatment of FLT3-mutated AML. UK MRC = United Kingdom Medical Research Council. CALGB = Cancer and Leukemia Group B. AMLSG = AML Study Group. BMT-CTN = Bone Marrow Transplant Clinical Trials Network.

Trial Name	Sponsor	Treatment	Population	Clinical trial number	Accrual start date
204	Cephalon	Chemotherapy +/- lestaurtinib	Relapsed FLT3-mutated AML	NCT00079482	Completed
AML15/17 I	UK MRC	Chemotherapy +/- lestaurtinib	Untreated FLT3-mutated AML	ISRCTN17161961 SRCTN55675535	Completed
CALGB10603 (RATIFY)	CALGB/Novartis	Chemotherapy +/- midostaurin	Untreated FLT3-mutated AML	NCT00651261	Completed
QUANTUM-R	Daiichi- Sankyo	Chemotherapy vs. quizartinib	Relapsed FLT3-ITD AML	NCT02039726	April 2014
'Admiral'	Astellas	Chemotherapy vs. gilteritinib	Relapsed FLT3-mutated AML	NCT02421939	October 2015
'Lacewing'	Astellas	Azacitidine +/-gilteritinib	Untreated FLT3-mutated AML, less fit	NCT02752035	June 2016
'Gossamer'	Astellas	Giltertinib maintenance	Untreated FLT3-mutated AML	NCT02927262	October 2016
AMLSG 19-13/ARO-007	AMLSG/Arog	Chemotherapy +/- crenolanib	Relapsed FLT3-mutated AML	NCT02298166	January 2017
BMT-CTN1506/'Morpho'	BMT-CTN, Astellas	Giltertinib maintenance	FLT3-mutated AML s/p allo transplant	NCT02997202	May 2017

Acute myeloid leukemia - Section 2

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EUROPEAN HEMATOLOGY ASSOCIATION

Acute myeloid leukemia - Section 3

3+7 and beyond

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Take-home messages

- 3+7 is the standard of care for AML a is used as a chemotherapy backbone
- Many new therapies have recently emerged which target cell surface markers, mutated genes, deregulated pathways or immune response
- Variety and complementarity of their mechanisms of action make it possible to action several cellular pathways (eg. apoptosis or signaling) or cell populations (eg. LSCs or immune effectors) and to envisage the development of multi-agent combination regimens.
- They may challenge the current paradigm of AML therapy and open prospects for chemo-free therapies.

Introduction

'3+7' generally refers to the combination of an anthracycline (daunorubicin or idarubicin given over 3 days) to cytarabine (given at conventional doses of 100 to 200 mg/m²/day over 7 to 10 days) that is the mainstay of acute myeloid leukemia (AML) induction chemotherapy since the last 30 years. Improvements on the original combination regimen have been the goal of many clinical trials conducted by cooperative groups all over the world during this period. This research has been productive as an international consensus has been achieved.[1] On the other hand, limited progress has been made contrasting with those achieved in other hematologic malignancies such as lymphoma, myeloma or chronic leukemias during the same period of time. Up to now, most of the attempts to improve on 3+7 were based on dose intensification or the addition of new drugs. However, this paradigm may be challenged because: 1) new potentially active therapies with various and original mechanisms of action (MoA) have emerged, and 2) the acute promyelocytic leukemia (APL) model demonstrates that 'chemo-free' treatment approaches (combination of retinoic acid and arsenic) are more effective than conventional treatments. Thus, we may wonder if time has come to change our paradigm and get rid of 3+7.

The strengths and weaknesses of 3+7

3+7 can induce complete remission (CR) in 60-80% of younger adults and in 40-60% of older adults that are fit for intensive therapy.[2] Blast clearance is generally obtained in few days and approximately two-third of the patients have empty marrow by day 15. This may be beneficial in AML and especially in forms with hyperleucocytosis associated with life-threatening complications. 3+7 may therefore represent an optimal debulking strategy. However, relapse that occur in the majority of patients remains an issue as well as toxicity which limits the applicability of 3+7 in elderly frail patients. Attempts to improve on 3+7 have included: 1) intensification of the anthracycline dose; 2) the use of high or intermediate doses of cytarabine (HIDAC); 3) double induction strategies;and 4) the addition of a third drug to the 3+7 'backbone'. Although still a matter of debate, the findings of several large multicenter randomized trials indicate that optimal daunorubicin doses range between 60 to 90 mg/m² while the benefit of replacing daunorubicin by idarubicin or conventional doses of cytarabine by HIDAC is not yet established.[1] Several attempts have been made to add a third drug to 3+7. The anti-CD33 antibody-drug conjugate (ADC) Gemtuzumab ozogamycin (GO) showed conflicting results, but a meta-analysis demonstrated a survival improvement in patients with non-unfavorable cytogenetics.[3] Cytotoxic agents such as clofarabine,[4,5] cladribine,[6] or lomustine,[7] gave conflicting or not-yet-confirmed results. The lack of consistent effects across trials reflects the weak activity of these drugs. Another issue is toxicity that balances the benefits of attempts to increase chemotherapy dose-intensity or to add a third drug. Finally, 3+7 is a 'one-size-fits-all' approach that disregards the molecular and genetic heterogeneity of AML.[8] This underlines the need for new agents with new MoAs and greater therapeutic index.

Acute myeloid leukemia - Section 3

The addition of new drugs to reinforce 3+7

Many new drugs are currently in clinical development and are summarized in Table 1. This list is not meant to be exhaustive but rather to give a snapshot of the variety of therapeutic classes and MoAs. It also deliberately omits FLT3 inhibitors that are discussed in the previous chapter of this review (see Section 2 - Targeting mutated FLT3 in acute myeloid leukemia).

New cytotoxic drugs may be used instead of or be added to 3+7. CPX-351 is an encapsulation in nano-scale liposomes of cytarabine and daunorubicin and has shown superior CR and survival as compared to 3+7 in elderly patients with secondary

Table 1. New agents in clinical development.

Therapeutic class	Drug	Patients	Status
Cytotoxic agents			
Liposomal D+A	CPX 351	HR elderly AML frontline	Rando Phase 2
Topo-II inhibitor	Vosaroxin	R/R AML	Phase 3 R/R
Monoclonal antibodies			
AntiCD33 mAb	Iintuzumab	Misc.	Phase 3
AntiCD33 ADC	GO	frontline	Phase 3
	SGN-33A	R/R AML +frontline	Phase 3 combo
AntiCD33/CD3	AMG330	R/R AML	Phase 1 single agent
Anti-CD123 mAb	Talacotuzumab	R/R AML	Phase 2 combo
Anti-CD123 ADC	SGN-CD123A	R/R AML	Phase 1 single agent
Anti-CD3/CD123	MGD006	R/R AML	Phase 1 single agent
	JNJ-63709178	R/R AML	Phase 1 single agent
Apoptosis targeting agents			
BCL2-i	Venetoclax	R/R AML	Phase 2 combos
	S55746	R/R AML	Phase 1
MCL1-i	S64315	R/R AML	Phase 1
MDM2-i	Idasanutlin	R/R AML	Phase 3 combo
Kinase/Cell cycle-i			
PIM kinase-i	CLGH447	R/R AML	Phase 1 combo
MEK-i	Cobimetinib	R/R AML	Phase 1 combo
PI3K/RAS-i	Rigosertib	R/R AML	Phase 1
CDK-i	Palbociclib	R/R AML	Phase 1
Epigenetic drugs			
Oral azacitidine	CC486	Frontline	Phase 3 combo
Decitabine prodrug	SGI-110	Frontline elderly	Phase 3
Bromodomaine-i	OTX015	R/R AML	Phase 1
DOTL1-i	EPZ-5676	R/R MLL AML	Phase 1
Immunotherapy			
ICB			
Anti-CTLA4	Ipilimumab	R/R AML	Phase 1-2
Anti-PD1	Nivolumab	R/R + frontline AML	Phase 1-2 combo
Anti-KIR	IPH2101	R/R AML	Phase 1
	Lirilumab	frontline elderly AML	Phase 2-3
Anti-NKG2A	Monalizumab	Maintenance post allo	Phase 1
CAR-T cells			
Anti-CD33	CART33	R/R AML	Phase 1
Anti-CD123	CART123	R/R AML	Phase 1
Anti-CD133	CART133	R/R AML	Phase 1
Others			
Nuclear export-i	selinexor	Frontline AML	Phase 3
Hedgehog-i	Sonidegib	Frontline R/R AML	Phase 1 combo
	PF-04449913	Frontline AML	Phase 2

AML.[9] Vosaroxin is a quinolone derivative shown to improve survival in elderly patients with refractory/relapsed AML when given in combination with IDAC[10] and is currently being evaluated in addition to 3+7.

Cell surface antigens such as CD33 and CD123 represent attractive targets for monoclonal antibodies (mAb), ADCs, bispecific antibodies or chimeric antigen receptor T-cells (CART). The prior experience with GO has validated the therapeutic relevance of CD33.[3] CD123 is expressed by the Leukemic Stem Cell (LSC) compartment. CD123 directed therapeutic strategies have therefore the potential to target LCS which may persist after remission and represent a relapse reservoir.

Small molecules can target products of mutated genes such as FLT3 (see Section 2) or IDH1 and 2, of overexpressed proteins involved in a variety of pathways including signaling (RAS, KIT, PIM), apoptosis (BCL2 family members, TP53) or of epigenetic regulators (DNA methytransferases, bromodomaines or DOT1L). Some of them have shown encouraging clinical activity in phase 1 and 2 studies and are currently in phase 3, like venetoclax, a BCL2 inhibitor,[11] idasanutlin a MDM2 inhibitor[12] or Selinexor (a nuclear transport protein exportin (XPO1) inhibitor). Trials of their combination with 3+7 frontline are underway (NCT02545283, NCT02403310).

Finally, immunotherapeutic approaches are appealing in AML for which a body of evidence supports the rationale for a role of immune effectors (T-cells and NK-cells) in the control of the disease.[13,14] Anti-KIR[15] and anti-NKG2A mAbs may block inhibitory signals and therefore promote anti-leukemic NK-cell cytoxicity. Immune checkpoint blockade using anti-PD1/PDL1 or anti-CTLA4 are currently being investigated in AML.(13) Preliminary congress reports suggest limited single agent clinical activity and combination studies are underway.

Classical clinical development paths involve the addition of new agents to a 3+7 backbone, and many of the above-mentioned drugs are currently tested accordingly. However, their wide variety of MoAs opens new perspectives, including the design of chemo-free therapies that follow an 'APL paradigm'. The molecular heterogeneity of AML predicts the failure of single-target approaches and future work should focus on the clinical evaluation of combinations, the rationale of which is already supported by many preclinical evidences. For instance, preclinical data suggest synergism between BCL2-inhibitors and MCL1-inhibitors or IDH1/2 inhibitors while ICP blockade might reinforce T-cell activation following treatment with bispecific anti-CD3 antibodies. In this perspective, new agents may be viewed as part of therapeutic platforms targeting actionable biological functions such as apoptosis (BCL2 family members inhibitors, MDM2 inhibitors), immune response (ICP blockade, anti-KIR) and signaling (multi-kinase-inhibitors, anti-FLT3, anti-MEK, etc.) or leukemic sub-populations like LCS (anti-CD123, Hhg inhibitors).

In this perspective, the future of AML therapy -beyond 3+7- might be the development of chemo-free strategies built on the right combination of the various therapeutic platforms, like the blocks of a building game, adapted to the various patient population and leukemia subgroups. Moving fast from utopia to reality, we have already embarked in these new approaches and several multi-agent combinations trials are underway.

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Acute myeloid leukemia - Section 3

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Aggressive lymphoma

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Introduction

Our understanding of the molecular pathogenesis of the various types of aggressive lymphoma has increased markedly over recent years. The use of gene expression profiling helps define the different molecular pathways that the various disease sub-types are dependent upon. The importance of this with respect to prognostic and potential therapeutic implications has been recognized within the revised 2016 WHO classification and with the advent of multiple novel targeted agents this offers a real opportunity for new treatment approaches. Currently R-CHOP remains very much the standard of care for the commonest aggressive lymphomas, despite recent attempts to improve on this. The challenge remains in delivering effective curative therapy to older patients and those with co-morbidities. Part of the answer to this is in effectively assessing the physical health of the patient in a structured way in order to define the appropriate intensity of therapy that the patient can receive. This session will provide insights into the management of aggressive non-Hodgkin's lymphoma (NHL) in the elderly as well as patients with relapsed disease and in addition will explore the new pathological basis of these diseases and how this might help in the development of new and improved approaches.

Learning goals

1. ***To better understand the molecular pathogenesis of aggressive NHL.***
2. ***To review the approach to the management of aggressive NHL in the elderly patient.***
3. ***To review the current approach to managing relapsed aggressive NHL and the potential new emerging therapies.***

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Aggressive lymphoma - Section 1

The biological basis of aggressive lymphoma

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Take-home messages

- Aggressive mature B-cell lymphomas are a heterogeneous group of diseases with different clinical and biological features that may be related in part to the diverse pathogenic molecular mechanism.
- The identification of molecular subtypes and specific genetic alterations in aggressive DLBCL are clinical relevant and may guide therapeutic strategies.

Aggressive mature B-cell lymphomas are a heterogeneous group of diseases with different clinical and biological features.[1] The most common subtype accounting for approximately 80% is diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS), a category that includes tumors that cannot be recognized in any of the other more specific entities. Other aggressive B-cell lymphomas are Burkitt lymphoma and different subtypes of large B-cell lymphomas that may be grouped in those originated in specific topographic sites, associated with EBV and/or HHV8 infection, and expressing a terminal B-cell differentiation phenotype among others. The updated WHO classification includes a provisional category of high grade B-cell lymphomas (HGBL) that highlights the relevance of *MYC* together with *BCL2* and/or *BCL6* translocations. The marked diversity of all these tumors is related to different pathogenic mechanisms.

Diffuse large B-cell lymphoma, NOS is very heterogeneous. One of the major advances understanding its diversity was the recognition of two molecular subtypes based on their gene expression profiling (GEP) related to the different cell of origin (COO) in germinal center B-cells (GCB) or activated B-cells (ABC).[2] In addition to the GEP these two molecular subtypes differ in the activation of different molecular pathways, profile of chromosomal alterations and somatic mutations (Table 1). These biological differences translate into different outcome of the patients with most of the studies showing worse prognosis for ABC than GCB-DLBCL. GCB tumors rely preferentially on the activation of the PI3K pathway whereas ABC tumors have a constitutive activation of the NFkB pathway through different mechanisms including the activation of the BCR signaling pathway. Genetic alterations in GCB include *BCL2* translocations, activating mutations in the histone modifier *EZH2*, loss of function mutations of *GNA13* that regulates the topographic location of germinal center B-cells and inactivating mutations of *TNFRSF14* that lead to a cell-autonomous activation of B cell proliferation. ABC tumors have frequent activating mutations in the BCR and TLR pathways including *CD79a, CARD11,* and *MYD88*, inactivating mutations in inhibitors of NFkB such as *TNFAIP3*, and mutations preventing the terminal B-cell differentiation of B-cells such as inactivating mutations of *PRDM1*.[2,3] In spite of these different alterations, GCB and ABC-DLBCL share also common alterations including alterations in *TP53*, histone modifiers (*CREBBP, KMT2D*), *FOXO1*, *BCL6* translocations and point mutations, and inactivating mutations in genes related to immunesurveillance (e.g. *B2M*). Given the interest in defining different therapeutic strategies the recognition of these GCB and ABC DLBCL molecular subtypes is now recommended in the clinical practice and can be performed using different immunohistochemical algorithms or gene expression based assays.[4,5]

Primary mediastinal large B-cell lymphoma (PMBL) usually presents in young females with a large mediastinal mass that may infiltrate surrounding structures. Gene expression profiling has identified a specific signature that may be useful to differentiate PMBL from DLBCL, NOS involving the mediastinum or to recognize these tumors in locations outside the thorax (Table 1).[6] The genetic profile differs from DLBCL, NOS with frequent translocations inactivating *CIITA*, and activation of the NFkB and JAK/STAT pathways due to several genetic alterations in regulatory genes such as *TNFAIP3* and *SOCS1* and *PTPN1*, respectively.[6]

Burkitt lymphoma (BL) is a well defined entity genetically characterized by *MYC* rearrangements. In the last years NGS have revealed the profile of somatic mutations with frequent mutations in *TCF3* and *ID3* that are very uncommon in DLBCL and lead to the activation of the PI3K pathway. Activating mutations of *CCND3* are found in 30% of the

Aggressive lymphoma - Section 1

Table 1. Distinctive molecular characteristics of aggressive B-cell lymphomas.

	GCB	ABC	PMBCL	HGBL-DH
Cell of origin	Germinal Ctr cell	Activated B cell	Thymic mature B cell	Germinal Ctr cell
Chromosomal alterations	BCL2-R BCL6-R MYC-R	3q, 18q, 19q gains 9p del BCL6-R MYC-R	9p24 gain/amp CIITA	MYC-R and BCL2 and/or BCL6-R
Somatic mutations	EZH2, GNA13, TNFRSF14	MYD88, CARD11, CD79B, A20, PRDM1	JAK, SOCS1, PTPN1, A20	EZH2, CREBBP, ID3, CCND3,
Altered pathways	PI3K GNA13	NFkB BCR	NFkB	JAK/STAT

R: rearrangements.

tumors. An unresolved issue is the existence of true BL without *MYC* translocation. Recent studies have identified cases with similar morphology and phenotype but negative for *MYC* rearrangements that have 11q alterations with proximal gains and telomeric losses. These cases have been named Burkitt-like lymphoma with 11q aberrations and have more frequent nodal presentation and complex karyotypes.[1] The information on these cases is still limited.

In the last years, the possibility to study MYC protein expression and gene alterations in routine cases has expanded the knowledge of *MYC* driven aggressive lymphomas. *MYC* rearrangements can be found in virtually all BL, 10-15% DLBCL, NOS, 50% of plasmablastic lymphomas and in around 50% of HGBL.[7] The updated 2016-WHO classification has considered the provisional category of HGBL with *MYC* and *BCL2* and/or *BCL6* rearrangements that includes all large B-cell lymphomas with these alterations, independently of their morphology.[1] These cases have been also named "double hit" HGBL (HGBL-DH).[8] Cases with blastoid morphology or with features intermediate between DLBCL and BL without translocations are considered HGBL, NOS. DLBCL with high expression of MYC and BCL2 protein without the double genetic hit alterations are called "dual-expressors" (DLBCL-DE).[1,9] This DE is considered an adverse prognostic factor but these tumors are not included in the HGBL category since the outcome does not seem so adverse. The biology of HGBL-DH is complex and not yet well understood.[8,9] The presence of *MYC* rearrangements is the determinant factor but there are several additional elements that modulate their biological relevance.[8-10] The association with *BCL2* translocations usually confers an adverse prognosis but the role of *BCL6* is still controversial.[9,10] Other modulators of *MYC* rearrangements are the translocated partner. IG-*MYC* rearrangements induce higher levels of MYC expression than non-IG partners and this may explain the worse prognosis of the former. The MYC and BCL2 protein expression levels may be also different in cases with translocation probably be due to additional phenomena such as amplification of the translocated allele, mutations of *MYC* or others. The cell context may also be a modifier with DLBCL cases having a better outcome than tumors with blastoid or DLBCL/BL intermediate morphology. The clinical features of the patients seem also important since patients with high-risk clinical features have more adverse evolution than patients with low-risk factors.[9,10] Mutations in other genes may also contribute to the aggressiveness of these tumors (Table 1).[11,12]

In summary, the increasing knowledge of the molecular pathogenesis of aggressive B-cell lymphoma is providing a better understanding of the clinical heterogeneity of these tumors and provide solid basis for new therapeutic approaches. The significance of the new HGBL-DH category is clinically relevant because most of the tumors have a very aggressive behavior and standard DLBCL treatments are considered insufficient. However, the particular relevance of some of these alterations, the possible relationship between the different molecular alterations, and how they should guide clinical intervention are still open questions that require further studies.

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Aggressive lymphoma - Section 1

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Aggressive lymphoma - Section 2

Relapsed aggressive lymphoma: Can we optimize the therapy

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Take-home messages

- Prognostic factors in relapse/refractory patients with DLBCL have been identified and may help to optimize their management.
- The standard salvage approaches in transplant eligible and non-eligible patients have not markedly improved in recent years.
- New agents, from targeted therapies to immune-based approaches, are currently developed, and it will be important to define priorities in their evaluation

Introduction

The outcome of patients with DLBCL has been markedly improved with the introduction of rituximab but about 40 to 50% of them still experience either disease refractoriness or relapse after having achieved response to first line therapy. The prognosis of patients with refractory disease or early relapses is usually very dismal, while a sizable minority of patients with late disease recurrence may still have a chance to be cured, especially when eligible for autologous transplant. In this context, the development of new therapies is urgently needed, and some promising approaches have been recently explored. In this review, we will discuss new data regarding the characteristics of these relapsed/refractory (R/R) patients and their prognosis. The results and limitations of current treatment options in transplant eligible and non-eligible patients will be discussed, before addressing some of the new options under investigation.

Characteristics and prognosis of R/R DLBCL patients

Logically, the population of patients failing first line treatment is enriched in patients with adverse clinical and biological prognostic features at diagnosis, including older age, advanced Ann Arbor stage, elevated LDH, extranodal disease and poor performance status. Furthermore, the proportion of patients with biological characteristics such as a non-GCB subtype, the presence of double-hit translocations involving *MYC*, or the expression of Myc and Bcl2 proteins also appears to be increased. Previous administration of rituximab during first line therapy is also associated with a worse outcome, as shown in prospective studies evaluating different strategies in both transplant eligible and non-eligible patients,[1,2] although this finding was not reproduced in some retrospective studies.[3] Overall, with the improvement made in the optimal management of patients from diagnosis (rituximab being given to all patients and potential personalized treatment in the near future), R/R DLBCL patients will represent more and more a very difficult to treat population.

This group of R/R patients is still heterogeneous (Table 1). Among the different prognostic criteria identified, delay from initial therapy and International Prognostic Index (IPI) at time of failure remain the major prognostic parameters for those patients before they start any second line therapy.[1] Patients with early failure (occurring within 6 to 12 months after the end of first line therapy) appear to have a similar prognosis to those unable to achieve a response at the end of this treatment, or who experience disease progression during first line treatment, although some data suggest that this latter group has an even worse prognosis. Recently, a group of US investigators defined a "ultra-high risk" group of patients (overall survival at 2 years of 13%) as those having at least one of the 3 following criteria: primary progression, high NCCN-IPI index[4] at time of treatment failure or the presence of a translocation involving *C-MYC*.[5] The unfavorable outcome of patient carrying a *C-MYC* translocation was already reported in the CORAL study.[6] The role of other biological tumor characteristics remains unclear. Some studies did not find outcome differences according to the cell of origin classification of DLBCL,[5-7] but most series were relatively small. Interestingly, in the CORAL study,[8] the outcome of patients with a GCB phenotype (but no those with a non GCB or ABC phenotype) appeared influenced by the regimen used for salvage: GCB

patients having received a high-dose cytarabine and cisplatin combination (R-DHAP) had a better outcome than those having received an ifosfamide/etoposide/carboplatin regimen (R-ICE). One report also indicated that patients with R/R DLBCLs expressing both the Bcl-2 and the Myc protein also had an inferior survival.[9] Interesting findings regarding the clonal evolution of patients after treatment suggest that different clonal evolution patterns may be found uin relapsed DLBLC.[10]

Results and limitation of current salvage regimens

The possibility to deliver a curative treatment in R/R DLBCL patients is greatly influenced by the patient ability to tolerate an efficient salvage therapy followed by a consolidation usually consisting in an autologous stem cell transplant (ASCT). Age and comorbidities are hence major parameters taken into consideration when choosing the second line strategy. The use of various combinations including several components such as cytarabine or gemcitabine, platinum salts, other alkylating agents (ifosfamide) or eventually different anthracyclins (with potentially less cardiotoxicities, i.e. mitoxantrone, pixantrone) were assessed in many small series, but only few trials have evaluated their respective benefits.

For transplant eligible patients, R-DHAP, R-ICE or R-GDP regimens are all able to bring about half of the patients to an optimal response before ASCT.[1,11] It has been suggested that some more intense (and complex) salvage regimens before ASCT might eventually improve outcome.[12] It is important to try to develop newer regimens with an increased efficacy, and evaluation of new targeted molecules combined with standard salvage regimens is underway. There has been no substantial progress in the ASCT conditioning regimen in recent years, even with use of radio-immunotherapy,[13] and rituximab maintenance was not able to diminish the risk of relapse after ASCT in DLBCL.[14] Of note, some other lessons may have been retrieved from the CORAL study. While many physicians will advocate that failing a first salvage regimen identify truly refractory patients that are incurable, Van den Nest and colleagues reported that some patients might achieved a new CR with another alternate regimen, and if offered ASCT, may be cured, especially if they had a low IPI index at relapse.[15] A quite small group of a patients relapsing more than 12 months after ASCT might be eligible for a second attempt of effective treatment with allogeneic stem cell transplant (allo-SCT).[16] Finally, recent reports regarding the respective role of auto-SCT and allo-SCT in relapsing DLBCL did not indicate that an allogeneic approach would be better than ASCT.[17] Allo-SCT indications remain then limited, probably for patients who failed after ASCT or for highly selected patients.

For transplant ineligible patients, many regimens were used over the last 20 years, with a median progression free survival unfortunately not exceeding 6 months and a minority of patients (10-15%) still alive after 5 years.[5] Recently, bendamustine was reported to have some efficacy in small series,[18] but discordant data were reported and comparative studies are lacking. For transplant ineligible patients, although commonly used by many physicians, the role of rituximab in the second line remains undetermined, especially in those with refractory disease or early failure.

Future directions in DLBCL patients with R/R disease

Many new targeted agents are currently being developed in lymphoma.[19,20] Some of them, such as antibody drug conjuga-

Table 1. Prognostic factors in patients with R/R DLBCL and consequences.

In all series	
- Age	eligibility for ASCT (also depends on comorbidities)
- IPI or NCCN-IPI at relapse	poor outcome for higher scores, consider clinical trials +++
- Delay between first line and disease progression	although variably defined, early relapses have a very poor prognosis
- C-MYC translocation	poorer outcome; consider clinical trials +++
In some reports	
- PET before ASCT transplant	usually recommended to perform ASCT in PET negative patients[21]
- GCB/non-GCB tumor phenotype	might consider different regimen or targeted agents
- Bcl2 and Myc protein co-expression	to be confirmed in future studies
- Refractoriness during first line therapy	ultra-high risk patients; consider clinical trials +++

IPI, International Pronostic Index; NCCN-IP, National Comprehensive Cancer Network-IPI; PET-CT, Positron emission tomography–computed tomography; GCB, Germinal Center B-cell; ASCT, Autologous Stem Cell Transplant.

tes (against CD22, CD79) or bispecific antibodies, may be used in all DLBCL subtypes, while other, such as IMIDs, inhibitors of the BCR (BTKi and PI3Ki), inhibitors of EZH2, inhibitors of the bromodomain or inhibitors of Bcl2 may only be effective in certain patients with specific tumor characteristics. Likely, these drugs need to be combined with standard cytotoxic regimens or eventually with each other. Immune checkpoint blockers have a limited effect as single agents in DLBCL, but a large randomized European study will soon be launched evaluating nivolumab in combination with R-GemOx. But the preliminary results of engineered CAR-T cell appear to open new avenues in the management of R/R DLBCL.[20] Even if optimizing the efficacy and tolerability CAR-T remains necessary, it is likely that these new cellular therapy approaches will play a role in certain group of patients with R/R DLBCL, eventually as a new option at first salvage therapy.

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EUROPEAN HEMATOLOGY ASSOCIATION

Aggressive lymphoma - Section 3

Treatment of aggressive lymphomas focused on elderly patients

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Take-home messages

- Elderly patients present more often with known risk factors and DLBCL subtypes associated with poor prognosis
- Severity of frank pathologic dysfunction and comorbidity increase with age and many chemotherapies cannot be given at dose intensities possible in young patients, thus further worsening the outcome of elderly DLBCL patients.
- Curative treatment should be pursued for the majority of elderly patients with special emphasis on supportive measures.

Abstract

Elderly patients are often diagnosed with more aggressive diffuse large B-cell lymphoma (DLBCL) subtypes and present with more clinical risk factors than younger patients. Comorbidities and organ dysfunctions often prohibit the consequent adherence to standard immunochemotherapy regimens. Specific supportive measures, however, can improve the tolerability for intensive treatments and increase the cure rates of elderly patients with DLBCL.

Introduction

Age is the most prominent factor for survival after the diagnosis of lymphoma and is recognized in the International Prognostic Index for diffuse large B-cell lymphoma (DLBCL). Though 60 years is the cut-off point in IPI, the cut-off point between young and elderly patients in prospective trials is usually set between 60 and 65 years, even though the more clinically relevant breakpoint is closer to 75 years, where comorbidity, dependency and geriatric symptoms become more prevalent. Elderly patients present more often with other commonly accepted risk factors like advanced stage, multiple extralymphatic sites of involvement, elevated LDH and poor performance status. Moreover, the morphologically defined immunoblastic variant which is associated with a poor prognosis is also more frequent in elderly patients as are the prognostically inferior DLBCL cases derived from activated B-cells (ABC subtype) in contrast to the GC (germinal center) cell-derived DLBCL. Moreover, BCL-2 expression, and cytogenetic complexity increase with age at diagnosis, and EBV+ DLBCL, an EBV+ clonal B-cell lymphoid proliferation with pour prognosis rarely occurs in patients <50 years.[1]

Diagnosis and staging: Because aggressive lymphoma is life-limiting also for elderly patients, but at the same time is curable in a significant proportion of elderly and very old patients, the guidelines developed for younger patients regarding diagnosis and staging of DLBCL must be consequently adhered to,[2] except in patients with a clearly palliative situation.

General treatment approach

Severity of frank pathologic dysfunction or comorbidity increases with age. The association between comorbidity and survival was demonstrated by Charlson[3] who showed that comorbidities are independent predictors of survival. Therefore, a basic geriatric screening is indicated in all patients >70 year old. Comorbidities and polymedications for the treatment thereof can further compromise the tolerability of therapy. The hematopoietic reserve is often reduced and a decrease in liver function can alter the metabolism of many drugs in elderly patients. Many older patients have a decreased glomerular filtration rate and a delay in drug excretion, necessitating adaption of cytotoxic drugs to creatinine clearance. The physiological increase of body fat and reduction in lean body mass also contribute to an increased toxicity. Many elderly patients have a reduced emotional tolerance to stress and need closer guidance in order to maintain treatment compliance, in particular with oral anti-cancer drugs. All these facts explain why many chemotherapies cannot be given to elderly patients at doses and treatment intervals for young patients thus compromising the responses of and worsening the outcome of elderly DLBCL patients.

Aggressive lymphoma - Section 3

Specific measures

Of particular importance are specific supportive measures for elderly patients. All elderly patients should receive prednisone 100 mg p.o. for several days (depending on the tumor load) as a "prephase" treatment to avoid tumor lysis syndrome and ameliorate the side effects of the first chemotherapy cycle which has the most pronounced myelotoxicity and is associated with the highest treatment-related death rate. Dose reductions due to hematological toxicity should be avoided, and hematopoietic growth factors should be given to all elderly patients starting with the first CHOP. In addition to floxazines during days of severe neutropenia, anti-infective prophylaxis with acyclovir is recommended which significantly reduced the rate of severe infections and treatment-related deaths of elderly DLBCL in an ongoing trial of the DSHNHL (Figure 1).[4] Finally, patients with severe fatigue between treatment cycles after tapering of prednisone should receive hydrocortisone substitution.

Treatment regimens

All principles of curative DLBL treatment must be applied to elderly patients and treatment strategies should be stratified according IPI. Whether less intensive / shorter chemotherapy can be given to the subgroup of elderly patients without bulky disease and with no IPI risk factor other than age is addressed in ongoing prospective trials. Best results have been obtained with 8 cycles of R-CHOP-21 and six cycles of R-CHOP-14 +2R. Both regimens have equal efficacy and acute toxicity.[5] 6xR-CHOP-14+2R[6] has the advantage over 8xR-CHOP-21 of a much shorter duration under chemotherapy (71 vs. 149 days), which has particular psychological importance for elderly patients, and of less (2/3) cumulative drug exposure compared to 8xR-CHOP-21, which is relevant with respect to the rate of cardiomyopathies and second neoplasms. Whether extended rituximab exposure by increasing intervals between rituximab applications later in the treatment course[7] can indeed improve the outcome of elderly DLBCL patients, is the objective of an ongoing trial.

Radiotherapy

Radiotherapy to bulky disease is recommended, because it eliminates bulky disease as a risk factor. Abandonment of radiotherapy to bulky disease led to an inferior outcome, including a significant reduction of overall survival in elderly patients with bulky disease.[8] Early results from a prospective

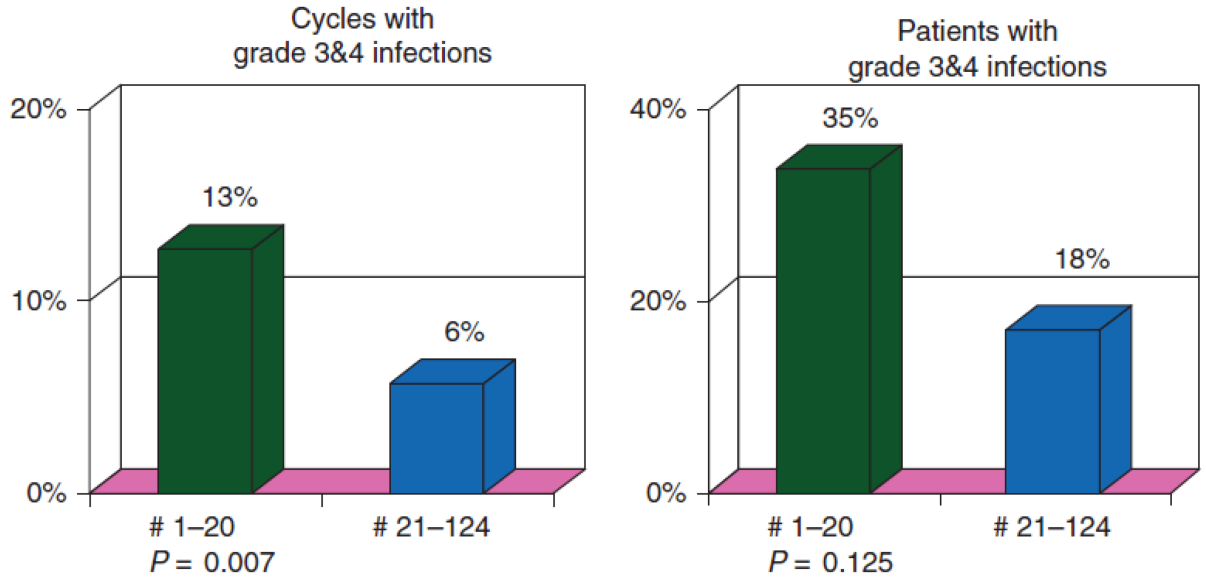

Figure 1. Grade 3 and 4 infections without and with anti-infective prophylaxis in the DENSE-R-CHOP-14 trial.[15] Starting with patient #21 (light grey (blue online) columns), patients received anti-infective prophylaxis with aciclovir and cotrimoxazole. This resulted in a significant reduction of chemotherapy cycles with grade 3 and 4 infections (left graph), and a considerable decrease of patients with grade 3 and 4 infections (left graph). Adapted from Murawski *et al*. Ann Oncol 2014;25:1800–6; with permission.

trial suggest that radiotherapy can be spared in patients with a negative PET after immunochemotherapy.[9]

Patients aged more than 80 years

A geriatric assessment in order to ascertain comorbidities and functional decline is recommended to help choice of treatment in these patients.[2] R-CHOP treatment can usually be given to fit patients up to 80 years of age. The combination of rituximab with attenuated chemotherapy, such as R-mini-CHOP, can induce complete remissions and long-term survival in fit patients older than 80 years.[10] Whether substitution of doxorubicin by gemcitabine, etoposide or liposomal doxorubicin, or even its omission, can be considered in patients with cardiac dysfunction or those who are otherwise unfit, must be shown in larger prospective studies. Alternative chemotherapy regimens such as GemOx[11] or CEMP,[12] which have proven efficacy in the treatment of elderly patients with relapsed and refractory DBCL, can also be considered in such situations.

Elderly patients with refractory / relapsed DLBCL

Elderly patients or those with comorbidities who are not candidates for high-dose chemotherapy face a dismal prognosis. ESMO guidelines recommend treatment of such patients in prospective trials addressing new drugs and treatment strategies. Outside such trials, immunochemotherapy with low-toxicity regimens such as GemOx (gemcitabine, oxaliplatin)[11] is an option, with pixantrone achieving some responses in heavily pretreated patients[13] Numerous conventionally dosed chemotherapies for relapsed or refractory aggressive lymphoma have been studied, most of them in a limited number of patients, but no standard has been established for elderly DLBCL patients in a randomized trial. R-ICE and R-DHAP might be too toxic for many of the elderly patients, but gemcitabine, dexamethasone and cisplatin (GDP), which was compared with R-DHAP in a large randomized NCIC-CTG LY.[12] trial and proved to be as efficacious, but considerably less toxic than R-DHAP, might be an option.[14]

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Bleeding disorders

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Introduction

The structure and function of the blood clot has been associated with altered risk of thrombosis. Dense fibrin structures with small pores increase the risk of thrombosis, and have major functional consequences by increasing the resistance to fibrinolysis and altering the visco-elastic properties of the clot. Dr. Whyte and Dr. Mutch will discuss biological and clinical relevance of fibrin clot structure. In the second chapter, Dr. Squizzato will describe the diagnosis and management of disseminated intravascular coagulation (DIC). This hyperfibrinolytic disorder is an acquired syndrome caused by infectious and non-infectious insults. The main pathophysiological mechanisms of DIC are inflammatory cytokine-initiated activation of tissue factor-dependent coagulation, insufficient control of anticoagulant pathways and plasminogen activator inhibitor 1-mediated suppression of fibrinolysis. Together, these alterations lead to endothelial dysfunction and microvascular thrombosis, causing organ dysfunction. In the last chapter, Dr. Kenet will describe how to diagnose and manage patients with Rare Bleeding Disorders (RBDs), a heterogeneous group of coagulation disorders characterized by fibrinogen, prothrombin, factors V, VII, X, XI, or XIII, and the combined factor V + VIII and vitamin K-dependent proteins deficiencies, representing roughly 5% of all bleeding disorders. Patients affected with RBDs may present a wide range of clinical symptoms, varying from mucocutaneous bleeding, to the most life-threatening symptoms such as central nervous system and gastrointestinal bleeding. Treatment of these disorders is mainly based on the replacement of the deficient factor, using specific plasma-derived or recombinant products when is available.

Learning goals

1. ***The composition of a thrombus is fundamental to its stability against mechanical and fibrinolytic degradation. The nature of the fibrin network is altered by numerous factors and clots generated in distinct parts of the vasculature have different cellular compositions and fibrin mesh.***
2. ***Patients with disseminated intravascular coagulation may present with bleeding, organ dysfunction, thrombosis of large vessels, or can be totally asymptomatic despite laboratory test abnormalities.***
3. ***Recommendations for hemostasis control include mainly replacement of the missing coagulation factors (unless presence of inhibitors renders it impossible).***

EUROPEAN HEMATOLOGY ASSOCIATION

Bleeding disorders - Section 1

Biological and clinical relevance of fibrin clot structure

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Take-home messages

- Fibrin clot stability is directly influenced by the overall network structure and cellular composition.
- Patients with thromboembolic diseases exhibit abnormal clot structure.
- Shear rate alters clot composition and stability.

Introduction

Activation of the coagulation cascade ultimately results in formation of a three-dimensional cross-linked fibrin network which directly adheres to the platelet 'plug'. Thrombin cleaves fibrinopeptides from circulating fibrinogen, altering the conformational and electrostatic nature of the molecule and allowing generation of half-staggered double-stranded protofibrils. Lateral aggregation of protofibrils forms fibers (reviewed in Weisel *et al.*[1]) which are cross-linked via the action of the transglutaminase enzyme factor XIIIa. Dissolution of a clot is necessary to maintain vessel patency, failure to do so leads to occlusion (thrombosis). Fibrin degradation is attained through the action of the fibrinolytic system. The central enzyme, plasmin, is formed by activation of the circulating zymogen plasminogen by tissue plasminogen activator (tPA) or urokinase (uPA). Cleavage by plasmin releases fibrin degradation products that are readily cleared from the circulation. The visco-elastic properties of the fibrin network and its resolution are governed by numerous factors, several of which will be highlighted in this review.

Current state of the art

The structural properties of a fibrin clot, such as fiber thickness, and cellular composition, influence its stability and susceptibility to breakdown, with abnormal fibrin structure directly linked to a number of thromboembolic diseases. Clot fractal dimension (*df*), has been proposed as a potential marker for abnormal clot microstructure and predictor of recurrent venous thromboembolism.[2] The location of clot formation within the vasculature influences clot composition, with thrombi from coronary arteries being composed of thinner fibers and a higher platelet count than those from peripheral arteries.[3] Individually, thin fibrin fibers are lysed more rapidly than thick fibers, however, network composition alters this arrangement with clots composed of thick fibers being degraded faster than those of thin fibers.[4] These differences arise due to packing of these fibers, with thin fibers being more tightly packed with smaller pores, while networks of thick fibers have a looser conformation.[4] Therefore, one must consider the fibrin network as a whole when considering susceptibility to fibrinolysis. A study of lysis of single fibers indicates that susceptibility to lysis is dependent on the strain in the fiber. In response to plasmin, individual fibers either elongate or lyse transversely. Thicker fibers formed at low thrombin concentrations tend to elongate and lose the intrinsic strain assimilated during polymerization.[5]

Numerous factors are known to influence the structure of a forming fibrin network, particularly thrombin and fibrinogen. The relative levels of these proteins dictate polymerization rate, fiber thickness and porosity thereby influencing clot stability. Our work demonstrates that platelet-derived polyphosphate (polyP), a highly charged biomolecule, delays fibrin polymerization and alters the rheological and structural properties of the fibrin.[6,7] PolyP containing clots are composed of dense fibrin aggregates interspersed by pores which are resistant to tPA-mediated fibrinolysis.[7] Inhibition of platelet-derived polyP in a flow model altered fibrin structure, impeded clot retraction and conferred resistance to lysis.[8]

A number of congenital fibrinogen abnormalities exist altering both the quantity (afibrinogenemia and hypofibrinogenemia) and quality (dysfibrinogenemia and hypofibrinogenemia) of circulating fibrinogen which impacts directly on clot structure. Interestingly, patients with congenital afibrinogenemia are at high risk of arterial and venous thromboembolic events.[9] Consistently, mice lacking fibrinogen and von Willebrand fac-

tor form abundant unstable platelet-rich thrombi in arterioles that embolize readily provoking vessel occlusion.[10] These data demonstrate the need for an optimal fibrinogen concentration *in vivo* to stabilize thrombi and to scavenge free thrombin but not tip the balance towards thrombus persistence. The lack of fibrinogen may contribute to undesirable thrombotic events in these patients. The rate of fibrin polymerization and the structural features of the fibrin network help predict the clinical phenotype of patients with congenital dysfibrinogenemia. A recent study provided evidence that bleeding correlates with delayed fibrin polymerization, with clots exhibiting thinner fibers and increased permeability.[11] Fibrinogen γ', a splice variant of the fibrinogen γ-chain, interferes with fibrin polymerization leading to tightly knitted fibrin structures interspersed by large pores.[12] These effects were independent of thrombin despite the increased binding of thrombin to this variant. Recent experiments, performed under hydrated conditions, suggest that γ'-fibrinogen alters clot stiffness by changing protofibril packing.[13]

Platelets and red blood cells (RBC) influence clot structure and regulate contraction and fibrinolysis. Contracted clots are more resistance to lysis, due to extrusion of proteins and tightening of the fibrin network. New evidence demonstrates that platelets and fibrin(ogen) exert contractile forces, segregating and compressing RBCs into tightly packed polyhedrocytes within clots.[14] Interestingly, the ratio of platelets to fibrinogen influences the degree of clot contraction, with reduced contraction evident at high fibrinogen concentrations.[15] In whole blood flow models fibrin(ogen) is visualized to emanate from platelet aggregates (Figure 1). Elegant *in vivo* studies demonstrate that a dense core of highly activated platelets forms at the site of vascular injury. Fibrin and thrombin accumulate in the core of the thrombus where the rate of solute transport is low.[16,17] The core region is shrouded by a shell of loosely packed platelets displaying only transient increases in intracellular calcium with negligible degranulation.[17] This suggest

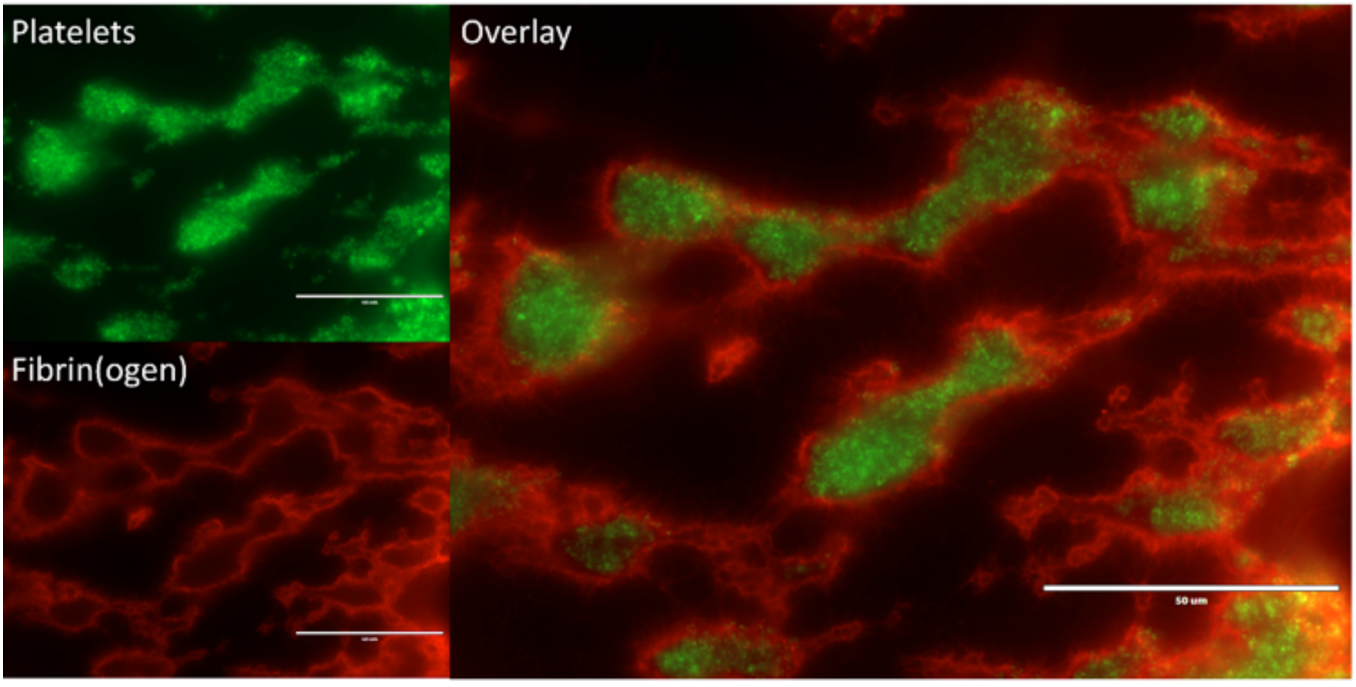

Figure 1. Thrombus formation under physiological shear rates. Platelet-fibrin thrombi were formed from whole blood. Blood containing DiOC6 (0.5 mg/mL) to label platelets (green) was re-calcified (7.5 mM CaCl2 and 3.75 mM Mg Cl_2) and perfused at 1000 s^{-1} over a collagen (100 ng)/thrombin (2 fmol)-coated surface for 6 min. HEPES buffer (10 mM Hepes (pH 7.45) 136 mM NaCl, 2.7 mM KCl, and 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA) containing fibrinogen-AF546 (75 µg/ml; red) was then perfused for 7 minutes. Images were taken on an EVOS FL imaging system with a x 60 1.42 oil immersion objective. Scale bars: 50 µm. C.S. *Whyte, G.B. Morrow and N.J. Mutch, unpublished data.*

that the granular content of platelets is largely released into the body of the thrombus, where local concentrations of platelet-derived proteins likely define the hemostatic balance. Circulating plasminogen accrues on platelet-associated fibrin, with a smaller pool directly associated with the activated platelet membrane where its local concentration will govern the rate of fibrin degradation.[18]

A large body of evidence indicates that the composition and structure of the fibrin network is modulated by shear rate, which in turn dictates susceptibility to lysis. Application of strain to fibers exposes hydrophobic residues resulting in expulsion of water and an increase in order and alignment.[19] The fibrin network primarily determines the response of forming clots to physiological stress.[20] Augmented thrombin generation under flow conditions may account for the structural changes observed in fibrin.[20] In human thrombi, internal fibrin fibers exhibit a random orientation, whereas, exterior fibers align in the direction of flow, are thinner and less porous.[21] Mechanical stretching replicates the appearance of fibers on the exterior surface of thrombi resulting in impaired fibrinolysis as a result of attenuated plasminogen activation and reduced plasmin sensitivity.[21]

Future perspectives

The composition of a thrombus is fundamental to its stability against mechanical and fibrinolytic degradation. It is now well established that the nature of the fibrin network is altered by numerous factors and that clots generated in distinct parts of the vasculature have different cellular compositions and fibrin mesh. Indeed, even within a thrombus exterior fibers exposed to shear stress may exhibit entirely different structure to those internal to the thrombus. It is clear from the literature that fibrin structure is an important determinant of normal hemostasis and inexplicitly linked to disease state, perhaps indicating that mechanisms to prevent deleterious thrombus formation should be tailored to the specific thrombus environment.

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Bleeding disorders - Section 1

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Diagnosis and management of disseminated intravascular coagulation and primary hyperfibrinolysis

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Introduction

Disseminated intravascular coagulation (DIC) is the systemic activation of coagulation that leads to a widespread intravascular fibrin formation in small- to medium-sized vessels with concomitant hyperactivation of the fibrinolytic system.[1,2] This severe imbalance of the hemostatic system can cause bleeding and organ dysfunction.[1,2] Poor prognosis of DIC has prompted some authors to read the acronym DIC as 'death is coming'.

DIC is always the final stage of a coagulopathy caused by an underlying condition: unprovoked DIC has never been described till now.[1-4] Severe infections, neoplasia, and obstetric complications are the major underlying cause of DIC. DIC affects almost 30% of patients with severe sepsis and 50% of patients with obstetric complications, such as abruptio placentae and amniotic embolism. Moreover, nearly 90% of patients with acute promyelocytic leukemia have DIC.

In patients with sepsis, systemic inflammatory response liberates intracellular proteases and produces cytokines that activate the coagulation system by increasing generation of tissue factor (TF), inhibiting natural mechanisms of anticoagulation and reducing the expression of thrombomodulin on the endothelium.[5] Increased levels of NETs induce apoptosis of vascular endothelial cells and degrade tissue factor pathway inhibitor, which promote platelet aggregation and increase hypercoagulability, respectively. Finally, increased plasminogen activator inhibitor 1 (PAI-1) levels inhibit fibrinolytic response.

Solid tumors may both activate the coagulation system by procoagulant molecules, such as TF, and inhibit the fibrinolytic system[6]. Some cancer cells, such as in prostatic adenocarcinoma, may express plasminogen activators molecules that can directly activate fibrinolysis and cause a primary hyperfibrinolysis syndrome. Extensive tissue damage, such as in burns, increase TF levels that lead to an uncontrolled generation of thrombin. A localized DIC is typical of some vascular abnormalities, such as giant hemangioma (the so called 'Kasabach-Merritt syndrome') and large aortic aneurysms, in which deposition of fibrin is limited to abnormal vessels and not disseminated.[4]

Current state of the art

Patients with DIC may present with bleeding, organ dysfunction, thrombosis of large vessels, or can be totally asymptomatic despite laboratory test abnormalities.[4,7] There are four main clinical phenotypes of patients with DIC: 'bleeding patient', 'patient with severe bleeding', 'patient with organ dysfunction' and 'asymptomatic patient'.[4] Clinical phenotypes are the results of the relative imbalance of the coagulation and fibrinolytic system.[4] When secondary hyperfibrinolysis is prevalent, such as in some patients with acute promyelocytic leukemia and obstetric complications, the first clinical manifestation is bleeding. When hypercoagulability overcomes hyperfibrinolysis, such as in patients with sepsis, organ dysfunction is the main clinical manifestation. When both coagulation and fibrinolytic system are only minimally altered, such as in some cancer patients, mild bleeding or asymptomatic coagulation test abnormalities are the typical clinical presentation. DIC may be also defined as acute or chronic: patients with acute DIC have major bleeding and/or organ dysfunction as main clinical presentation; patients with chronic DIC have usually mild bleeding or are completely asymptomatic. Different underlying pathophysiological processes and clinical phenotype suggest that the acronym DIC may be also read as 'disease-induced coagulopathy' and, therefore, new acronyms can be proposed for each underlying DIC causes (Table 1).

Diagnosis of DIC and identification of the underlying cause should be the most rapid as possible: if left untreated, patients have an unavoidable poor prognosis.[8-11] Unfortunately, no gold standard for the diagnosis of DIC exists, not a single laboratory test has shown sufficiently high diagnostic accuracy to establish or rule out the diagnosis of DIC, and it is not possible to diagnose DIC in the early phases.[7,12,13] A combination of the following altered laboratory tests suggests DIC: reduced

platelet count, prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT), reduced fibrinogen and increased D-dimer.[2] None of these tests is specific, as each abnormal value may be explained by other conditions and by the underlying disease itself. A reduction of natural anticoagulants such as antithrombin and protein C is common, and may be useful to support DIC diagnosis when rapidly available.

Several diagnostic score have been proposed to increase diagnostic accuracy.[7,12,14] The International Society of Thrombosis and Hemostasis (ISTH) score was extensively investigated in cohort studies and was recommended by national and international guidelines. Recently, DIC subcommittee of the Japanese Society on Thrombosis and Hemostasis proposed new diagnostic criteria for DIC and an extensive list of alternative diagnoses.[14] Indeed, differential diagnosis includes other conditions that can cause blood test abnormalities such as liver disease, thrombotic microangiopathy and primary hyperfibrinolysis.[2,1]. Clinical suspicion of primary hyperfibrinolysis should be high in cases in which bleeding continues despite hemostatic replacement therapy, platelet levels are relatively conserved but fibrinogen levels are disproportionately low, and D-dimer levels are disproportionately high for DIC. [2] Inherited primary hyperfibrinolysis is a rare condition associated with congenital deficiency of α_2 plasmin inhibitor (α_2-PI) and PAI-1.[15] In cancer-associated primary hyperfibrinolysis, there are often reduced levels of α_2-PI and thrombin activatable fibrinolysis inhibitor, and an impaired hepatic clearance of tissue plasminogen activator. In acute promyelocytic leukemia-associated primary hyperfibrinolysis, PML-RAR-α fusion protein enhances the expression of a S100 protein, which forms a heterotetrameric complex with annexin-A2 that promotes plasminogen activation and protect plasmin against inhibitors.[16]

DIC invariably worsens prognosis of the underlying disorder and can anticipate the fatal outcome if patients are left untreated.[17] In patients with severe sepsis and DIC, mortality is about twice that in patients with sepsis without DIC.

Future perspectives

There are three possible theoretical therapeutic strategies: treatment of the underlying disorder, interruption of fibrin deposition with anticoagulant drugs and prevention/treatment of clinical manifestations (the so called 'supportive therapy').[8-11,18-19] Only treatment of the underlying disorders has been proved to improve survival. Available evidence does not support a routine use of anticoagulant agents (such as antithrombin, protein C and therapeutic dose of unfractionated heparin [UFH] or low-molecular-weight heparin [LMWH]). Recombinant thrombomodulin is under investigation with promising results.

Platelet transfusion should be given to maintain platelet count

Table 1. Underlying causes of disseminated intravascular coagulation (DIC).

Main subgroups and example of specific causes	Proposed new acronyms
Severe infection	
- Severe sepsis	
- Malaria	SIC: Sepsis-induced coagulopathy
Obstetrical complication	
- Abruptio placentae	
- Amniotic fluid embolism	PIC: Pregnancy-induced coagulopathy
Hematological neoplasia	
- Acute promyelocitic leukemia	LIC: Leukemia-induced coagulopathy
Solid tumor	
- Prostate adenocarcinoma	
- Gastric adenocarcinoma	CIC: Cancer-induced coagulopathy
Toxic or immunological reactions	
- Snake bites	
- Transfusion reactions	TIC: Toxic-induced coagulopathy
Other causes	
- Acute pancreatitis	
- Burns	
- Hypoxia	OIC: Other-induced coagulopathy
Vascular abnormalities (localized DIC)	
- Large aortic aneurysm	
- Hemangioendothelioma (Kasabach-Merrit syndrome)	VIC: Vascular-induced coagulopathy

>50×10^9/l in case of bleeding while a lower threshold of 20 to 30×10^9/l may be used in DIC without bleeding.[19] Fresh frozen plasma should be considered when PT ratio is more than 1.5 and/or aPTT ratio is more than 1.5 and/or fibrinogen level is less than 1 g/l. Thromboprophylaxis with UFH or LMWH is advised until bleeding ensues or platelet count drops below 30×10^9/l. When a venous thromboembolic event occurs is indicated to place a vena cava filter in bleeding patient just waiting to introduce unfractionated heparin or low molecular weight heparin as soon as the bleeding stops.[19]

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Recent international consensus on supportive management of DIC patients.

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Bleeding disorders - Section 3

Rare bleeding disorders - Diagnosis and treatment

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Take-home messages

- Among rare bleeding disorders apart from congenital hemophilia, observational findings from international registries provide the only feasible large-scale data source.
- Laboratory assessment and especially molecular techniques enable accurate diagnosis.
- Recommendations for hemostasis control include mainly replacement of the missing coagulation factors (unless presence of inhibitors renders it impossible).
- Future gene therapy is promising and novel disruptive alternatives may be interesting for treatment and prophylaxis of patients with RBD as well, following clinical trials.

Introduction

Rare diseases are defined as life-threatening or chronically debilitating conditions that have a prevalence of fewer than 1:2000 according to the European Union or 1:1250 according to the USA federal Hemophilia Treatment Center Network.[1] Rare bleeding disorders (RBDs) are reported in most populations, with incidences varying from 1:5000 for hemophilia A (HA), to 1:30,000 for hemophilia B (HB), to the much rarer 1:500,000 for FVII deficiency, and 1-3:1,000,000 for prothrombin or FXIII deficiency).[1,2]

The European network RBD project confirmed that FVII and FXI deficiencies comprise most (39%-29%) of the RBDs, whereas fibrinogen (8-9%), FXIII (6%), FV+FVIII (3%) and prothrombin (1%) disorders are rarer. Most RBDs are inherited as autosomal recessive, however, heterozygous carriers may confer an unpredictable propensity for bleeding.[3]

Current state of the art

Severe RBDs often present with acute bleeding symptoms that may occur either spontaneously or following a minimal trigger. In neonates, these include cephalhematomas, skin bleedings and injury-related bleeding following invasive procedures (e.g., circumcision) or sites of peripheral venipunctures. Persistent oozing from the umbilical stump is typical of defective fibrinogen or FXIII deficiency. Hemarthroses, which are typical for severe hemophilias, rarely occur before ambulation. A small proportion of infants with RBDs present with intracranial hemorrhage (ICH), as frequently reported in FVII, FX and FXIII deficiencies (prevalence up to 25%) and rarely in afibrinogenemia, FII, FV and vitamin K-dependent coagulation factor deficiencies.[2,4]

In contrast, some patients with contact pathway deficiencies, e.g., FXI deficiency, may present only after adulthood with post-trauma or surgical bleeding typical for tissues in which there is high fibrinolytic activity (e.g., teeth extractions, urinary and prostate surgeries).[1,3]

Combined FV+FVIII deficiency is associated with a mild bleeding phenotype, whereas combined vitamin K deficiency resulting from inherited defects in activation (γ-carboxylation) of the vitamin K-dependent factors (FII, FVII, FIX, and FX) presents early with serious bleeding events, including ICH.[2,5]

Women with RBDs may present with menorrhagia, bleeding ovarian cysts or corpus luteum, post-partum hemorrhage or other obstetric complications, including recurrent miscarriages due to the roles that deficient factors (e.g., FXIII) play in placental implantation and pregnancy maintenance.[1,3,5]

Critical and unique bleeding manifestations of various RBD are summarized in Table 1.

When acute severe new bleeding symptoms appear unexpectedly at older ages, acquired coagulation inhibitors should be ruled out.

Laboratory diagnosis

The diagnosis of RBD is usually straightforward, requiring standard coagulation tests preceded by special assays for relevant factors activity. Antigenic assays are essential for diagnosis, classification and treatment, especially for patients with

dysfibrinogenemia and dysprothrombinemia, both associated with an increased thrombotic risk.[1] Normal prothrombin time (PT) and partial thromboplastin time (aPTT) screening test results do not exclude defective FXIII or thrombocytopathy. Notably, patients may also present with bleeding symptoms and a prolonged PTT due to the presence of autoantibodies against FVIII. In these rare cases, aPTT does not correct after mixing with normal plasma. When a patient's plasma is incubated with FVIII, its residual activity defines the FVIII inhibitor level, as measured by Bethesda units.

Thrombin generation (TG) and rotating thromboelastogram (ROTEM) are complementary global clotting tests, which may be used as indicators of overall hemostasis. TG was studied in RBDs as a predictor for bleeding risk and as a tool for monitoring patients. Notably, test standardization is still required before it will be applicable for widespread clinical use. ROTEM, which evaluates clot kinetics and fibrinolysis in whole blood, enables the assessment of fibrinogen, FXIII deficiency and platelet disorders that are sometimes misdiagnosed by other assays.[6-8] In an era in which genetic knowledge is growing, identification of the causative mutation of RBDs is becoming the definitive diagnostic method.[9] In an attempt to prevent future morbidities, proper molecular diagnosis enables prenatal familial counseling by the use of pre-genetic determination together with in vitro fertilization to select healthy embryos prior to implantation.[10]

Table 1. Special characteristics of rare bleeding disorders.

	Known gene defect	Potential gene therapy	Unique serious manifestation	Replacement therapy	Non-replacement therapy and future options
Fibrinogen deficiency	FGA, FGB, FGG(4q28)	N/A	Thrombosis Bleeding from umbilical stump	Pd Fibrinogen concentrate/FFP/Cryo	
Prothrombin deficiency	F2(11p11-q12)	N/A	Mucosal bleeding Hemarthrosis ICH	PCC/ FFP	
FV deficiency	FV (1q24.2)	N/A	Epistaxis umbilical stump bleeding Muscle hematoma Hemarthrosis	FFP/FV (clinical study)	Platelet transfusion
FVII deficiency	FVI(I 13q34)	Yes* *No human trials	ICH hemarthrosis	rFVIIa /PCC/FFP/ PdFVII	
FVIII deficiency (hemophilia A)	FVIII(Xq28)	Yes	ICH Bleeding at circumcision Hemarthrosis	PdFVIII/rFVIII/ EHL FVIII	Concizumab (anti-TFPI)/Fitusiran(siRNA) /emicizumab
FIX deficiency (hemophilia B)	FIX(X- long arm)	Yes	ICH Bleeding at circumcision	PdFIX/rFIX/EHL FIX	Concizumab (anti-TFPI)/Fitusiran(siRNA) Hemarthrosis
Hemophilic patients with inhibitors		No	ICH Bleeding at circumcision Hemarthrosis	FAIBA/rFVIIa	Concizumab (anti-TFPI) /Fitusiran(siRNA) /emicizumab HA.
Combined FV and FVIII deficiency	LMAN1(18q21.3-q22) MCFD2 (2p21-p16.3)	N/A	Bleeding at circumcision Post-surgery/ trauma bleeding	FFP- rFVIII	DDAVP
FX deficiency	FX(13q34)	N/A	GI bleed ICH Umbilical stump bleeding	PCC/PdFX	
FXI deficiency	FXI(4q35.2)	N/A	Cases of MI and DVT have been reported	PdFXI	
FXIII deficiency	FXIII(6p24-p25) FXIIIB(1q31-q32.1)	N/A	Delayed wound healing Miscarriages ICH	rFXIII A subunit/ PdFXIII /FFP/cryo	
Vitamin K-dependent coagulation factors deficiency	GGCX(2p12) VKORC1(16p11.2)	N/A	Skeletal abnormalities ICH Umbilical stump bleeding	Vitamin K/PCC/FFP	

R- recombinant; PCC- prothrombin complex concentrate; FFP- Fresh-frozen plasma; Pd- plasma-derived; cryo- cryoprecipitate; ICH- intracranial hemorrhage; HA- hemophilia A; HB- hemophilia B; GI- gastrointestinal; DVT- deep vein thrombosis; MI- myocardial infarct.

Treatment

Current management of RBDs is mainly based on replacement therapy. In countries with abundant resources, the majority of RBDs are treated by specific factor concentrates, mostly plasma-derived, although recombinant products are also available[11] (Table 1). Adjuvant therapies, such as antifibrinolytics, used alone or in combination with replacement, as well as estrogen/progestin preparations can be considered for milder mucosal hemorrhages or heavy menstrual bleeding.

In some RBDs, such as FXI deficiency, treatment may be required only directly following trauma or during surgical procedures. On the other hand, standard of care of other RBDs, such as HA, HB, FXIII and severe FVII deficiency, is regular prophylactic therapy. Extended half-life coagulation products that were recently developed for hemophilia may improve hemostatic efficacy and patients' adherence to therapy.[12] Formation of antibodies directed against the missing factor concentrate is a complication that may render factor replacement therapy ineffective.[13] Acquired hemophilia A (AHA) is a severe bleeding disorder caused by autoantibodies against clotting FVIII. With an estimated annual incidence of 1.3 to 1.5 per million, AHA is also a rare (although not a congenital) bleeding disease.[14] Treatment may involve the use of bypass agents to control hemostasis, while immunomodulation (with an increasing role for rituximab) may be required to eradicate the inhibiting antibodies.

Most congenital RBDs are monogenic diseases, and even a small increase in factor activity levels can profoundly improve the disease phenotype. This makes RBDs ideal candidates for gene therapy. The greatest progress has been achieved in hemophilia B,[15] whereas the more challenging therapy for HA is currently being tested in clinical trials.[16,17]

Other new treatments are represented by non-replacement therapies. Emicizumab, a bispecific antibody binding FIXa and FX, was effective in HA patients with or without inhibitors,[18] although its combined use with bypass agents should be handled with caution.

Another approach is the inhibition of inhibitors of coagulation cascades, such as Concizumab (anti-TFPI by Novo Nordisk), that showed promising phase 1 results.[19] Fitusiran is a synthetic small interference RNA therapeutic molecule designed to suppress liver production of antithrombin. Its monthly subcutaneous administration improved TG and hemostasis.[20]

Future perspectives

The development of novel alternative therapeutics has not been studied in patients with other RBDs. Novel therapies are not expected to change the standard of care in RBDs that involve factors "downstream" from the coagulation cascade (e.g., dysfibrogenemia/FXIII deficiency) although, theoretically, there may be a potential role for them in the treatment of FVII/FXI/FX deficiency.

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Bleeding disorders - Section 3

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Challenges in blood transfusion

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Introduction

This year selected topics consist of novel and/or urgent transfusion questions: the significance of extracellular vesicles (EV) in blood products, red blood cell (RBC) cross-match problems and possible solutions in myeloma patients after anti-CD38 treatment and the lack of data to recommend RBC transfusion triggers in hematology patients. The session starts with an overview of EV in blood products. Besides the physiological generation of EV *in vivo* resulting from shear stress in the circulation, blood withdrawal, preparation and storage of donor blood, affects the amount and size of EV in blood products. The measurement of quantity and nature of EV in blood products and unravelling their possible role in unwanted transfusion complications such as TRALI and TRIM (transfusion related immunomodulation) are suspected but not yet studied. The session continues with cross-match problems caused by therapeutic (monoclonal) antibodies intended to target antigens expressed by malignant cells such as CD38 expressed, but not exclusive, on malignant plasma cells. The company producing anti-CD38 provides warnings and solutions for hematologist which cannot always be performed by the transfusion laboratory. The last lecture reveals the shocking situation, that hematologist who use circa one-third of available blood products, in contrast to most other medical disciplines use no evidence–based indications. Attempts to investigate evidence–based RBC transfusion triggers included 255 hematological patients in contrast to over 20.000 patients involved in transfusion trigger studies in other patient categories such as major surgery and patients with critical illness.

Learning goals

1. ***Surveillance of possible pathological implications of extracellular vesicles in transfusion products needs adjustment of transfusion quality control parameters and hemovigilance reporting.***
2. ***The development of targeted immune therapy of malignancy can be associated with cross reactivity against non-malignant cells and tissues***
3. ***Hematology patients use approximately one-third of all available blood products, but in contrast to other medical disciplines do not use evidence based transfusion triggers.***

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Challenges in blood transfusion - Section 1

Cell-derived microvesicles/microparticles in blood components: Consequences for transfusion recipients

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Take-home messages

- Extracellular vesicles (EVs) released by platelets, red blood cells, lymphocytes, and endothelium cells in response to activation or apoptosis are abundant in the blood circulation and present in collected blood.
- Blood collection procedures, components preparation and storage may further exacerbate the generation of microvesicles exhibiting potent pro-thrombotic and pro-inflammatory properties.
- Hemovigilance, and pre-clinical and clinical studies should delineate objectively the pathological implications of transfusing microvesicles to patients at risk, and impact of blood processing methods including new technologies (such as pathogen inactivation treatments) on microvesicles content should be studied more systematically.
- Whether the presence of microparticles in blood components has deleterious clinical relevance needs to be further investigated.

Introduction

Most cells can release different classes of vesicles into their environment. These vesicles are released upon different stress signals and are categorized based on their intracellular origin. They include apoptotic bodies, exosomes and microvesicles. Apoptotic bodies, or vesicles, are 1-5 µm in diameter and released from the plasma membrane when cells go through apoptosis. Exosomes are 40-100 nm in size and originate from the internal bourgeoning of the late endosomal membrane of multivesicular bodies and formation of intraluminal vesicles. Finally, microvesicles (EVs; microparticles), which are addressed here, encompass an heterogenous population of 30 nm-1 mm lipid bilayer vesicles present in body fluids,[1] including peripheral blood where they circulate at a concentration exceeding 10^9/mL. Blood-borne EVs are released by platelets, red blood cells (RBC), white blood cells (WBC), and endothelial cells, due to apoptosis, activation (by agonists, other cells or other microvesicles), or shear stress. Platelet-derived EVs (PEVs) seem to represent a predominant population in blood (70% or more), at least for those >400 nm, but recent data suggest this is over-estimated.[1] A loss of asymmetry of parent cells membrane leads to the budding and release of EVs. EVs expose negatively charged phospholipids (e.g., phosphatidylserine), various functionally-active surface markers and have a composition in proteins, peptide, bioactive lipids, nucleic acids, that reflects that of the parent cells. EVs are active players of cell-cell communication and of disease propagation through interaction with ligands on target cells, transfer of surface antigens, modification of the microenvironment, activation of intracellular signaling pathways, or regulators of gene expression.[1,2] Links exist between EVs number in the circulation and some pathological conditions.[3]

State of the art

Blood collection methods influence EV content in blood components,[4,5] as illustrated in Figure 1. *Ex vivo* processing of blood generates EVs, an event first defined as "storage lesions". While EVs are present in all blood components, one can speculate that they are removed during plasma fractionation due to the complex combination of purification, viral inactivation and filtration methods in place.[6] RBC concentrates contain EVs derived mostly from erythrocytes and some from residual leukocytes and platelets.[7] RBC vesiculation increases exponentially after 10-28 days of storage,[7,8] in asso-

ciation with RBC physiological aging and change from discoid to echinocytic and spherocytic shapes, with a higher proportion of larger size EVs being generated.[9] EVs accumulation during storage can gradually reach a level capable to trigger inflammatory, transfusion-related acute lung injury (TRALI), procoagulant, immunosuppressive, or hemolytic reactions, or alloimmunization in transfused patients, as a consequence of the enrichment in complement system, phosphatidylserine, immunoglobulins, and various biological modifiers and antigens.[5] In animal models, RBC EVs (REVs) can prime and activate neutrophils[10] and induce TRALI.[11] The responsibility of EVs in any increased risk of transfusion reactions associated with older blood components in some patient groups deserves attention. REVs exert proinflammatory and prothrombotic actions and may cause postoperative thrombosis, transfusion-related immunomodulation (TRIM), and mediate non-immune TRALI.[7,12] Platelet EVs (PEVs) are present in high number in platelet concentrates. They have a negatively charged pro-coagulant surface that can support the binding of coagulation factors leading to the formation of the prothrombinase and tenase complexes.[2] Phosphatidylserine pro-coagulant phospholipids may be a main contributor to the increased 50~100-times thrombin generation capacity of PEV membranes compared to resting platelets.[13] In addition, PEVs, through interactions with membrane surface markers like $CD61^+$ (GPIIIa), may reinforce the polymerization and strength of the fibrin clot, and enhance the thrombin generation.[14] Due to the expression of P-selectin (CD62P), they can interact with P-selectin glycoprotein ligand-1 (PSGL-1) present on leukocytes. Binding and activation of neutrophils in pulmonary tissues through P-selectin-PSGL-1 interaction may lead to TRALI.[7] PEVs can activate and aggregate monocytes *in vitro* and stimulate the release of EVs expressing tissue factor, a main activator of coagulation.[7] In addition, PEVs are a reservoir of biological response modifiers (anti-leukocyte antibodies and lipids) in particular soluble (s) CD40L

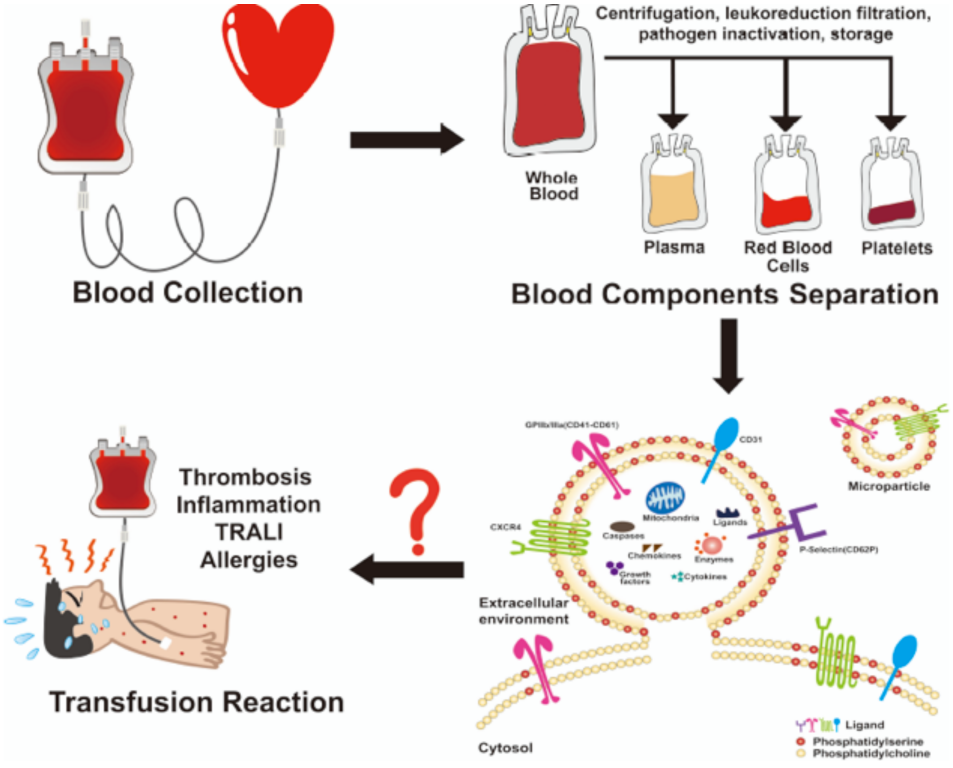

Figure 1. Possible role of microvesicles generated during blood processing and components preparation on transfusion reactions in recipients.

(sCD154), a pro-inflammatory mediator, and is associated with adverse transfusion reactions and TRALI.[5] Experimentally, sCD40L can interact with CD40 and prime human polymorph nuclear leukocytes, inducing PMN-mediated cytotoxicity of human pulmonary microvascular endothelial cells and damage of endothelium triggering occurrence of TRALI.[15] Apheresis platelet concentrates were shown to contain a relatively high concentration of sCD40L depending upon preparation methods and duration of storage.[15] PEVs can contain platelet mitochondria and release mitochondrial DNA that may trigger pro-inflammatory events following transfusion.[16] Studies are needed to explore further the circulating half-life of various types of EVs present in platelet concentrates. A half-life ranging from 10 minutes to 5.5 hours has been determined for PS-exposing PEVs,[3,17] but additional studies using specialized exploratory techniques are needed. Plasma for transfusion contains a variety of EVs, reflecting their heterogeneity in circulating blood and the generation taking place during blood collection and processing. EV generation is enhanced by freeze/thaw of plasma containing residual blood cells. While EVs may contribute to the hemostatic effect of plasma transfusion, an excess of PEVs may cause the thromboembolic complications seen in some patient groups.[18] EVs are concentrated in cryoprecipitate due to co-precipitation during centrifugation and/or interactions with fibrinogen, fibronectin, and vWF. A clinical dose of blood bank cryoprecipitate contain a quantity of EVs equivalent of $4x10^9$ platelets.[19] The belief that EVs contribute to the clinical hemostatic activity of plasma and cryoprecipitate is counter-balanced by the fact that plasma and cryoprecipitate subjected to solvent-detergent/oil treatment and 0.2 µm-filtration (expected to dissolve and remove EVs, respectively) still exert good hemostatic efficacy due to their content of coagulation factors.[20]

Future perspectives

EVs are increasingly seen as potential thrombotic bombs and "pathogenic particles" that can be detrimental when administered in large quantity to severely compromised patients.[4] The most serious events likely triggered by EVs include thromboembolism, inflammatory and immune reactions, and, most particularly TRALI. A main obstacle in dimensioning the true clinical impact of EVs in transfusion medicine is a lack of understanding of the content and type of EVs present in blood components at the time of transfusion. Laboratory, pre-clinical and clinical research is needed to i) develop, or improve, transfusion medicine-relevant EVs detection methods, especially in the smaller, less explored, size range (<100 nm);[21] ii) further assess the impact of blood/plasma collection and processing methods (including filtration and pathogen inactivation) on functional activity of EVs *in vitro;*[4] iii) develop and study the impact of EVs and removal methods (e.g., washing, filtration) in experimental models of thrombosis, inflammation, and TRALI; and iv) consider the role of EVs in hemovigilance programs overviewing transfusion reactions.

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Challenges in blood transfusion - Section 1

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Challenges in blood transfusion - Section 2

Challenges in typing and matching strategies in patients with hematological malignancies in the era of immunotherapy

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Take-home messages

- CD38 is weakly expressed on human erythrocytes. Therapeutic CD38-targeting antibodies interfere with routine pre-transfusion laboratory tests, complicating the selection of compatible red blood cells (RBCs) for transfusion.
- Reported mitigation strategies to overcome the interference have different advantages and disadvantages.
- The provision of RBCs can be significantly delayed if protocols are not in place to communicate this interference with compatibility testing to patients, laboratory staff, and physicians in a timely manner.

Introduction

Multiple Myeloma (MM) is a plasma cell malignancy that represents approximately 1% of all neoplasia and about 15% of hematological cancers.[1,2] It is characterized by the proliferation of monoclonal plasma cells in the bone marrow, with a consequent increase in monoclonal immunoglobulins in the serum and/or urine and organ damage including bone lytic lesions, renal impairment, hypercalcemia or anemia.[2] Over the last decade, the survival of MM patients has significantly improved due to the application of autologous stem cell transplantation, the introduction of proteasome inhibitors and immunomodulatory drugs. However, most patients die from refractory disease.[3,4]

Innovative treatments with little toxicity and favorable tolerability are needed and immunotherapeutic strategies are emerging as therapeutic approaches in MM, with several monoclonal antibodies (mAbs) targeting cell surface markers such as SLAMF7 (CS-1) and CD38.[4] An important advantage of mAbs is their specific targeting. However, since many laboratory tests are also based on specific antibody-antigen interactions, mAb interference in laboratory medicine is considered an increasing problem.[5] In trials with the anti-CD38 mAb daratumumab, all patients demonstrated panreactivity in red blood cell (RBC) panel testing,[5,6] complicating the selection of compatible RBCs for transfusion.

Current state of the art

Daratumumab (Darzalex™), developed in 2012, is a humanized mAb that binds CD38-expressing malignant cells with high affinity, inducing tumor cell death through diverse mechanisms of action.[3,4,7] Intravenous daratumumab has been approved for patients with MM who have received at least three prior lines of therapy, including a proteasome inhibitor and immunomodulatory agent or who are double-refractory to both. In addition to daratumumab, two other CD38-specific antibodies are in clinical development: isatuximab and MOR202.[3]

Oostendorp *et al*.[5] and others[6,8] showed that treatment of MM patients with daratumumab results in false positive indirect antiglobulin tests (IATs) for 2-6 months after infusion (Figure 1). Daratumumab causes agglutination in a dose and interval dependent manner, also observed with isatuximab and MOR202.[5] This interference is due to weak expression of CD38 on erythrocytes.[5,6] Adsorptions using enzyme-treated or untreated RBCs fail to remove the interference, putatively due to low expression of intact CD38 antigen on the adsorbing RBCs.[6] Contradictory results were reported for direct coombs testing (DAT) in daratumumab-treated patients, some reporting only negative DATs[5] (suggesting that RBC's with sufficient IgG coated on the surface have been removed from the circulation) others reporting patients with IgG positive DATs.[6,8] However, no laboratory signs of chronic hemolysis are found in daratumumab-treated patients.[6]

Daratumumab infusion results in a mild, temporal hemoglobin decrease of approximately 1.6 g/dL and an increase in reticulocyte count, but no relevant anemia.[5] This is likely not due to complement-mediated lysis, but due to Fc-receptor-mediated clearance in the spleen.[5] It has been hypothesized that only a small number of RBCs have sufficient CD38 density to allow relevant daratumumab binding, resulting in *in vivo* clearance

and *in vitro* IAT interference.[5,9] Detection of irregular antibodies in the plasma of daratumumab-treated patients is masked for up to six months after the last infusion. It therefore hinders routine pre-transfusion testing and complicates the selection of suitable RBC units.[5,6] However, thus far no major transfusion related events have been observed in daratumumab-treated patients.[5]

Since the first reports on anti-CD38 interference, different solutions have been presented, each with its own (dis)advantages.[10] Oostendorp *et al*.[5] reported the use of an in-house developed sCD38 extracellular domain protein (sCD38) as a generic mitigation option to prevent false-positive IATs. sCD38 was shown to block daratumumab and interference by other anti-CD38 mAbs, and allowed correct identification of known irregular antibodies. Addition of excess in-house developed anti-idiotype daratumumab antibody to both daratumumab-spiked plasma and plasma of daratumab-treated patients, also abrogated the interference and successfully restored antibody screening and identification.[5] An advantage of these neutralization methods is that, if freely available, they provide a fast and uniform way to deal with the interference. Suitable for every laboratory, since routine techniques for antibody screening, identification and crossmatching can be used. Disadvantages are higher reagent costs and yet a lack of widespread availability of the reagents.[6] In addition, a more thorough clinical validation of these assays is needed.

CD38 on RBCs is sensitive to denaturation by dithiothreitol (DTT) and Chapuy *et al.*[6] showed that treating reagent RBCs with DTT negates the daratumumab interference and allows alloantibody identification. Because DTT also denatures Kell antigens, K negative units should be selected for these patients.[6] An advantage of this method is that DTT is inexpensive and already used in immunohematological reference laboratories.[6] Drawbacks are the disruption of a limited number of blood group antigens[6] and difficulties performing this method in routine laboratories.[8]

Schmidt *et al*.[11] were able to rule out significant RBC antibodies in daratumumab-treated patients by use of cord RBC panels. They concluded that these cells have extremely low CD38 on their membrane and are thus useful for antibody screening

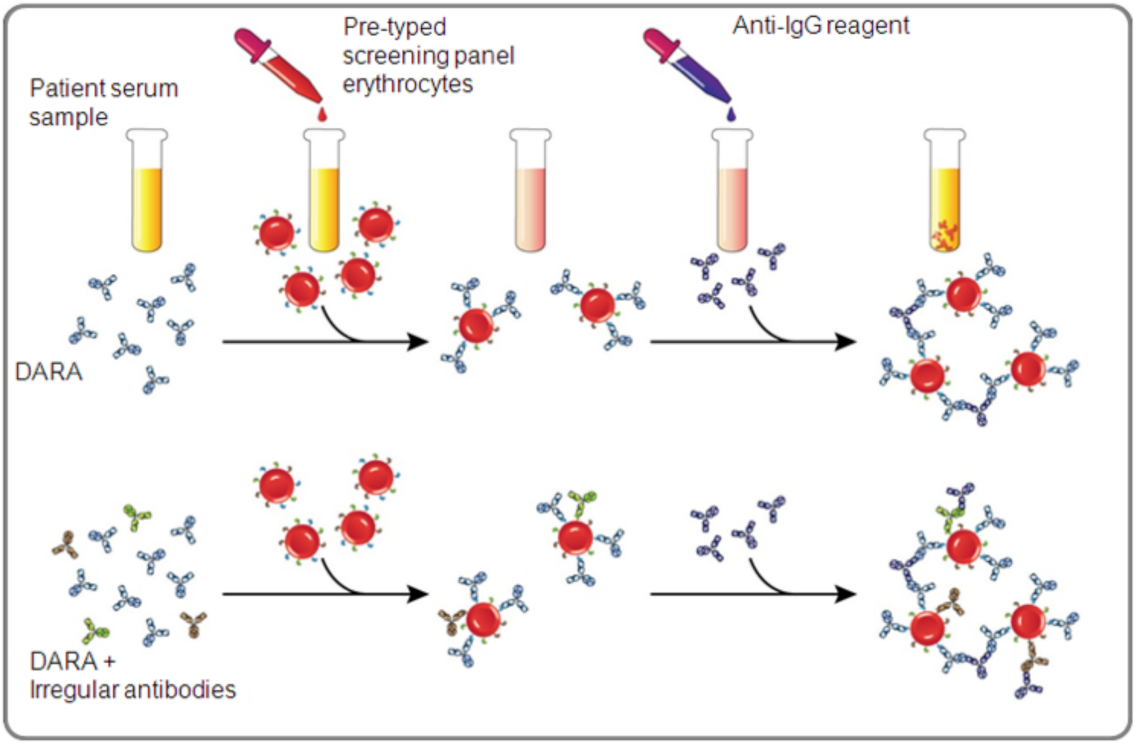

Figure 1. Interference of daratumumab in indirect antiglobulin test. Daratumumab (DARA) in the patient's serum binds to the test RBCs. After adding the anti-IgG reagent, RBC agglutination is observed, thereby generating a false positive result. The presence of irregular antibodies is masked by the presence of daratumumab.[5] Reproduced from Oostendorp *et al.* Transfusion 2015;55:1555-62; with permission.

in daratumumab-treated patients.[11] However, this method depends on the availability of cord blood RBCs, which could be a problem in some countries.[9]

Hannon *et al*.[8] described the successful application of antigen typing, allowing for selection of antigen-matched units, in a clinical daratumumab trial with six patients requiring transfusion. Although this strategy prevents mismatching for and irregular antibodies against the most common blood groups, it is time consuming. Often only a limited number of matching donors are available, resulting in shortage of compatible RBC units if the blood loss is too extensive. Furthermore, the presence of other irregular antibodies cannot be excluded due to anti-CD38 mAb induced positive cross-matching results.[10]

Besides implementation of mitigation strategies in the laboratories, patients should be provided with a blood group card alerting physicians on their anti-CD38 use to prevent unnecessary delays.[5,6,9,11,12] It may be prudent to have clinicians notify the blood bank (whether or not by HIS/LIS connection) when patients receive daratumumab, to prevent the laboratory from spending unnecessary time and resources in evaluating these samples.[11] In addition, before starting daratumumab, a serum screen for irregular antibodies is recommended.

Future perspectives

Immunotherapeutic strategies are emerging as promising therapeutic approaches in MM, with several monoclonal antibodies being in advanced stages of clinical development. CD38-targeting antibodies interfere with blood compatibility testing and thereby complicating safe transfusion. The development and availability of a neutralization reagent will probably help routine laboratories most, since it can be integrated into standard serological techniques. The provision of RBC units can be significantly delayed if protocols are not in place for communicating this interference to patients, laboratory staff, and physicians in a timely manner. Additionally, laboratories should have a protocol on how to deal with the interference and select compatible RBC units. As CD38 antibodies may have a role in the treatment of diseases beyond hematological malignancies, including solid tumors and antibody-mediated autoimmune diseases[3], many physicians and laboratory staff are likely to encounter this issue in the near future if they have not done so already.

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In this excellent review the clinical efficacy of mAbs targeting CD38 and SLAMF7 in MM is summarized. In addition, the emerging laboratory interferences they cause, with respect to immunofixation, serum protein electrophoresis assays, flow cytometric evaluation and blood compatibility testing are extensively discussed.

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Challenges in blood transfusion - Section 3

Red blood cell transfusion: When to transfuse in patients with hematological malignancies?

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Take-home messages

- High quality evidence is lacking for guiding red cell transfusion in patients with hematological malignancies.
- New randomized controlled clinical trials of enough power are needed to definitively establish the best strategy to indicate RBC transfusion in this population of patients.

Introduction

In the European Union, 18.3 millions of red blood cell (RBC) units were transfused in 2013.[1] In most of the countries of EU, about 40% of them are transfused to patients suffering from neoplasm and blood diseases.[2] Since 1999 high quality evidence has been published to guide RBC transfusion in a wide variety of clinical scenarios such as intensive care units (adult and pediatric),[3,4] sepsis,[5] orthopedic[6] and cardiac surgeries or gastrointestinal bleeding[7]. The evidence suggests that a restrictive hemoglobin threshold for indicating a RBC transfusion is at least as safe and efficacious as hemoglobin concentrations of 90-100 g/L. The restrictive threshold would be hemoglobin concentrations of less than 70 g/L for stable, adult inpatients including those in the intensive care unit and hemoglobin levels of less than 80 g/L for a group of postsurgery patients or those with preexisting cardiac disease.[8,9]

Current state

The patients suffering from hematological malignancies often require RBC transfusion as a consequence of the anemia developed by the disease itself or by the intensive treatment in form of chemotherapy or hematopoietic stem cell transplantation used. Unfortunately, in this group of patients, there are no currently published randomized controlled trials of enough power that help attending physicians to establish the best strategy to indicate RBC transfusions. Thus, to decide the RBC transfusion policy in this type of patients we have to rely on the existing published evidence. However, patients being treated for hematological malignancies represent a unique group of patients with differential characteristics that have to be taken into account when extrapolating the evidence found in other group of patients, such as longer term transfusion dependence.

Probably the most striking difference of patients being treated with intensive chemotherapy /radiotherapy combined or not to hematopoietic progenitor cells transplantation for hematological malignancies with patients included so far in the studies of RBC transfusion threshold is the frequent presence of profound thrombocytopenia (less than $20x10^9$/L) associated with the anemia that hematology patients present in the evolution of its disease. Some *in vitro* research data indicate that platelet interaction with vascular subendothelium is affected by the amount of RBC present is the circulating blood. Escolar *et al.*, using an *in vitro* perfusion system reported that in blood with $200x10^9$/L platelets, the percentage of perfused subendothelium covered by platelets decreased a 44% when the hematocrit was reduced from 41% to 19%.[10] Valeri *et al.* showed in healthy volunteers that the removal of two units of RBC decreased the hematocrit by 15% and the platelet count by 9% and at the same time a 60% increase in the bleeding time. Reinfusion of the two units of RBC previously removed, restored the bleeding time in the donors.[11] It has been suggested that hemorheological factors might explain, at least in part, this observation. The RBC would circulate at the center of the flow, pushing the smaller platelets to the periphery of the flow facilitating its interactions with the subendothelium of the vessels.[12]

The potential effect of low hemoglobin levels in the bleeding of anemic and thrombocytopenic patients with hematological malignancies has been studied in some small randomized controlled trials. Webert *et al.*[13] reported a pilot study where patients with acute leukemia receiving induction chemothera-

py or patients undergoing hematopoietic progenitor cell transplantation were randomized to receive 2 RBC units when hemoglobin level was less than 80 g/L or when the hemoglobin was less than 120 g/L. Sixty patients were enrolled in the study. The proportion of patients presenting clinically significant bleeding and the time to first bleed was not significantly different between the control and experimental group. The experimental group received more RBC transfusions compared to the study group (0.233 *vs* 0.151 patients/days with RBC transfusion, p=0.003). The authors concluded that it was feasible to enroll the needed patients for a large randomized controlled trial to investigate the effect of hemoglobin level on bleeding risk.

Interestingly, Robitaille *et al*. published in 2013 that a randomized controlled trial where two hemoglobin thresholds (70 *vs* 120 g/L) as triggers for RBC transfusion in children undergoing allogeneic bone marrow transplantation were being studied, was closed by the Data Safety Monitoring Board after enrolling 6 patients, because the 3 patients enrolled in the 120 g/L threshold arm developed veno-occlusive disease, while none of the 3 patients in the 70g/L arm did.[14]

Another pilot study was recently published where the feasibility of performing a randomized controlled trial of a low hemoglobin (70 g/L) RBC transfusion trigger versus a high (80 g/L) trigger in patients receiving treatment for acute leukemia. Ninety patients consented and were randomly assigned to each of the study arms. The authors concluded that the primary objective of feasibility was met. Regarding secondary outcomes, there was no statistically significant differences in bleeding events or neutropenic fevers between study arms. The mean number of RBC units transfused to patients in the low arm was 8.2 units per patient and 11.3 units in the high arm (p=0.0003).[15] In addition to those already published, there is one study registered at the Clinical.Trials.gov (NCT01237639) which studied the RBC transfusion triggers in patients undergoing hematopoietic stem cell transplantation (TRIST);[16] the study has recently completed the enrolment of patients but has not yet reported the results.

Table 1. Randomized controlled trials (RCT) on the red blood cell (RBC) transfusion threshold in patients with hematological malignancies.

Author, year, reference	Type of study	Patient population, n	Hemoglobin threshold compared	Primary outcomes	Secondary outcomes	Comment
Webert *et al.* 2008[11]	Multicenter, pilot-randomized controlled	Adult (>16 years) with acute leukemia, induction or consolidation. 60 patients	80 g/L *vs* 120 g/L	Feasibility of conduction a large RCT in this population	Clinically significant bleeding, RBC and platelet transfusions	Promyelocytic leukemia excluded.
Robitaille *et al.* 2013[12]	Multicenter, randomized, controlled	Children (<16 years), allogeneic bone marrow transplantation, 6 patients	70 g/L *vs* 120 g/L	Time to neutrophil recovery	Platelet recovery, number of RBC and platelet transfusions, hospitalization length, overall survival, transplantation related mortality, relapse	Study stopped by Data Safety Monitoring Board after 100% of the 3 patients enrolled in experimental arm developed eno-occlusive disease and none of 3 patients in the control arm
DeZern *et al.* 2016[13]	Pilot, randomized, controlled	Adult (>18 years), acute leukemia, 89 patients	70 g/L *vs* 80 g/L	Feasibility of conducting a large randomized trial	Fatigue, bleeding, response to therapy, vital status, length of hospital stay and number of RBC and platelet transfusions	2:1 (70 *vs* 80 g/L) randomization ratio.
Tay *et al.*[14]	Open-labeled, multicenter, pilot study	Adult, autologous or allogeneic hematopoietic stem cell transplantation, 100 patients	70 g/L *vs* 90 g/L	Feasibility of conducting a large randomized trial	Transfusion requirements, transplant related mortality, acute graft *vs* host disease, veno occlusive disease	Study completed, results not yet reported

Challenges in blood transfusion - Section 3

Future perspectives

New randomized controlled clinical trials of enough power are needed in patients with hematological malignancies to definitively establish the best strategy to indicate RBC transfusion in this population of patients.

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Chronic lymphocytic leukemia

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Introduction

Chronic lymphocytic leukemia (CLL) is a very heterogeneous disease. Beyond the two subtypes of CLL with mutated versus unmutated IGHV genes, microenvironmental factors as well as genetic aberrations define multiple subtypes of the disease, which may even occur in a single patient. In the first part Prof. Federico Caligaris-Cappio will present data regarding the role of microenvironment in CLL development, particularly the role of tumor-associated macrophages (TAMs). Because TAMs critically support the survival and proliferation of CLL cells, research and increasing knowledge about these cells is essential in order to understand not only the mechanism of disease, but also potential therapeutic targets. Prof. Sarka Pospisilova will discuss the predictive and prognostic value of different parameters in CLL patients. Besides clinical and serum parameters, genetic aberrations play a major role in development and further progression of the disease. Some of these parameters are crucial for treatment decision as particularly *TP53* disruption. Prof. Clemens Wendtner will not only highlight genetic aberrations, but also clinical factors as age and comorbidity status for the choice of frontline therapy as well as sequence of therapy. All presentations show that the field of CLL is currently undergoing a rapid development in basic and clinical research towards longer lasting disease control, if not cure.

Learning goals

1. ***To understand the relevance of the microenvironment for the development of CLL as well as for further progression of the disease.***
2. ***To learn about the most important prognostic and predictive factors and their relevance for clinical practice.***
3. ***To learn about considerations regarding the choice of frontline therapy as well relapse therapy including chemoimmunotherapies as well as novel targeted therapies.***

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Chronic lymphocytic leukemia - Section 1

Relevance of the microenvironment in chronic lymphocytic leukemia

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Take-home messages

- Tumor associated macrophages (TAMs) are key cells in the CLL cell microenvironment.
- Extracellular vesicles contribute to the inflammatory and immune-protected tumor-supportive niche.
- It will be important to confirm in human samples the findings obtained in (xenogeneic) mouse models.

Malignant chronic lymphocytic leukemia (CLL) clones are genetic mutineers that develop and thrive within permissive specialized tissue microenvironments provided by secondary lymphoid organs and bone marrow (BM) where the stimulation of the B-cell receptor (BCR) has a pivotal role (*Stevenson FK et a.l, Semin Hematol. 2014*). CLL can be classified into two major subsets that carry unmutated (U-CLL) or mutated (M-CLL) IGHV genes. These CLL subsets are biologically distinct and have different prognosis, but share the common feature of chronic antigen exposure in tissue sites where proliferative events occur (*Stevenson FK et al., Seminars Hematology 2014*) and where leukemic cells entail a bi-directional dialogue with a host of non-malignant elements including cells of both innate and adaptive immunity (*Caligaris-Cappio et a.l, Seminars in Cancer Biology, 2014*). Cell-cell interactions and soluble factors favor malignant cell growth, survival, drug resistance and prevent anti-tumor response. Thereby CLL cell/microenvironment cross talk is a major challenge for effective anti-CLL treatment (**Burger et al., Blood 2009*).

Only a small fraction of CLL cells are able to divide in the so-called "proliferation centers" of primary and secondary lymphoid tissues, where contact with antigen (Ag) and CD4(+)CD40L(+) T cells occurs. The mechanisms leading to CLL proliferation are still uncertain. A significant role appears to be exerted by the subset of T follicular helper (Tfh) cells (*Pascutti MF, et al Blood 2013*) which require BCL-6 and Il-21 (*Jogdand GM et al., Frontiers in Immunology 2016*). A critical aspect of CLL clonal expansion is the incapacity of leukemic cells to differentiate into antibody-producing cells that might be able to neutralize the stimulating Ag. In mouse models the B-cell maturation block appears to be not CLL-cell inherent but microenvironment dependent, a sort of non-classical germinal center–like reaction which takes place in tissue proliferation centers through the activity of Th1-polarized T-bet+ T cells (*Patten et al., JCI Insight 2016*).

As all CLL relevant events occur in tissue microenvironments it is relevant to consider the role of cell traffic and migration, a crawling journey accounted for by the cytoskeleton reorganization of malignant cells. Investigating the incompletely understood mechanisms of malignant cell egress from tissues into peripheral blood (PB) (*Patrussi L et al., Cancer Res 2015*) as well as those allowing tissue entry and re-entry (*Lafouresse F et al., Blood 2015*) starts shedding some light onto the rules that govern the circulation of tumor cells. The core of the problem is which events occur within the involved tissues and how the pro-tumorigenic and immune-protected habitats of CLL microenvironments are established.

Key players in the CLL microenvironment include T cells, stromal cells and nurse-like cells (NLC) (*Figure 1*). A role for monocyte/macrophage cells in CLL had already been suggested by a number of *in vitro* and correlative studies (*Reinart et al., Blood 2013*). As an example NLC are induced upon co-culturing PB or spleen monocytes with CLL cells *(Burger et al., Blood 2000*) and have been identified as CLL-specific tumor-associated macrophages (TAMs) *(Filip et al., Blood Cells Molecules and Diseases, 2013*). Furthermore, the gene expression profile (GEP) of CLL cells exposed *in vitro* to NLCs is remarkably similar to that of CLL cells isolated from lymph nodes *(Herishanu et al., Blood 2011*).

Recently the mechanisms through which TAMs regulate leukemic cell growth *in vivo* have been investigated by means of different CLL transplantation mouse models and TAM depletion strategies (**Galletti et al., Cell Reports 2016; Hanna et al., Leukemia 2016; *Nguyen PH et al., Cancer Cell 2016*). The results demonstrate that TAMs critically support the survival and proliferation of CLL cells *in vivo* and suggest therapeutic strategies based upon manipulating TAM/CLL-

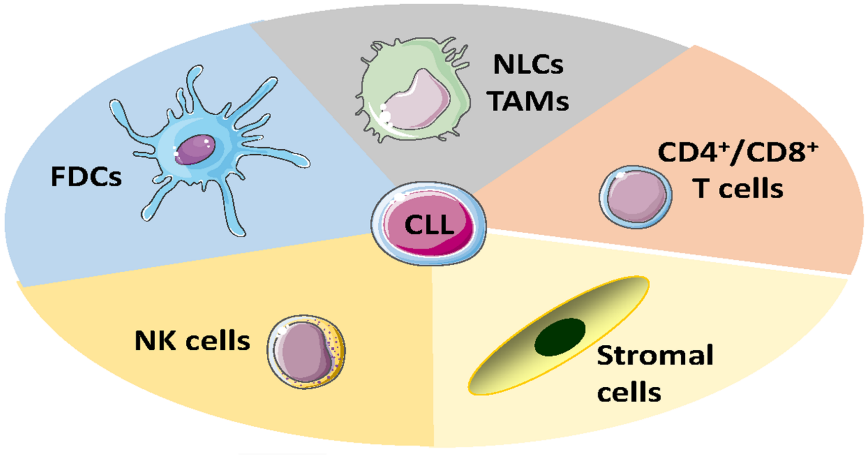
Chronic lymphocytic leukemia - Section 1

cell interactions (*Galletti et al., Leukemia 2016*). GEP shows that BM monocytes/macrophages exposed to leukemic cells *in vivo* are enriched for specific genes involved in a number of monocyte/macrophage functions (**Galletti et al., Cell Reports 2016*) and that also the transcriptome of leukemic cell is modified underlying the cross talk bi-directionality. The scenario emerging from mouse findings emphasizes that the microenvironment provides critical niches where the engraftment and progression of leukemic clones occur with the help of monocytes/macrophages and that at the same time the leukemic infiltration modifies the function of normal myeloid cells during leukemia development and progression. It will be important to explore whether the information obtained from mouse models can be extrapolated to patients with CLL and to elucidate to what extent this scenario is influenced by the stimulating role of (auto)antigens.

A critical molecule on the surface of monocyte/macrophages is colony-stimulating factor-1 receptor (CSF1R). The therapeutic anti-CSF1R monoclonal antibody (moAb) emactuzumab prevents the formation of new macrophages by inducing apoptosis or inhibiting monocyte differentiation and is an emerging clinical tool to target macrophages *(Ries et al., Cancer Cell 2014; Ruffell and Coussens, Cancer Cell 2015*). In mouse models the anti-CSF1R moAb has been shown to impair CLL cell engraftment and to associate with a striking anti-leukemic effect significantly improving mouse survival (**Galletti et al., Cell Reports 2016*). Mechanistically macrophage targeting sensitizes leukemic cells to apoptosis via induction of TNF signaling pathway and triggers their death through a TNF-dependent mechanism. A critical intracellular signaling molecule has been shown to be Lyn (**Nguyen PH et al., Cancer Cell 2016)*. Evidence has been provided in the Eμ-TCL1 mouse model (**Nguyen PH et al., Cancer Cell 2016)* that, while Lyn deficiency in murine CLL B cells does not influence the malignancy evolution, it supports CLL pathogenesis by operating in the leukemia microenvironment, especially through macrophage function as the loss of Lyn in the macrophages fails to support CLL growth. This mouse finding may be highly significant considering that Lyn had been found to be overexpressed and constitutively activated in human CLL *(Contri et al., J Clin Invest 2005)*.

Within the general context of the interactions between macrophages and leukemic cells it will be relevant to understand if and how the new drugs (e.g., kinase inhibitors) might be influencing *in vivo* macrophages besides exerting their effect on tumor cells.

Key mediators of intercellular communication between CLL cells and the microenvironment are cell-cell contacts through ligand/receptor interactions and exchange of soluble factors. An emerging unanticipated complexity of the mechanisms that account for intercellular communication is exemplified by the cell-cell signalling within the immune system represented by the shedding of extracellular vesicle (EV) (*Robbins and Morelli, Nat Rev Immunol 2014*). EV include exosomes and



FDC: follicular dendritic cells
NLCs: nurse-like cells
TAMs: tumor-associated macrophages

Figure 1. The key cells in the CLL cell neighborhood.

larger micro vesicles: their precise categorization as well as the specific biological significance of the different forms of EV are still problematic. Even with these caveats it has been found that CLL-produced exosomes induce the transition of stromal cells into cancer-associated fibroblasts (CAFs) (*_Paggetti et al., Blood 2015_*) which contribute to an inflammatory tumor-supportive microenvironment creating a niche that promotes CLL development/progression by favoring cell adhesion, survival, and growth. EV may also be involved in shaping an immune-protected niche. The transfer of CLL EV into autologous T cells has been shown to increase T-cell motility, improve the function of immunological synapse and promote the interactions of T cells with leukemic cells (*_Smallwood et al., Blood 2016_*), suggesting that the yet incompletely defined EV molecule cargo might have important immunomodulatory implications.

Taken together these findings shed some light onto which are the key cells in the CLL cell neighborhood and how they communicate. Conceivably the improved understanding of the microenvironment complexity (Figure 1) together with the role of antigen stimulation will soon become clinically meaningful.

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Chronic lymphocytic leukemia - Section 2

Prognostic factors in chronic lymphocytic leukemia: When, which and how?

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Take-home messages

- CLL has a very heterogeneous clinical outcome depending on many factors; the prognostic markers thus play an important role in disease management and assist in the selection of the best treatment option.
- The key prognostic and predictive factors influencing treatment decisions are *TP53* gene aberrations with increasing significance in the era of novel therapies.
- The introduction of high-throughput genomic approaches has led to the identification of novel genetic abnormalities that could contribute to improved risk stratification of CLL patients, while also enable the tracking of leukemic clone(s) evolution.

Introduction

CLL displays very variable clinical behavior distinctly dependent on a variety of biological, biochemical and genetic features of the disease. Classical prognostic factors such as age, gender, clinical stage, concentration of serum β2-microglobulin and serum thymidine kinase, status of IGHV gene somatic hypermutations (SHM) or chromosomal aberrations still hold their prognostic significance and are incorporated into up-to-date scoring systems.[1,2] Recent tremendous developments in genomic approaches, particularly in next-generation sequencing (NGS), have enabled a deeper insight into the molecular background of the disease and to discover novel markers with a potential role in disease prognostication and therapy response prediction.[3,4] Characterization of the CLL genomic landscape and identification of recurrent driver mutations associated with disease development and progression may improve patient stratification and optimize treatment decisions. Nevertheless, only certain novel genomic abnormalities have been proven to display a clear prognostic and predictive significance, and intensive efforts to elucidate the importance of specific biomarkers are ongoing.

Current state of the art

Since many patients suffering from CLL live for years without clinical symptoms while others require early therapeutic intervention and achieve variable treatment response, they apparently differ in a variety of prognostic and/or predictive factors. Numerous prognostic factors providing information on the likely outcome of the disease, and predictive factors providing information on the likely treatment benefit have been described in CLL, but only a few have been validated by multivariate analyses and prospective clinical studies so far.
To define the highly important factors with the greatest prognostic or predictive power, several attempts to develop a new scoring system for CLL patients have been made. Recently, an international group of CLL investigators published a meta-analysis including data from 8 controlled, randomized, prospective clinical trials and identified five independent prognostic factors: *TP53* gene deletion and/or mutation, IGHV gene SHM status, serum β2-microglobulin concentration, clinical stage and age.[2] In addition, many newly identified markers are being assessed for their clinical applicability.

Clinical prognostic factors

Clinical stage (Rai 0–IV, Binet A-B-C) importance arises from its direct impact on treatment decisions. However, clinical staging does not identify patients with an incipient disease and high probability of progression and also does not predict a treatment response. Age (having a borderline at 65 years) showed a significant prognostic impact on overall survival and is also considered as a prognostic factor.[2]

Chronic lymphocytic leukemia - Section 2

Serum parameters

From biochemical markers, mainly serum β2-microglobulin concentration is widely used holding its significance in CLL prognostication as an independent biomarker and has become part of the patient risk stratification system.[5]

Genetic markers

Immunoglobulin heavy variable (IGHV) gene somatic hypermutation (SHM) status has been proven in many studies to be a robust prognostic marker associating unmutated/minimally mutated IGHV sequences with unfavorable disease prognosis.[6,7] IGHV gene SHM status is unaffected by disease progression and its analysis can be performed at any stage throughout the disease course, according to the ERIC recommendations.[8,9] B cell receptor (BcR) immunogenetic characteristics beyond IGHV gene SHM status also appear to be prognostically relevant in the era of targeted therapy using BcR signaling inhibitors. Indeed, one-third of CLL patients express stereotyped B-cell receptors[10] which are grouped into distinct subsets displaying consistent biological characteristics and a clinical course ranging from very indolent (subset 4) to aggressive (subsets 1 and 2) disease.[11] Different spectra of recurrent gene mutations in CLL subsets harboring stereotyped B-cell receptors have recently been described showing a subset-biased acquisition of gene mutations.[12] Detection of chromosomal abnormalities using fluorescent *in situ* hybridization (FISH) has an essential role in CLL prognostication. According to the type of genomic aberrations, the Döhner's classification has defined five categories: del(17p), del(11q), 12q trisomy, normal karyotype, and del(13q) as the sole abnormality, with patients carrying del(17p) having the shortest median treatment-free interval. Locus 17p13 encodes the antioncogene *TP53* and its inactivation by deletion is frequently associated with mutation of the second allele; however, *TP53* mutations also occur independently of del(17p). *TP53* gene alterations are the most important genetic prognostic and predictive marker in CLL associated with very poor prognosis and resistance to chemoimmunotherapy, and should always be analyzed before a therapeutic decision is made.[13] Moreover, even low-burden CLL clones carrying *TP53* mutations detected by ultra-deep NGS could predict an inferior outcome.[14-16] Deletion 11q22-23 involving the ataxia-telangiectasia mutated (*ATM*) gene is also known to provide a negative impact on disease prognosis having the importance mainly in elderly patients. CLL patients with biallelic *ATM* defects have even shortened progression-free survival (PFS) and an adverse impact on overall survival (OS) has been documented.[17]

Complex karyotype, defined as the presence of three or more chromosomal abnormalities, has recently been shown to have a prognostic and predictive significance due to its negative influence on TTFT and OS in CLL patients treated with ibrutinib.[18] In addition, many novel genes identified using NGS technologies are potentially applicable for CLL prognostication.[3,4] *NOTCH1* and *SF3B1* gene mutations led to a shorter OS in CLL patients treated within clinical studies.[19] The importance of these alterations, including mutations in *BIRC3* and *MYD88* genes, has been included in the genetic prognostic model.[20,21] Prognostic or predictive significance of some other markers, such as CD38, ZAP70, peripheral lymphocytosis, bone marrow infiltration and serum soluble CD23, has been rather overcome by more robust novel genetic parameters.[1]

Future perspectives

The heterogeneous clinical course of CLL could likely be explained by the differences in underlying immuno-, cyto- and molecular- genetic prognostic factors. Analysis of these molecular factors at diagnosis and/or disease progression (before frontline therapy) and/or relapse (before subsequent

Table 1. Evaluation of genetic prognostic factors in chronic lymphocytic leukemia patients.

	Complex karyotype	Chromosomal aberrations (FISH cytogenetics)				*TP53* mutations	IGVH gene mutation status
		del(11)	trisomy 12	del(13)	del(17)		
Initial diagnosis	Optional	Yes	Yes	Yes	Yes	Yes	Yes
Disease progression / Before frontline therapy	Yes	Optional	Optional	Optional	Yes, unless detected before	Yes, unless detected before	Yes, unless performed before
Relapse / Before subsequent therapies	Yes	Optional	Optional	Optional	Yes, unless detected before	Yes, unless detected before	Yes, unless performed before
Prognostic significance	Yes*	Yes	Yes	Yes	Yes	Yes	Yes*

**Predictive significance for BcR signaling inhibitors.*

therapy) is strongly recommended for proper disease prognostication and assessment of the therapeutic outcome (Table 1). The application of modern genomic technologies, in particular targeted amplicon based NGS, enables us to further decipher the leukemic cells' molecular heterogeneity and clonal evolution[22] and becomes a part of routine CLL prognostication. Increasing whole genome and exome sequencing possibilities would facilitate 'personalized' CLL patient management and the choice of an optimal treatment strategy in the near future.[23]

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Chronic lymphocytic leukemia - Section 3

Prioritizing therapies in chronic lymphocytic leukemia

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Take-home messages

- For CLL patients we have to prioritize treatment options based on clinical and novel molecular markers.
- Chemoimmunotherapy remains the standard-of-care for the majority of CLL patients in the frontline setting.
- Novels drugs like ibrutinib, idelalisib and venetoclax are nowadays treatment standards for CLL patients with relapsed/refractory disease.

In the last few years we have faced major breakthroughs in the understanding of the molecular pathways and mechanisms in malignant B cells that resulted in the development and partially also approval of new classes of drugs in chronic lymphocytic leukemia (CLL). Therefore, it is important to prioritize treatment options available based on clinical and molecular characteristics of the individual patient.

First, we have to be aware that the majority of patients still benefits from a watchful waiting in early stages of the disease. If the indication for treatment is given, we have to select those patients with an ultra-high risk profile, independent of age and fitness: patients with a 17p deletion and/or a TP53 mutation should be offered nowadays treatment with the BTK inhibitor ibrutinib.[1] If there is a contraindication for the use of ibrutinib, the PI3K inhibitor idelalisib (in combination with rituximab) could be applied.[2] As an alternative, the BCL2 inhibitor venetoclax has recently been approved for patients being unsuitable for the use of a B-cell receptor inhibitor (BCRi).[3]

The majority of patients will not show an aberration of the TP53 gene, neither a deletion nor a mutation. Here we have to adapt the therapy based on classic criteria, i.e. age and fitness. It becomes apparent that more and more the IGHV status might help to choose for the most adequate treatment for specific subgroups. Nowadays, for fit patients (based on CIRS score) that are not older than 65 years, a combination treatment based on fludarabine, cyclophosphamide and rituximab (FCR) would still be the standard-of-care. Especially patients with a mutated IGHV and no further high risk features besides 17p-/TP53mut will statistically have a long-term progression-free survival, based on several independent trials by different study groups.[4,5] Nevertheless, we have to await the results of the FLAIR trial by the UK CLL Study Group that will challenge the FCR standard in comparison to a combination of ibrutinib and rituximab. If a patient has been defined to be fit, but is older than 65 years we would rather recommend a treatment based on the doublet of bendamustine plus rituximab (BR). Here the CLL10 trial of the GCLLSG has shown that BR resulted for fit elderly patients in similar efficacy compared to FCR, but significantly less toxicity.[6] The prioritization of therapies in the elderly non-fit patients without ultra-high risk features is more difficult because we do have several options that have not been compared to each other within controlled clinical trials. Based on the COMPLEMENT-1 trial the combination of chlorambucil plus ofatumumab has been shown to be superior to a classic monotherapy with chlorambucil.[7] Furthermore, the CLL11 trial conducted by the GCLLSG has proven that chlorambucil plus obinutuzumab is the treatment of choice in less fit elderly patients, this in comparison to a chlorambucil monotherapy and a doublet based on chlorambucil plus rituximab.[8] The MABLE trial has recently been presented with preliminary data showing a superiority of BR compared to chlorambucil plus rituximab.[9] Finally, ibrutinib has been approved in the frontline setting based on a phase III trial (RESONATE-2) that included patients above the age of 65. This trial demonstrated significant PFS and OS advantages for ibrutinib when being compared to chlorambucil

Chronic lymphocytic leukemia - Section 3

(without anti-CD20 mAb).[10] Despite the limitation of the comparator arm, ibrutinib monotherapy received the EMA label for frontline treatment in CLL, irrespective of age and fitness. Nevertheless, the hematology community is awaiting the data of the ILLUMINATE trial that performs a head-to-head comparison of ibrutinib to chlorambucil, both drugs being used in combination with obinutuzumab. Since subgroup analysis have shown that ibrutinib has the same efficacy independent from the IGHV status, ibrutinib frontline treatment could be prioritized for elderly non-fit patients with an unmutated IGHV in countries where ibrutinib is available in this setting.

While the options in the first-line treatment setting seem to be well defined (see Figure 1), the therapeutic armamentarium in the relapsed setting (see Figure 2) is less clear due to the fact that only few randomized trials have been performed in the past years. There is a consensus that chemoimmunotherapy can be repeated in patients that have been in remission for at least three years after frontline therapy, however BCR inhibitors could be used alternatively, notably after testing again for unfavorable genetic markers such as TP53 aberrations. While the option of reinduction with classic chemoimmuntherapy in case of late relapses might be real in a younger and fit patient, it is becoming more and more theoretical in elderly patients since toxicity of chemotherapy might become a burden with increasing age. Especially in elderly late relapsing patient, but also in cases of early relapse or even for patients with refractory disease after frontline chemoimmunotherapy, we should go for one of the new agents like ibrutinib or idelalisib (the latter in combination with rituximab). Ibrutinib has been randomized to ofatumumab in the RESONATE trial and has demonstrated a PFS and OS advantage.[11] Idelalisib (plus rituximab) was shown to be superior to a rituximab monotherapy comparator arm.[12] Both drugs have been approved by the EMA in the relapsed setting, after at

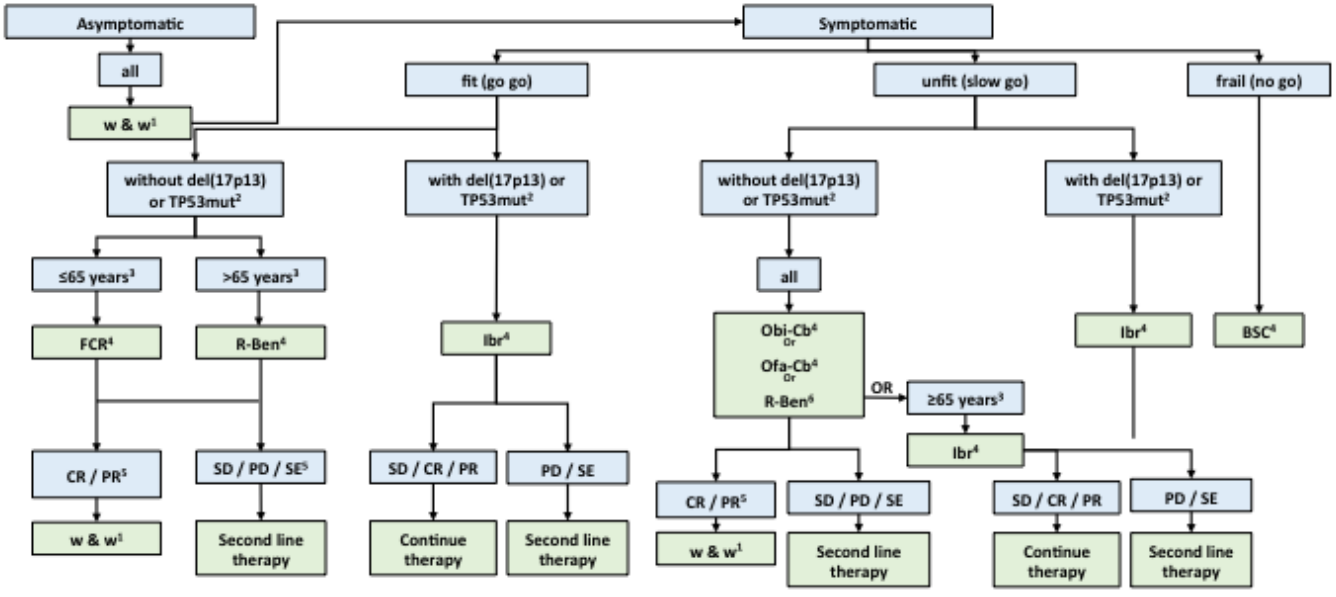

Figure 1. 1st line treatment recommendations for CLL

Adapted from Wendtner CM, et al. Onkopedia January 2017. Available at: https://www.onkopedia-guidelines.info/de/onkopedia/guidelines/chronische-lymphatische-leukaemie-cll
[1]w & w – watch and wait; [2]based on FISH (17p-) and Sanger sequencing (TP53mut); [3]Based on the inclusion criteria of the underlying studies; Therapy based on comorbidity and less on age [4]Therapy: Ben - bendamustine, BSC - Best Supportive Care, C - cyclophosphamide, Cb - chlorambucil, F - fludarabine, Ibr - Ibrutinib, Obi - obinutuzumab, Ofa - ofatumumab, P - prednisone, R - rituximab; [5]CR – complete remission, PD – progressive disease, PR - partial remission, SD - stable disease, SE – Side effects. [6]Dose reduction of bendamustine to 70 mg/m² (day 1 +2) in patients in unfit state (slow go).

Chronic lymphocytic leukemia - Section 3

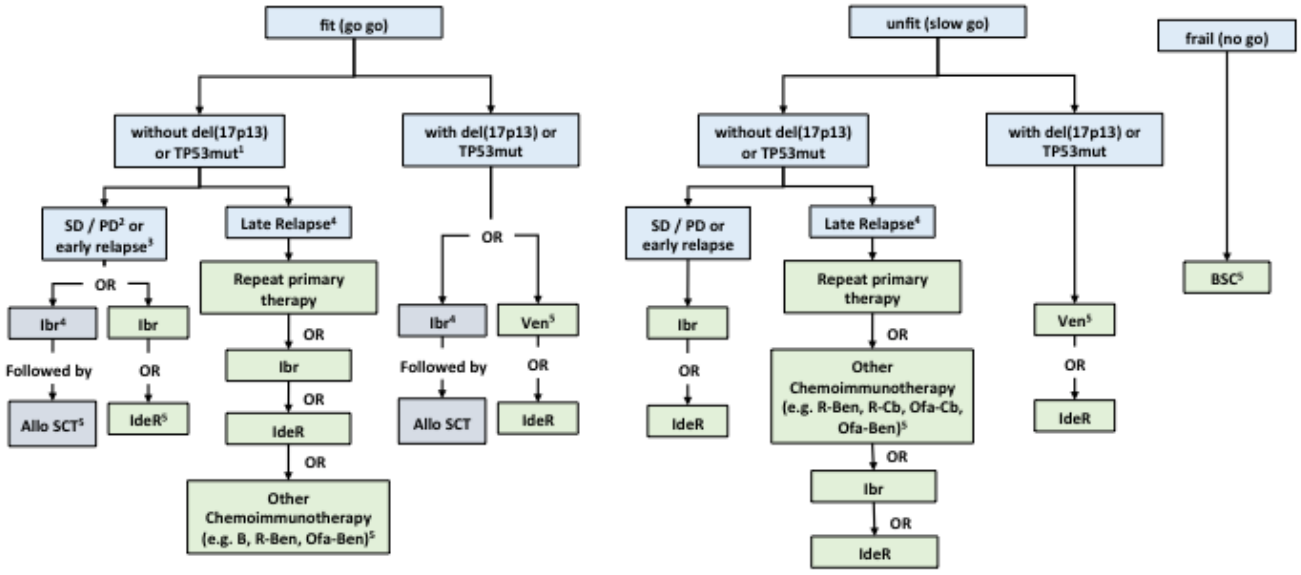

Figure 2. 2nd line treatment recommendations for CLL

Adapted from Wendtner CM, et al. Onkopedia January 2017. Available at: https://www.onkopedia-guidelines.info/de/onkopedia/guidelines/chronische-lymphatische-leukaemie-cll
[1,2]based on FISH (17p-) and Sanger sequencing (TP53mut); [2]PD – Progressive disease, SD – Stable disease. [3]Early relapse – within 2-3 years; [4]Late relapse – later 2-3 years; [5]Therapy: allo SCT – allogenic stem cell transplant, Ben - Bendamustine, BSC – Best Supportive Care, Cb – Chlorambucil, Ibr – Ibrutinib, Ide – Idelalisib, Obi – Obinutuzumab, Ofa – Ofatumumab, P – Prednisone, R – Rituximab, Ven – Venetoclax.

least one line of prior therapy. For patients with a ultra-high risk aberration (17p-, TP53mut) in the relapsed/refractory setting both drugs are the treatment of choice. If a patient with a 17p-/TP53mut feature has seen one of these BCR inhibitors before, one option would be to switch from a BTK inhibitor to a PI3K inhibitor or vice versa. Presumably a better option, although never proven in a randomized fashion, would be to offer the BCL2 inhibitor venetoclax in case of a BCRi failure.[13] Venetoclax is also approved for patients with failure to chemoimmunotherapy and BCRi, irrespective of the TP53 status. Current and future trials will analyze whether we can limit the exposure to treatment, also including new drugs that have otherwise to be used indefinitely, by combining different drug classes with each other in order to improve the quality of life for our patients without taking the risk of an uncontrolled disease.

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In case of failure of a BCR inhibitor, the BCL2 inhibitor venetoclax is an effective treatment choice.

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Chronic myeloid leukemia

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Introduction

Three generations of tyrosine kinase inhibitors (TKIs) are now available for the treatment of chronic myeloid leukemia (CML) patients. None of them, however, is capable to eradicate leukemic stem cells (LSCs), whose persistence is though to be the main obstacle to successful treatment discontinuation and may, in some patients on therapy, trigger disease progression. Intense research has thus been devoted, over the past years, to the understanding of how LSCs survive, how we can identify and count them, and which therapeutic strategies may realistically be devised to eliminate LSCs while not impairing excessively treatment tolerability and patients' quality of life. Another field of active research aims to identify (pre-treatment) biomarkers enabling us to predict which patients will do well, which patients will not, which patients will be able to stop treatment without experiencing disease recurrence. While work is in progress, thorough molecular monitoring of patients remains the key to therapeutic success and novel technologies might further improve accuracy and sensitivity. Choice between imatinib, dasatinib and nilotinib in newly diagnosed CML cases should be the result of a patient- and disease-centered algorithm taking into account age, risk, comorbidities, treatment end-points, costs. Change of the TKI might be needed in case of intolerance or resistance; in some cases, transplant is still an option. New treatment modalities are still being explored in clinical trials: a novel inhibitor with allosteric binding mode (ABL001) appears to be the most promising one.

Learning goals

1. ***To review the key mechanisms underlying LSC persistence in CML and how we can therapeutically interfere with them.***
2. ***To discuss the diagnostic work-up for newly diagnosed CML patients, the role of predictive and prognostic factors and the clinical value of molecular monitoring with current and novel technologies.***
3. ***To review the factors to be considered when choosing the TKI and how toxicity and resistance should be managed.***

EUROPEAN HEMATOLOGY ASSOCIATION

Chronic myeloid leukemia - Section 1

Novel approaches to eradicate chronic myeloid leukemia stem cells

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Take-home messages

- CML stem cells utilize multiple cell-intrinsic pathways, together with microenvironmental and immune cell interactions to evade current therapies.
- Identification of clinically relevant targets on CML stem cells, e.g. PPARγ, p53, c-MYC, BCL-2 and EZH2, is likely to lead to improved therapies for patients with all phases of CML in the future.

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder, derived from a hematopoietic stem cell (HSC), which acquires the *BCR-ABL* fusion oncogene. Despite the huge success of tyrosine kinase inhibitors (TKIs) in treating CML, resulting in the majority of patients obtaining a major molecular response (MMR) on sustained therapy,[1-3] there is strong evidence that these drugs are ineffective against the CML leukemic stem cell (LSC).[4-6] This can lead to molecular disease persistence and relapse, both on TKI therapy and after TKI cessation. It is clear from TKI discontinuation studies, e.g. STIM, TWISTER and EUROSKI,[7-9] that only a minority of optimally responding patients can safely stop their TKI without evidence of molecular recurrence. A number of recent studies have identified novel approaches that may eliminate CML LSCs. This short review will evaluate some of these potential LSC eradication strategies (Figure 1).

Current state of the art

LSC quantification

Two recent studies have focused on estimating the size of the LSC population in CML, and how this alters with therapy. Werner *et al* presented a mathematical model which describes the relative increase in LSCs over time on therapy in comparison to overall tumur burden, together with the slow decline in absolute LSC numbers.[10] Thielen *et al.* used multiparameter flow cytometry and fluorescence in situ hybridization for *BCR-ABL* to correlate LSC numbers (CD34+38-) with established prognostic markers and response to therapy.[11]

LSC heterogeneity

Evidence is accumulating that CML LSCs are heterogeneous. In a murine model of CML, Zhang *et al* described the heterogeneity of leukemia-initiating capacity of CML LSCs, with high levels of MPL expression correlating with superior engraftment and enhanced leukemogenesis[12] Our own recent studies in myeloid blast-phase CML clearly show that multiple non-hierarchically arranged immunophenotypically-defined stem and progenitor cell populations have functional LSC capacity.[13] Furthermore, blast-phase-associated additional chromosomal abnormalities are detected in all stem and progenitor cell populations.

The importance of the stem cell niche in CML

In addition to CML LSC-intrinsic factors, it is becoming increasingly clear that the immune system and bone marrow (BM) microenvironment have very important roles in CML LSC persistence.[14-18] CML LSCs have altered proliferation, differentiation and localization within the BM niche. While aberrant cytokine expression gives CML LSCs a growth advantage, abnormal localization of LSCs is due, at least partly, to reduced CXCL12 expression. Studies have shown that TKI treatment only partially corrects these alterations in the CML BM microenvironment, and targeting these microenvironmental pathways can enhance LSC eradication.[14,15] Recently, the importance of gonadal adipose tissue as an LSC niche has been described,[16] supporting LSC metabolism and enhancing chemoresistance, particularly in CML LSCs expressing the fatty acid transporter CD36.

Chronic myeloid leukemia - Section 1

Aberrant interleukin-1 signaling

Two recent studies have identified the interleukin 1 receptor (IL-1R) pathway as a potential therapeutic target in CML LSCs. Increased expression of the IL-1R complex, via upregulation of survival pathways including NF-kB, JNK and p38MAPK, promotes the growth and survival of CML LSCs.[19,20] These two preclinical studies adopted different, but effective, strategies for eliminating CML LSCs. The first used an IL-1R antagonist (used clinically for the treatment of rheumatoid arthritis[21]) to inhibit growth of CML LSCs and increase sensitivity to nilotinib.[19] The second utilized antibody-dependent cellular cytotoxicity against IL-1 receptor accessory protein (IL-1RAP), a surface molecule shown previously to be expressed on CML LSC but not normal HSC,[22] and demonstrated increased survival in murine xenograft models of chronic- and blast-phase CML.[20]

Targeting alternative cell surface molecules

Other groups are also seeking to exploit aberrant expression of cell surface molecules on CML LSCs to improve therapeutic responses. CD26 (dipeptidylpeptidase IV), CD33, and CD93 have all been shown to be overexpressed on CML LSC,[23-25] and have the potential to be exploited therapeutically. CD26 expression discriminates CML LSCs from normal HSCs and, furthermore, numbers of CD26+ LSCs correlate with response to TKI therapy.[26] Preclinical *in vivo* studies indicate that gliptins, a family of anti-diabetic drugs, may reduce CML LSCs.[24] CD33+ CML LSC may be targeted by the antibody-drug conjugate gemtuzumab ozogamicin,[23] but clinical utility is likely to be limited by the unacceptable side effect profile for most patients with CML. Although CD93 is overexpressed on CML LSC compared to normal HSC, it is also expressed on endothelial cells, platelets and more mature myeloid cells,[27] making it a less attractive option for therapy.

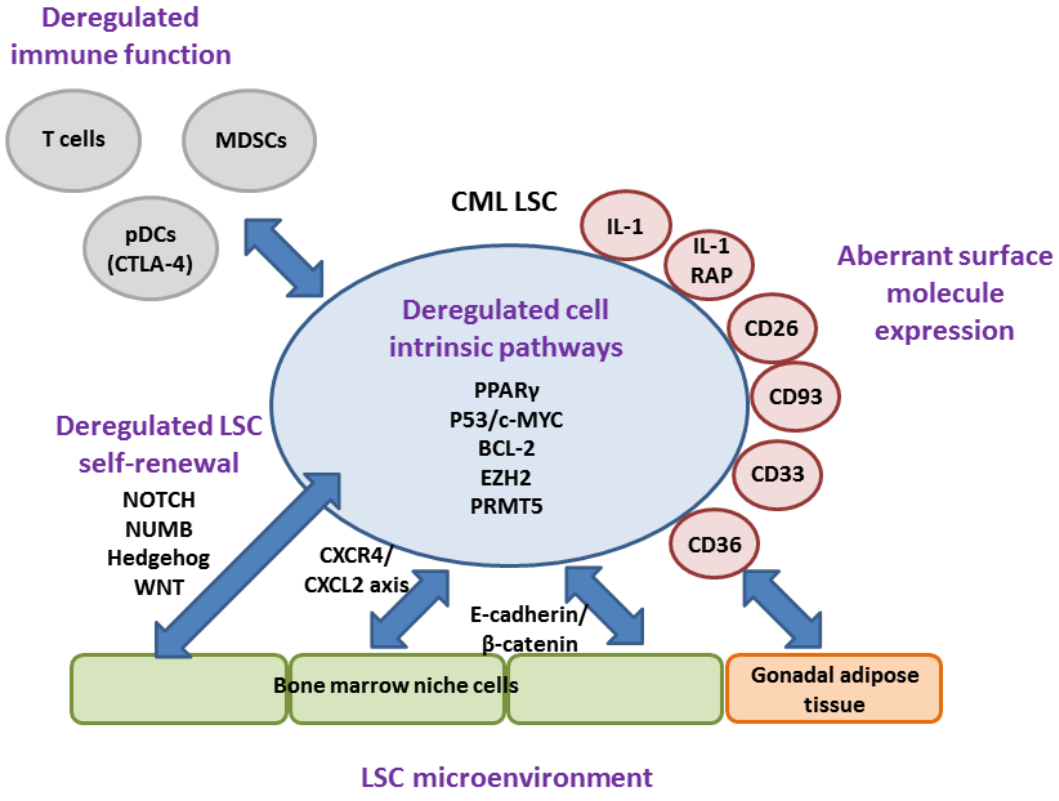

Figure 1. Potential leukemia stem cell (LSC) eradication strategies. This review focuses on a number of recently published original articles exploiting different approaches for eliminating CML LSCs. Four different approaches are considered which focus on LSC microenvironment, aberrant cell surface marker expression, deregulated LSC self-renewal and deregulated cell-intrinsic pathways. MDSCs; myeloid-derived suppressor cells, pDCs; plasma dendritic cells.

Self-renewal pathways as therapeutic targets in CML LSC

Interest continues to focus on self-renewal pathways as a potential route to eliminate CML LSCs. We have recently shown that NOTCH is silenced in chronic-phase CML, with activation of NOTCH leading to reduced self-renewal capacity in CML LSCs.[28] Recent studies have also demonstrated that NUMB inactivation results in imatinib resistance in CML.[29] Thus, activation of NOTCH or NUMB may be potential therapeutic strategies against CML LSCs. Although the Hedgehog pathway is de-regulated in CML,[30] clinically available SMO antagonists have proven to have an unacceptable side effect profile when used alone or in combination with TKIs in clinical trials. Previous studies have shown that WNT signaling from the bone marrow niche contributes to LSC persistence.[15] Secreted WNT ligands are modified by the O-acyl transferase, porcupine (PORCN). Recently, Agarwal *et al.* demonstrated that the potent and selective PORCN inhibitor WNT974, either alone or in combination with nilotinib, effectively targeted CML LSCs.[31]

Targeting CML LSC-intrinsic pathways

A number of recent studies have described strong preclinical evidence for cell-intrinsic pathways that may be exploited to eradicate CML LSCs. Peroxisome proliferator-activator (PPAR)-γ agonists, including the anti-diabetic drug pioglitazone, reduce the CML LSC pool in preclinical studies by bringing quiescent cells into cell-cycle and rendering them sensitive to TKIs.[32] The ACTIM study, a proof-of-concept study, comparing imatinib plus pioglitazone with a historical imatinib-only cohort indicated a superior MR4.5 rate in the combination arm (56% *versus* 23%).[33] A randomized study to confirm this is now required.

Using a novel bioinformatics approach, incorporating proteomics, transcriptomics and network analyses in primary chronic-phase CML, Abraham *et al.* identified p53 and c-MYC as critical signaling hubs.[34] Upregulation of p53 using an MDM2 inhibitor, combined with downregulation of c-MYC using a BET inhibitor, led to selective and potent elimination of CML LSCs.

BCL-2 is over-expressed in advanced phase CML. The combination of BCL-2 inhibitor, ABT-199, with TKI-mediated inhibition of BCL-XL and MCL-1 effectively eliminated CML LSCs in murine models and blast-phase patient samples by inducing apoptosis of quiescent LSCs.[35]

There is an increasing focus on epigenetic therapies in leukemia. Two groups have recently described the over-expression of EZH2, the catalytic subunit of the polycomb repressive complex (PRC)-2 in CML LSCs.[36,37] These preclinical *in vitro* and *in vivo* studies have demonstrated that the combination of EZH2 inhibitor with TKI enhances eradication of CML LSCs. Further recent studies have also demonstrated that targeting the methyltransferase PRMT5, which is over-expressed in CML LSCs, reduced self-renewal, possibly via reduction of the WNT/β-catenin pathway molecule disheveled homolog 3 (DVL3).[38]

Conclusions/future perspectives

Remarkable progress continues to be made in defining and identifying potential therapeutic strategies to eliminate CML LSCs. It is likely that a number of the approaches described here will proceed to clinical trial and the long term goal is improved elimination of CML LSCs in all phases of CML; to reduce resistance in advanced phase patients and increase the number of optimally responding patients capable of permanently discontinuing TKI therapy.

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Chronic myeloid leukemia - Section 1

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Chronic myeloid leukemia - Section 2

Molecular work up and monitoring of chronic myeloid leukemia patients

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Take-home messages

- Standardized MRD analysis is the best molecular predictor of outcome in CML.
- Biomarkers for advanced disease and pharmacokinetic variables may help to predict response.
- Increasing evidence of the role of the immune system in the response of CML to treatment.

Diagnosis of CML

All cases of chronic myeloid leukemia (CML) are, by definition, positive for the *BCR-ABL* fusion. About 95% of cases exhibit a visible Philadelphia chromosome on conventional cytogenetic analysis, the smaller derivative of the t(9;22)(q34;q11), or a variant that typically involves one or more additional chromosomes. The remaining 5% of cases have a cytogenetically cryptic *BCR-ABL* fusion and often have a normal karyotype. Such cases may be picked up by FISH to detect aberrant juxtaposition of the *BCR* and *ABL* genes or RT-PCR to detect *BCR-ABL* mRNA. The great majority (97-98%) of CML cases expresses a chimeric mRNA in which *BCR* exon 13 or exon 14 is joined to *ABL* exon 2 (e13a2 and e14a2 fusions, respectively, also commonly referred to as b2a2 and b3a2). The remaining 2-3% of cases express diverse, atypical fusions involving other exons of *BCR* and/or *ABL*. For all cases, it is important to determine the *BCR-ABL* mRNA transcript type prior to treatment to enable effective molecular monitoring.

Definition of prognosis prior to treatment

Considerable efforts have been made to identify pre-treatment biomarkers that can distinguish patients destined to perform well on therapy from those who will perform poorly. These biomarkers can be considered within four basic categories: (i) intrinsic differences in disease biology, (ii) markers of disease progression (iii) pharmacokinetic variables that influence the effectiveness of therapy, and (iv) the immune environment.

Intrinsic disease biology

Although atypical *BCR-ABL* fusions and complex *BCR/ABL* rearrangements are not thought to be strong indicators of prognosis (except possibly the rare occurrence of the p190 fusion), it has been suggested that individuals expressing e13a2 *BCR-ABL* have marginally inferior cytogenetic and molecular responses to tyrosine kinase inhibitors (TKIs) compared to e14a2 cases. Although no effect is discernible on survival, differences in the rate and the depth of molecular response could potentially impact on TKI discontinuation.[1-4] Emerging data suggest that in some cases *BCR-ABL* may be acquired on a background of clonal hematopoiesis driven by mutations in genes such as *TET2* or *ASXL1*, but at the current time there appears to be no clear impact of this finding on clinical course or outcome.[5]

Disease progression

Advanced phase CML responds poorly to therapy and it is no surprise that markers of disease progression are associated with an adverse prognosis.[6] Detection of additional cytogenetic abnormalities, particularly major route abnormalities (+Ph, trisomy 8, isochromosome 17q or trisomy 19), suggests progression to accelerated phase or blast crisis and these abnormalities at diagnosis are associated with a negative impact on survival.[7] Gene expression profiling may indicate advanced disease in some individuals who would otherwise be categorised as chronic phase.[8] At the stem cell level, there is marked heterogeneity in the relative proportion of *BCR-ABL* positive and (presumed) normal stem cells, with a greater proportion of leukemic stem cells suggesting more advanced disease and correlating with an inferior outcome.[9]

Pharmacokinetics

Like all other drugs, the effectiveness of TKIs is influenced by ADME: absorption, distribution, metabolism and excretion. *CYP2C8* genotype significantly alters imatinib metabolism in patients through gain- and loss-of-function mechanisms.[10] Polymorphic variants, expression levels and, more convincingly, functional activity of the transporter OCT1 (encoded by *SLC22A1*), correlate with clinical outcome for patients treated with imatinib (but not other TKIs).[11] Similarly, high expression levels of *ABCB1* (which encodes the multidrug resistance protein MDR1 implicated in TKI export) has been linked to initiation of TKI resistance[12] and polymorphic variants of this genes linked to molecular response.[13]

Immune environment

There is increasing evidence that immunologic surveillance mechanisms impact on the response of CML to therapy. Killer immunoglobulin-like receptors (KIRs) profiles on natural killer (NK) cells have been shown to predict for response to TKIs and the polymorphic variants KIR2DL5B and KIR2DS1 are associated with outcome.[14,15] Low numbers of L-selectin (CD62L)-expressing CD4+ and CD8+ T cells correlated with adverse clinical features and both reduced CD62L expression on T cells and increased soluble CD62L levels predicted molecular response to TKI therapy.[16] Finally, a high proportion TNF-α/IFN-γ secreting mature NK cells is associated with successful imatinib discontinuation, whereas high expression of the CTLA-4 ligand CD86 on plasmacytoid dendritic cells is associated with a higher risk of relapse after TKI discontinuation.[17]

Despite these advances it is sobering to appreciate that no biomarker has thus far been proven to outperform simple, cheap clinical scoring systems (Sokal, Hasford, EUTOS, ELTS). In addition to the markers above, behavioral factors that influence treatment compliance are also relevant to prognosis.[18]

Definition of prognosis and response on treatment

Plasma levels of imatinib correlate with clinical response and changes in *ABCB1* expression may help to predict response,[13] but by far the strongest prognostic indicator is the measurement of residual disease levels on treatment by reverse transcription – quantitative PCR (RT-qPCR) using methods aligned to the International Scale.[19] Optimal response as defined by the European LeukaemiaNet is strongly associated with better outcomes, and rising *BCR-ABL* mRNA levels on sequential analysis suggest disease relapse, usually either due to biological resistance or inadequacy of therapy due to compliance issues. In routine practice, resistance is associated with secondary *BCR-ABL* mutations in up to a third of cases and the finding of such mutations may help to guide subsequent therapy. Mutations may be detected down to levels of 1-2% variant allele frequency (VAF) using next generation sequencing (NGS),[20] and with even greater sensitivity using targeted approaches, but it is not yet clear if these increased levels of detection afford any clinical advantage over standard Sanger sequencing, which detects mutations down to a level of 10-20%. Other mechanisms of resistance such as *BCR-ABL* overexpression and *LYN* kinase overexpression are difficult to discern on a routine basis.

Interpreting molecular responses is helped by assessing sequential trends rather than just considering specific time-dependent milestones. In this regard measurement of the rate of decline of *BCR-ABL* transcripts from months 0-3 on treatment may be useful to decide whether a patient should be considered as an early molecular response (EMR) failure or not. Standardized measurement of deep molecular responses (MR^4, $MR^{4.5}$ etc.)[19] is particularly important when considering stopping therapy. Digital RT-PCR may provide greater accuracy for measurement of low levels of disease but it is not yet clear if this is of any clinical benefit. Similarly, DNA based PCR approaches are of interest to increase the limit of detection of CML cells, but it seems unlikely that this will become routine practice.

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Chronic myeloid leukemia - Section 2

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Chronic myeloid leukemia - Section 3

How to treat chronic myeloid leukemia in 2017

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Take-home messages

- The advent of tyrosine kinase inhibitors has substantially changed biology and outcome of the disease.
- With optimal management, patients achieve an almost normal life expectancy which results in an important annual increase of the prevalence of CML.
- Treatment free remission is feasible for an important minority of patients but requires stringent surveillance.

Introduction

Overall survival (OS) in patients with chronic myeloid leukemia (CML) under treatment with imatinib approaches 90% at 5 years and 83% at 10 years.[1] Since the advent of second- and third-generation tyrosine kinase inhibitors (TKIs), faster and deeper remissions have been reported, including complete cytogenetic remission (CCyR), major molecular remission (MMR), and deep molecular response (MR^4, $MR^{4.5}$).[2,3] To date, none of the clinical trials involving new therapies have shown a survival advantage, although the ENESTnd trial[2] did demonstrate a favorable progression-free survival in CML patients treated with nilotinib.

A number of different TKIs are now available, giving many treatment options for CML. Evidence-based care requires an understanding of the optimal use of these drugs, their specific early and late toxicities, the prognostic significance of achieving treatment milestones, and the critical importance of molecular monitoring. Efficacy is important, but treatment choice does not depend on efficacy only. Choosing among various treatment options is informed by understanding the distinct benefits and risks of each agent, along with careful consideration of patient-specific factors, such as risk status, age, and comorbidities. After failure of first-line TKI, a switch to a second- or third-line therapy is recommended. As a result, the influence of a certain TKI therapy on OS has become more difficult to assess.[4,5]

Treatment aims

With a newly diagnosed patient, aims of therapy should be discussed since choosing the optimal first line therapy depends on the knowledge of the options and aims. Individual aims (according to age and social situation of the patient) consist of (i) the chance to maintain normal survival probabilities, (ii) reduction of the risk of accelerated phase and blast crisis, (iii) good tolerability of the therapy avoiding severe side effects, (iv) rapid cytogenetic and molecular response with (v) the chance of deep molecular remission allowing eventual treatment discontinuation, and (vi) preservation of fertility.

First line therapy

Proof of CML diagnosis depends on the detection of the t(9;22) translocation by cytogenetic analysis, the juxtaposition of BCR and ABL by fluorescent in situ hybridization (FISH) or (in most cases preferred and fastest) by multiplex RT-PCR. Immediate start of therapy may be required in case of very high leukocyte counts with the risk of leukostasis. In such cases, transient therapy with hydroxyurea (40 mg/kg body weight) may be required, until the BCR-ABL test result is available. Treatment should be accompanied by urine alkalization (pH 6.4-6.8) with sodium hydrogen carbonate. Allopurinol should be avoided due to xanthin accumulation with risk of renal failure. After confirmation of the BCR-ABL fusion, TKI therapy should be commenced at full dose. Pretherapy with hydroxyurea dose should be tapered in parallel.

Choice of first line therapy depends on the treatment aims of the patient. Imatinib (400-800 mg/d), nilotinib (300 mg twice daily) and dasatinib (100 mg/d) are available and licenced. Chance to achieve early molecular response, major molecular response and deep molecular response is higher with nilotinib or dasatinib, but survival probability is almost identical compared to imatinib. Vascular, cardiac, pulmonary or metabolic

comorbidities and risk factors should be considered in the choice of initial therapy. Imatinib is frequently accompanied by fluid retention and muscle cramps, nilotinib by hyperglycemia and hypercholesterolemia and cardiovascular events, dasatinib by pleural effusions and thrombocytopenia.
In order to reduce treatment costs, generic imatinib might be used as first line therapy. This approach should be balanced against the risk of progression according to the individual risk profile and the chance to discontinue therapy in case of durable deep molecular response.
Early treatment phase is frequently accompanied by transient cytopenias, mostly in patients with splenomegaly. Treatment interruption is recommended in case of grade 3 or 4 neutro- or thrombocytopenia only. In case of liver toxicity, gradual dose adjustment might be considered.

Treatment after intolerance

Intolerance to first line therapy should prompt symptomatic therapy, dose adjustment and/or change of the TKI, if required. Some adverse events are transient, e.g. cytopenias, diarrhea, rash, liver function abnormalities, other should prompt immediate stop of therapy, e.g. vascular events on nilotinib therapy. Recommended dose of alternative therapy after TKI intolerance is identical to the dose of the respective drug as first line therapy.[6]

Treatment after resistance

In case of hematologic, cytogenetic, or molecular resistance or relapse according to ELN criteria, change of therapy should be considered. Check of compliance, check of pharmacokinetic interaction, cytogenetic and BCR-ABL1 mutation analysis is recommended and should guide the choice of the alternative drug. In chronic phase, nilotinib should be administered at a dose of 400 mg twice daily, dasatinib at 100 mg/d, bosutinib with 400 mg/d start dosis and dose increase to 500 mg/d if tolerated and ponatinib at 30 mg/d with dose adjustments to 15 or 45 mg/d according to tolerability and efficacy.[7-10]

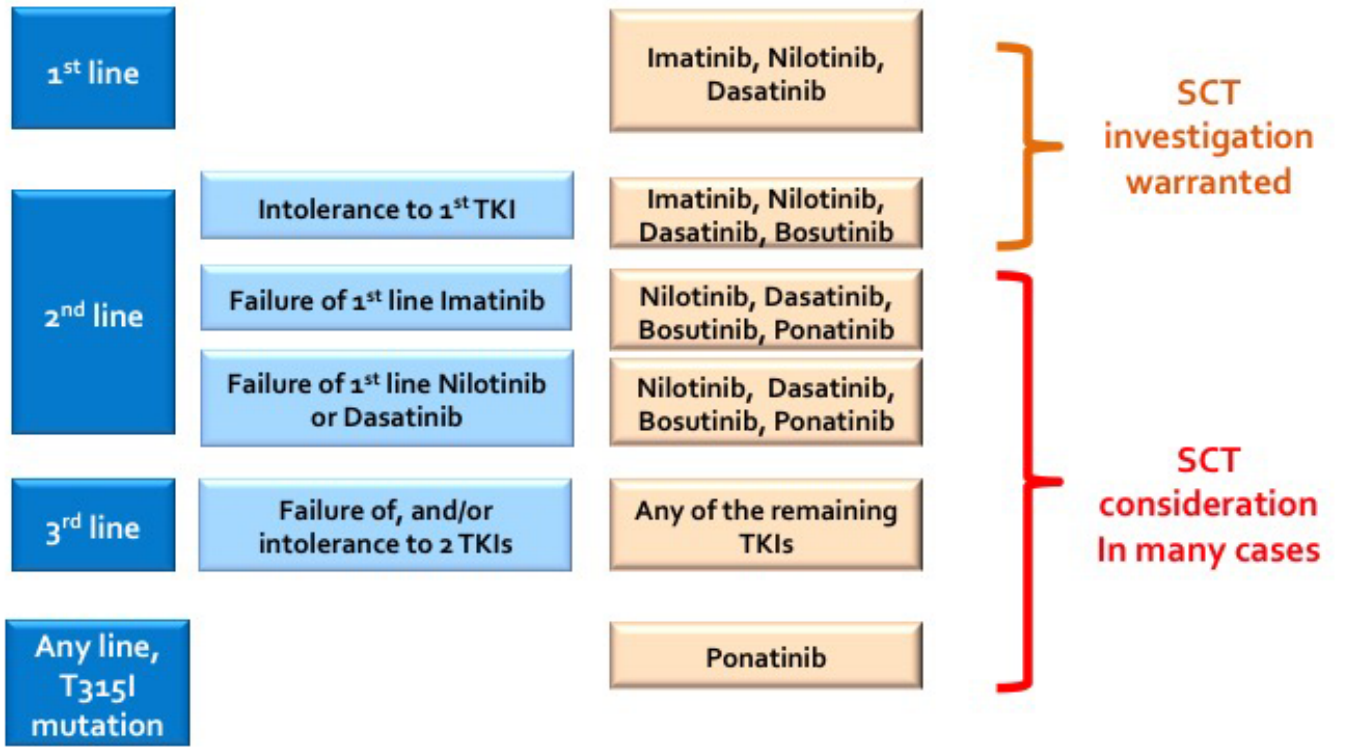

Figure 1. Recommended treatment options for CML patients in chronic phase. SCT = stem cell transplantation.

Chronic myeloid leukemia - Section 3

Allogeneic stem cell transplantation

Allogeneic stem cell transplantation remains an option for cure if available first- and second line drugs fail according to ELN criteria and the likelihood of response to salvage therapies is low. Since responses to second and later lines of therapy are limited and depend on the individual mutation profile, allogeneic stem cell transplantation should be considered on an individual basis for eligible patients.[11]

Treatment free remission

The likelihood of treatment free remission after stopping TKI therapy is 40-60% for patients in deep molecular remission after long term TKI therapy. Eligibility depends on the consolidated achievement of deep molecular remission determined in a standardized laboratory after long-standing TKI therapy. The impact of stopping strategies in CML will be enormous for patients, health care systems, and society at large. Studies are underway which will guide physicians in determining when it is safe and most promising to stop TKI therapy in CML patients. This will have a large economic impact on CML treatment. With the increasing prevalence of CML patients and the high costs of TKI treatment per year, stopping treatment in CML will result in a considerable and durable reduction of treatment costs world-wide. The important question of how to increase the proportion of patients is being addressed by treatment optimization studies.[12,13]

The impact of (pegylated) interferon alpha to improve the rate of patients without disease recurrent is currently being tested in a series of clinical trials using IFN in parallel with TKI therapy or as maintenance after TKI discontinuation.[14]

New treatment options

Combination trials investigate the impact of the inhibition of BCR-ABL1 independent pathways to target residual stem cells. The allosteric ABL1 inhibitor Asciminib (ABL001) demonstrates promising results in mono- and combination therapies and targets resistant disease including all known BCR-ABL1 ATP-binding site mutations.[15]

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Fertility preservation in patients with hematological malignancies

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Introduction

The advances in cancer therapy over the past decades have led to remarkable improvements in survival rates especially in childhood hematological cancer. This has resulted in a dramatic increase in the number of young adults experiencing late effects of treatment, among them premature ovarian insufficiency, testicular dysfunction and loss of fertility in both males and females are of major importance. It is highly important to understand the effects of different cancer treatment modalities on the gonads (ovaries and testes) and reproduction. At diagnosis, all patients should be consulted on the threat of compromising their fertility with planned cancer treatment. However, today only a small fraction of patients is actually referred to specialists to discuss the different options available to preserve fertility. This education module will focus on the effects of chemotherapy and radiotherapy on female and male reproductive system, in order to increase awareness among medical health care professionals and patients. The different options available to preserve fertility in females will be presented, advantages, method selections and outcome results will be discussed. In adult males, sperm freezing is well established, however there is no clinical solution for prepubertal boys at present. The current ongoing research for this population will be presented and discussed.

Learning goals

1. ***To provide current evidence for impaired testicular and ovarian function after chemotherapy and radiation.***
2. ***To present the effects of pelvic radiotherapy on the uterus.***
3. ***Fertility preservation options should be discussed with all young women having to face gonadotoxic treatment.***
4. ***To study and better understand the different available modalities to preserve fertility in women-oocyte, embryo and ovarian tissue cryopreservation. Semen cryopreservation for future use is effective in post-pubertal men, however, fertility preservation in prepubertal males remains experimental.***
5. ***To present the current research is focused on developing in vitro and in vivo strategies to preserve fertility for childhood cancer survivors.***

EUROPEAN HEMATOLOGY ASSOCIATION

Fertility preservation in patients with hematological malignancies - Section 1

The effects of chemotherapy and radiotherapy on reproduction

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Take-home messages

- More high quality research is required to provide the evidence for impaired testicular and ovarian function after chemotherapy and radiation.
- Conditioning treatments for BMT that include chemotherapy and or radiotherapy are likely to impair gonadal function irrespective of the age of the patient at treatment.
- Radiotherapy to a field that includes the uterus in females may impair uterine function with increased risk of miscarriage and preterm delivery.

Introduction

With increasing numbers of childhood cancer survivors, it has become important to understand the effects of successful treatment on the gonads (ovaries and testes) and reproduction. In the male treatment with chemotherapy, radiotherapy, or surgery that involves the testis can cause impaired spermatogenesis, testosterone deficiency, and physical sexual dysfunction in young adult cancer survivors. In the female, some patients are at risk of premature ovarian insufficiency (POI) as a direct consequence of successful cancer treatment and those that have been exposed to radiation to a field that includes the uterus, particularly in childhood, are at risk of miscarriage and preterm labor.[1-6]

The ovary

The human ovary establishes several million non-growing follicles (NGF) at around five months of gestational age, which is followed by a decline to the menopause when approximately 1,000 remain at an average age of 50-51 years. With approximately 450 ovulatory monthly cycles in the normal human reproductive lifespan, this progressive decline in NGF numbers is attributed to follicle death by apoptosis. Recently we have identified the first model of human ovarian reserve from conception to menopause that best fits the combined histological evidence. This model allows us to estimate the number of NGF present in the ovary at any given age (Figure 1) and suggests that 81% of the variance in NGF populations is due to age alone. We have also demonstrated than the rate of NGF recruitment increases from birth to age 14 years then declines with age until the menopause.[5]

Radiation and the ovary

The ovaries may be damaged by radiation to a field that includes the pelvis (e.g. total body irradiation (TBI), abdominal or pelvic irradiation) and the magnitude of the effect is related to the radiation dose, fractionation schedule and age at time of treatment.[7] The human oocyte is exquisitely sensitive to radiation, with an estimated LD_{50} (the lethal dose required to destroy 50% of oocytes) of less than 2 Gy. Using our understanding of the effect of radiotherapy on the human oocyte we can estimate the age at POI and the estimated sterilizing dose following any given dose of radiotherapy at any given age.[8] This will not only provide a useful basis for clinicians to provide accurate information when counselling women about fertility following treatment for childhood cancer, but also will help clinicians to select the patients at highest risk of POI for ovarian cryopreservation. Gonadotrophin insufficiency after cranial irradiation (>24 Gy in the treatment of brain tumors) will often be manifest as delayed onset of puberty or absent menses and can be treated by sex steroid replacement therapy. Gonadotrophin insufficiency can also be treated with gonadotrophin replacement which will restore fertility.

Chemotherapy and the ovary

Chemotherapy treatment in premenopausal women is associated with an increased risk of premature ovarian insufficiency

(POI) but the exact mechanism through which this occurs is uncertain.[8] Ovarian damage is drug and dose dependent and is related to age at time of treatment, with progressively smaller doses required to produce POI with increasing age. The age-related difference is most likely to be due to older women having a smaller primordial follicle reserve at the start of treatment compared to young women, so that loss from a smaller follicle pool is more likely to induce POI.[4]

Chemotherapy treatment that is gonadotoxic (e.g. alkylating agents) appears to have two distinct effects on ovarian function. A direct effect on the primordial follicle pool and an immediate effect on growing follicles. The first is immediate, occurring during treatment, and is characterized by amenorrhea and results from loss of the growing follicle population. However, provided that sufficient primordial follicles remain in the resting pool upon the cessation of treatment, the population of growing follicles will then be replenished, and menses resume. Depending on the extent of the loss of the primordial follicle pool, POI and amenorrhea may result at a later date.[9] Where there is only partial loss of primordial follicles, this longer-term effect may not manifest itself until years or even decades after treatment, when the patient then undergoes POI. If there is in addition a direct effect on the primordial follicle pool the patient undergoes POI manifest by permanent amenorrhea shortly after treatment.[10]

Radiation and the uterus

It is important to remember the uterus when discussing the effects of cancer treatment in young women. The uterus is at substantial risk of damage following radiation to a field that includes the pelvis, in a dose and age dependent manner. Uterine function may be affected by doses of 10-30 Gy probably as a result of a direct effect on the uterine vasculature and musculature elasticity. We have shown impaired uterine growth and blood flow after TBI (14.4Gy)[11] and a recent study has confirmed that survivors who received pelvic radiation are at increased risk of preterm delivery. Pregnancy in survivors of childhood cancer who have received radiotherapy to a field that includes the uterus should therefore be considered as high risk, essentially related to uterine dysfunction.

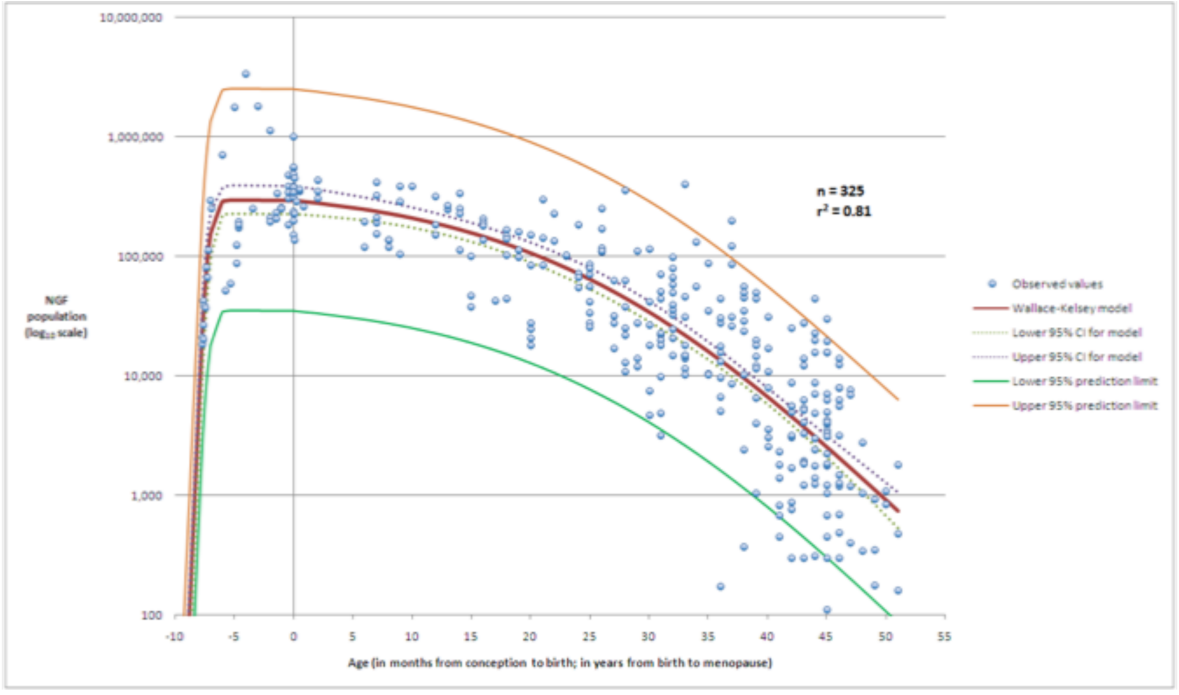

Figure 1. The best model for the establishment of the NGF population after conception, and the subsequent decline until age at menopause is described by an ADC model. The figure shows the dataset (n=325), the model, the 95% prediction limits of the model, and the 95% confidence interval for the model. The horizontal axis denotes age in months up to birth at age zero, and age in years from birth to 51 years.[6]

Fertility preservation in patients with hematological malignancies - Section 1

The testis

Normal physiology

The seminiferous epithelium of healthy infant and child testes consists of immature Sertoli cells and spermatogonia. Spermarche occurs at a median age of 13.4 years (range 11.7-15.3) at a time when median testicular size is 11.5 ml (range 4.7-19.6). The pre-pubertal testis is approximately 2 ml in volume. The onset of puberty begins with enlargement of the testis at approximately 11.4 years. The healthy adult testis volume is 15-25 ml. Azoospermia may be present if the volume of each adult testis is 10 ml or less in a patient treated in childhood. As the endocrine function of the testis (Leydig cell activity) is relatively independent of Sertoli cell function and spermatogenesis, spontaneous progression through puberty is not a guarantee of future fertility.[12]

Radiotherapy to a field that includes the testis

Radiation has adverse effects on gonadal function in all males irrespective of their pubertal stage at the time of treatment. The degree and persistence of the damage is dependent on the dose, the treatment field and the fractionation schedule. Spermatogenesis is susceptible to irreversible damage at very low doses of irradiation (>1.2 Gy). However, Leydig cells are more resistant to damage from radiation and is usually preserved in doses up 20 Gy in pre-pubertal boys and 30 Gy in sexually mature men.[13] Progression through puberty with normal serum testosterone is common, despite azoospermia or evidence of severe impairment to spermatogenesis. TBI as a conditioning regimen for stem cell transplantation causes azoospermia in approximately 80% of males.[14]

Chemotherapy and the testis

A recent systematic review[1] looked at the evidence for adverse effects of cyclophosphamide, chlormethine and procarbazine on spermatogenesis, and found that there was reasonable evidence for an increased risk of impaired spermatogenesis after treatment with busulfan and cyclophosphamide, or fludarabine and melphalan, hematopoietic stem-cell transplant (HSCT) conditioning, ifosfamide doses of more than 60 g/m^2. The risks of impaired spermatogenesis after treatment with cisplatin remain unclear, although a recent study suggests a significant detrimental effect on the chance of siring a pregnancy.[15] In this CCSS study of male survivors reduced likelihood of pregnancy was associated with upper tertile doses of cyclophosphamide (HR 0·60, 95% CI 0·51-0·71; p<0·0001), ifosfamide (0·42, 0·23-0·79; p=0·0069), procarbazine (0·30, 0·20-0·46; p<0·0001) and cisplatin (0·56, 0·39-0·82; p=0·0023).[15]

Fortunately, modern first line treatment of Hodgkin lymphoma in children and young people is unlikely to be sterilizing as most patients are no longer exposed to procarbazine and high cumulative doses of alkylating agents.

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Fertility preservation in patients with hematological malignancies - Section 1

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Fertility preservation in patients with hematological malignancies - Section 2

Fertility preservation in female patients

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Take-home messages

- Fertility preservation options should be discussed with all young women having to face gonadotoxic treatment.
- Oocyte cryopreservation can be proposed to post-pubertal patient who can afford a delay in the onset of chemotherapy (at least 10-12 days are necessary to allow time for ovarian stimulation and oocyte collection).
- Ovarian tissue cryopreservation can be proposed to prepubertal patients and all patients younger than 35 years of age, who cannot afford a delay in the onset of cancer treatment.

Introduction

The advances in cancer therapy over the past two decades have led to remarkable improvements in survival rates,[1] but treatments such as chemotherapy (especially alkylating agents), radiotherapy and/or surgery can induce premature ovarian insufficiency (POI) in some circumstances.[2] Fertility preservation (FP) is therefore a key challenge for these women. At diagnosis, all women affected by cancer should benefit from an informed consultation on the threat of compromising their fertility with planned cancer treatment. In case of total body irradiation, pelvic irradiation, bone marrow transplantation and aggressive chemotherapy with high dose of alkylating agents,[2-5] the risk is considered to be very high. However, only a small fraction of patients are actually referred to specialists to discuss FP prior to cancer treatments. The decision-making process is especially problematic since the long-term effects of cancer treatment have not been fully elucidated.[2-4] The prevalence of subfertility is nevertheless known to be increased, even when ovarian function is maintained.[5] The main issue is that health care workers are unfamiliar with the rapid advances taking place in FP research and their implementation in clinical practice.[2,6] Selection criteria need to be available not only to endocrinologists and gynecologists in reproductive medicine, but also pediatricians and oncologists. Moreover, informed discussion on a patient's fertility prognosis can be a positive experience, even if an FP procedure is not indicated (low risk) or possible.

Current state of the art

GnRH agonists for fertility preservation

According to ASCO[7] and ASRM[8] recommendations, evidence supporting the effectiveness of gonadotropin-releasing hormone (GnRH) agonists for FP is currently insufficient, although it is recognized that these agents might yield other medical benefits, such as reduced vaginal bleeding when patients have low platelet counts as a result of chemotherapy.
Reviews on the topic remain contentious, even if a randomized controlled trial (RCT) found that the ovaries are protected from depletion by administration of GnRH agonist in young women receiving cyclophosphamide.[9] As stressed by the authors themselves, the markers of ovarian reserve [like anti-Müllerian hormone (AMH) and antral follicle count (AFC)] were not evaluated. Moreover, the real benefits should not only be evaluated in terms of recovery of menses, but in terms of ongoing pregnancy and live birth rates.
A very recent RCT, clearly demonstrated the absence of any beneficial effect of GnRH agonists on future pregnancy rates.[10] Until definitive proof of efficacy has been clearly established, other FP approaches should be offered alongside GnRH agonist therapy.

Oocyte or embryo cryopreservation

Embryo cryopreservation is generally offered as the primary method of FP if the woman is postpubertal and if sperm is available. Nevertheless, we have to keep in mind that cryopreserved embryos are the joint property of the woman and her male partner.[2] Therefore, some centers propose oocyte cryopreservation instead of embryo cryopreservation, at least in the

context of FP. This was possible thanks to the excellent survival rates of oocytes after vitrification and warming. In the field of FP, oocyte cryopreservation gives women the possibility of reproductive autonomy. It should nevertheless be pointed out that, in young women, 15 vitrified oocytes are required to achieve a cumulative live birth rate of 85%, and this live birth rate decreases dramatically if the patient is over 36 years of age.[11] For oocyte vitrification in the case of cancer women, chemotherapy usually needs to be delayed by at least 10-12 days[6,11] to allow time for ovarian stimulation and oocyte collection. Also, specific ovarian stimulation protocols are used according to the steroid sensitivity of the cancer.[2] Importantly, the patient should be informed that there is no guarantee that good-quality oocytes will be collected.

Ovarian tissue cryopreservation

Ovarian tissue cryopreservation (OTC) is the only fertility preservation option available for prepubertal girls and women who cannot delay the start of chemotherapy.[2-4,12] Indications for OTC in our department are shown in Figure 1 (series of 600 patients). Hematologic malignancies represent the most frequent indication for OTC, as these represent 36% of all indications, and 48% in the subgroup of patients younger than 18 years of age**.** An age less than 35 years, a realistic chance of surviving for 5 years, and at least a 50% risk of POI are established selection criteria.[2,12] This risk of POI is directly related to the intensity of treatment received. The real concern is that it is impossible to predict exactly who will develop POI after aggressive chemotherapy.[2,4] Moreover, treatment protocols sometimes need to be adapted or become more aggressive, e.g. in case of relapse, and patient may change risk category of POI.[4,12]

Ovarian tissue reimplantation in an orthotopic site (namely inside the pelvic cavity, either to the ovarian medulla or inside a peritoneal window)[13] leads to restoration of ovarian endocrine activity with occurring of menses in more than 95% of cases after transplantation. The mean duration of ovarian function is 4-5 years, but this can vary according to follicular density at the time of cryopreservation, which depends on the patient's age at the time of cryopreservation and if she received or not already some chemotherapy before.

Taking into account all published series to date, the number of live births has now (January 2017) reached more than 110. An evaluation including patients from 5 renowned centers (n=111), yielded a pregnancy rate of 29% and live birth rate of 23%.[14] These rates were subsequently confirmed by other series with live birth rates of at least 30%.[15,16]

Transplanting ovarian tissue to heterotopic sites remains rather questionable, however, and only one pregnancy has been reported following this procedure.[17]

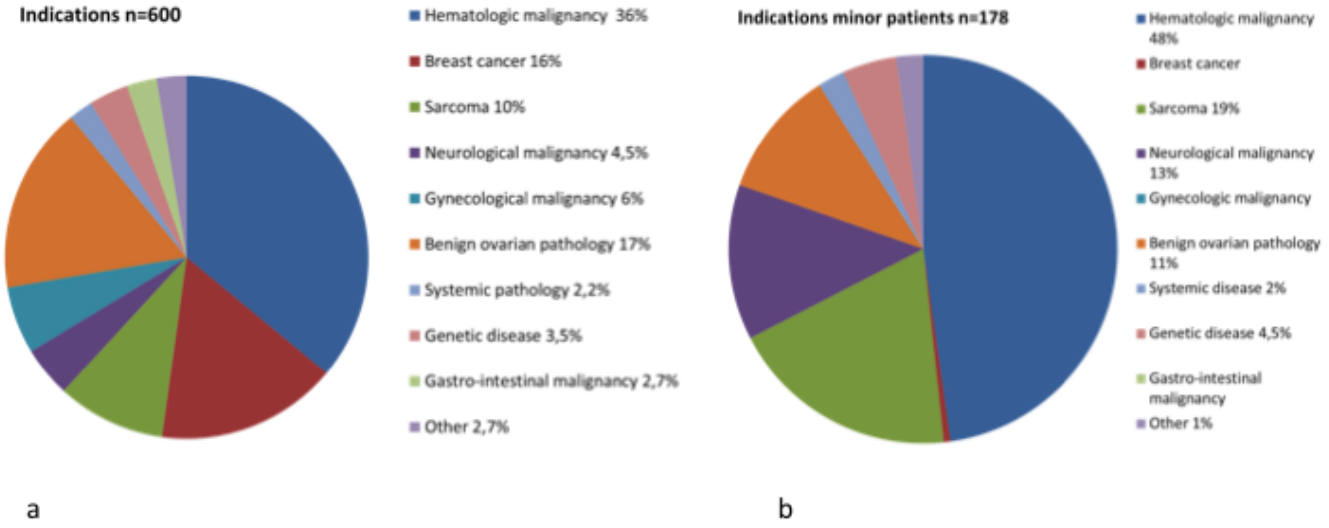

Figure 1. Indications for ovarian tissue cryopreservation in 600 women in our ovarian tissue cryobank (a) and in the subgroup of patients younger than 18 years of age (b).

Combined technique: a way to improve FP chances

A combination of OTC followed immediately by ovarian stimulation and ovum pick-up (with a view to vitrifying mature oocytes) does not impair oocyte number or quality, and could actually increase the efficacy of the procedure by giving young patients with cancer more chances of success.[18]

Future perspectives

A serious concern that must be addressed is the risk of reimplanting malignant cells together with the grafted tissue, especially in patients with leukemia,[19] which is the most common hematological cancer in women under 20 years of age. The risk is particularly high in women with acute leukemia and cannot be completely eliminated, even if the biopsy destined for cryopreservation is taken from patients in complete remission.[20]

One alternative to avoid reimplanting malignant cells is to obtain mature oocytes by means of the so-called transplantable artificial ovary. Isolation of primordial follicles from cryopreserved ovarian tissue and their transfer onto a scaffold to create this artificial organ will serve to eliminate the risk of transmission of malignant cells.[21] This option is also applicable to leukemic patients.[22] Another option is to obtain *in vitro* follicular growth through a dynamic multistep culture system[23] before fertilizing the oocytes *in vitro*. Both options are still experimental.

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Fertility preservation in patients with hematological malignancies - Section 3

Fertility preservation in pre-pubertal and adult males

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Take-home messages

- Semen cryopreservation for future use in ART is an effective means of preserving fertility in post-pubertal men at risk of infertility.
- Fertility preservation in prepubertal males remains experimental and currently there are no established clinical options to preserve fertility in this patient group.
- Current research is focused on developing *in vitro* and *in vivo* strategies to preserve fertility for childhood cancer survivors.

Introduction

Childhood cancer rates have increased dramatically (29% since the 1970s) over recent decades and currently 1 in 500 children in the UK will develop cancer.[1] Improved long-term survival (>80% 5 year survival) has resulted in a dramatic increase in the number of young adults experiencing late effects of treatment.[2] One of the most frequent effects is infertility which occurs in the majority of males receiving high-dose alkylating agents, commonly used in childhood cancer.[3] Unlike the situation in females and for adult men, there is currently no prospect of preserving fertility in prepubertal males at risk of infertility because mature gametes are not present until puberty (Table 1).[3] This short review will summarise the current state of the art for fertility preservation in males at risk of infertility as a result of cancer treatment and highlight progress towards potential future clinical approaches for prepubertal boys.

Current state of the art

For fertility preservation in adolescent and adult males at risk of infertility due to cancer treatment there is the established option of semen cryopreservation for future use for insemination, IVF and ICSI.[4] This approach is widely used, albeit with inter-center variation in provision. Despite the fact that in many cancer patients there is a decline in semen parameters, the success rates with *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are similar to those of standard IVF for infertile couples.[4] However, for many patients it is not possible to obtain a semen sample for a variety of reasons. These include religious, cultural and psychosocial factors and many adolescents and young adults face difficulties producing a semen sample as a result of emotional immaturity.[3] For these patients, it may be possible to perform surgical testicular sperm extraction (TESE) for cryopreservation.[4]

For prepubertal patients that have not yet achieved spermatogenesis, obtaining sperm for storage is not possible.[3] Currently, there are no established clinical options to preserve fertility in these patients. Potential approaches might involve protecting the gonad in-situ either by modifying treatment regimens[5] or alternatively by co-administering treatments that may prevent the damage.[6] A number of promising rodent studies have shown that hormonal (e.g. GnRH antagonists, sex steroids) manipulation can protect or restore of fertility; however, the limited evidence in humans does not support this approach for clinical applications.[5] Recent studies involving administration of granulocyte colony-stimulating factor (G-CSF) have demonstrated protection of fertility in animal models, including Rhesus monkeys; however, this approach has not yet been translated into humans.[6]

Over the last decade a number of centers have begun to cryopreserve testicular tissue from prepubertal boys prior to gonadotoxic cancer treatments.[4] This approach remains experimental in the absence of any clinical applications for the cryopreserved tissue.[7] Testicular tissue cryopreservation requires a surgical biopsy, ideally to coincide with a planned theatre procedure. Tissue may be stored according to a number of freezing protocols that have been described for prepubertal testis tissue which demonstrate viability of the spermatogonial stem cells (SSC) after thawing.[8-10] However, in the absence of a proven strategy to restore fertility using this cryopreserved tissue, the true functional capacity of the SSC within the

stored tissue is unknown.

Several approaches have been proposed for subsequent use of cryopreserved prepubertal testicular tissue to generate mature gametes and/or restore fertility.[5] This includes *in vitro* culture of testicular tissue and (xeno) transplantation of testicular tissue or SSCs. *In vitro* culture of neonatal mouse testis using a soft agar system has been shown to result in the generation of functional sperm from the spermatogonia, resulting in the generation of progeny following ICSI.[11] *In vitro* generation of sperm from human prepubertal testicular tissue has not been reported. The safety of *in vitro* generated spermatozoa (e.g. epigenetic stability) is an important consideration for *ex vivo* strategies and therefore transplantation methods may offer an alternative approach. Transplantation of SSC from neonatal mouse testis directly into the seminiferous tubules of a germ-cell ablated mouse has been successful in generating functional gametes that can produce progeny.[12] SSC transplantation has also been successful in Rhesus monkeys for generating sperm that are capable of fertilization using ICSI.[13] Similar to SSC transplantation, testicular tissue (xeno) grafting has also resulted in the generation of functional gametes in rodents[14] and monkeys;[15] however, for prepubertal human tissue xenografts, germ cell differentiation did not proceed beyond spermatocytes.[16] Both SSC transplants and testis tissue transplants have the potential to re-introduce malignancy, which may be a particular issue for hematological malignancies.[17] Therefore, development of these strategies for future clinical use would require robust systems to ensure this cannot occur.

Future perspectives

Over recent decades, significant progress has been made towards developing strategies to preserve fertility in young people treated for cancer. For prepubertal boys facing gonadotoxic treatments there are no clinical options to preserve fertility. The focus for future research should be on developing strategies to protect the gonad in-situ and also on techniques to generate viable gametes from cryopreserved testicular tissue. Generation of functional germ cells from pluripotent stem cells has recently been described[18] although this approach remains in its infancy. For any future strategy that is developed for clinical use, particular attention must be paid to avoidance of re-introduction of malignancy, ensuring the genetic and epigenetic stability of the germ cells, and careful follow-up of offspring.

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Table 1. Options for fertility preservation in males with cancer.

		Prepubertal	Adolescent	Adults
Established	Semen cryopreservation	×	✓*	✓
	Testicular sperm extraction	×	✓*	✓
Experimental	Protection of the *in situ* gonad	✓	✓	✓
	SSC transplantation	✓	✓	–
	Testicular tissue transplantation	✓	✓	–
	In vitro maturation	✓	✓	–
	Pluripotent stem cells	✓	✓	–

**Option for pubertal individuals.*

Fertility preservation in patients with hematological malignancies - Section 3

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Use of pluripotent mouse embryonic stem cells to generate germline stem cell-like cells capable of contributing to spermatogenesis.

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Hereditary hematological disorders

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Introduction

The development of next generation sequencing (NGS) for genetic studies is making these diagnostic tests broadly available.Through this approach, we are able to characterize genetically pathologies with high diagnostic difficulty due to the heterogeneity of the clinical presentation. Dr. A Shimamura in her excellent manuscript will address the diagnosis of a group of diseases characterized by its great heterogenicity: congenital bone marrow failure and myelodysplastic syndromes.These approaches also allow studies in patients with suspected risk for familiar cancer. Dr. C. Kratz reviews this group of diseases. Through the genetic confirmation we can better understand the risks, the age at diagnosis, or other clinical or biological characteristics of the subject that can help to establish the prognosis.However, despite these advances, there is still a high percentage of cases in which the diagnosis is not reached, since these approaches only allow us to diagnose those diseases in which the genes involved have been previously described. In the third talk, Professor Ouwehand will explain how to face these cases with an interesting approach based on the clinical and laboratory phenotypic information coded with Human Phenotype Ontology terms, searching for similar genetic bases after whole genome sequencing.

Learning goals

1. ***Genetic diagnosis for congenital hematological diseases is now broadly available based on NGS.***
2. ***Genetics diagnosis is mandatory for patients with congenital hematological diseases, since only by this approach, the diagnosis can be undoubtedly stablished, the best treatment can be offered, and the prognosis can be well defined.***
3. ***Exome sequencing should be performed in those patients without diagnosis after NGS. The use of Human Phenotype Ontology will help to classify these patients, grouped them, and guide the diagnosis.***
4. ***The NHS 100 000 Genomes Project should be an example of platforms that will allow the study of rare inherited hematological diseases in a feasible and scalable manner.***

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Syndromes predisposing to hematological malignancies

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Take-home messages

- Disruption of several biologic pathways leads to an increased leukemia risk.
- The underlying germline defects overlap with genes/pathways disrupted in sporadic leukemia.
- Patients with various leukemia predisposition syndromes differ substantially regarding cancer risk, spectrum, age of leukemia onset and other clinical or biological features.

Introduction

Genetic conditions predisposing to myeloid and lymphoblastic neoplasms can be grouped based on the underlying defect into seven distinct groups: (1) Li-Fraumeni syndrome; (2) transcription factor defects; (3) inherited bone marrow failure syndromes (IBMFS) / DNA repair defects / immunodeficiency disorders; (4) chromosomal anomalies; (5) Rasopathies; (6) defects of epigenetic regulation; and (7) other (new) entities. As illustrated in Figure 1, syndromes vary in terms of (*i*) hematologic cancer risk; (*ii*) age of onset of hematologic cancer; (*iii*) hematologic cancer spectrum (AML, ALL, MDS, MPN, HL/NHL); (*vi*) risk of developing non-hematologic cancers; (*v*) presence and severity of physical anomalies; (*vi*) benign hematologic anomalies; (*vii*) immunodeficiency; and (*viii*) somatic mutation signature. All syndromes have in common that affected individuals require special attention in the care for their malignant and non-malignant health related problems and psychosocial needs.

Current state of the art

Li-Fraumeni syndrome (LFS)

LFS is an aggressive cancer predisposition syndrome with a broad cancer spectrum caused by germline mutations of the tumor suppressor gene *TP53*. In children with low-hypodiploid ALL, ~40% of patients harbor a *TP53* germline mutation and *TP53* germline defects are associated with relapsed ALL. LFS patients are at increased risk of therapy related-MDS/AML, especially after being treated with alkylating drugs.[1,2]

Transcription factor defects

The same hematopoitic transtriction factors that are somatically altered in multiple hematopoietic neoplasms can be mutated in the germline resulting in leukemia predisposition. The domintantly inherited *PAX5*^{G183S} mutation has been described in familial precursor B-cell ALL. *IKZF1* germline defects occur in patients with immunodeficiency and ALL. Germline mutations of *ETV6* lead to an autosomal dominant syndrome with thrombocytopenia, red cell macrocytosis and cancer predisposition (precursor B-cell ALL, but also other leukemia and tumor types). *RUNX1* mutations cause a familial platelet disorder with associated myeloid malignancy. T-cell ALL and other cancers occur less frequently. *GATA2*-associated predisposition to MDS/AML is an autosomal dominant condition that can be associated with immune deficiency (MonoMAC syndrome) or lymphedema (Emberger syndrome). *GATA2* mutations are common in primary pediatric MDS, especially in adolescents with MDS and -7. ~50% of patients with *CEBPA*-associated predisposition to AML develop leukemia.[3-8]

IBMFS / DNA-repair defects / immunodeficiency

Constitutional mismatch repair deficiency (CMMRD) is caused by bi-allelic germline mutations of *MLH1*, *MSH2*, *MSH6*, or *PMS2* and leads to a highly penetrant, early onset predisposition to brain, gastrointestinal, and hematopoietic cancers (T-NHL most common). Fanconi anemia (FA) is a mainly recessive disorder with at least 21 associated DNA-repair genes (*FANCA-V*). The gene products repair DNA interstrand cross-links and interact with DNA damage response pathways. Dyskeratosis congenita (DC) is classically characterized by nail dystrophy, abnormal skin pigmentation, and oral leukoplakia and caused by mutations in telomere biology genes (X-linked: *DKC1*; autosomal dominant: *TERC*, *TINF2*;

Hereditary hematological disorders - Section 1

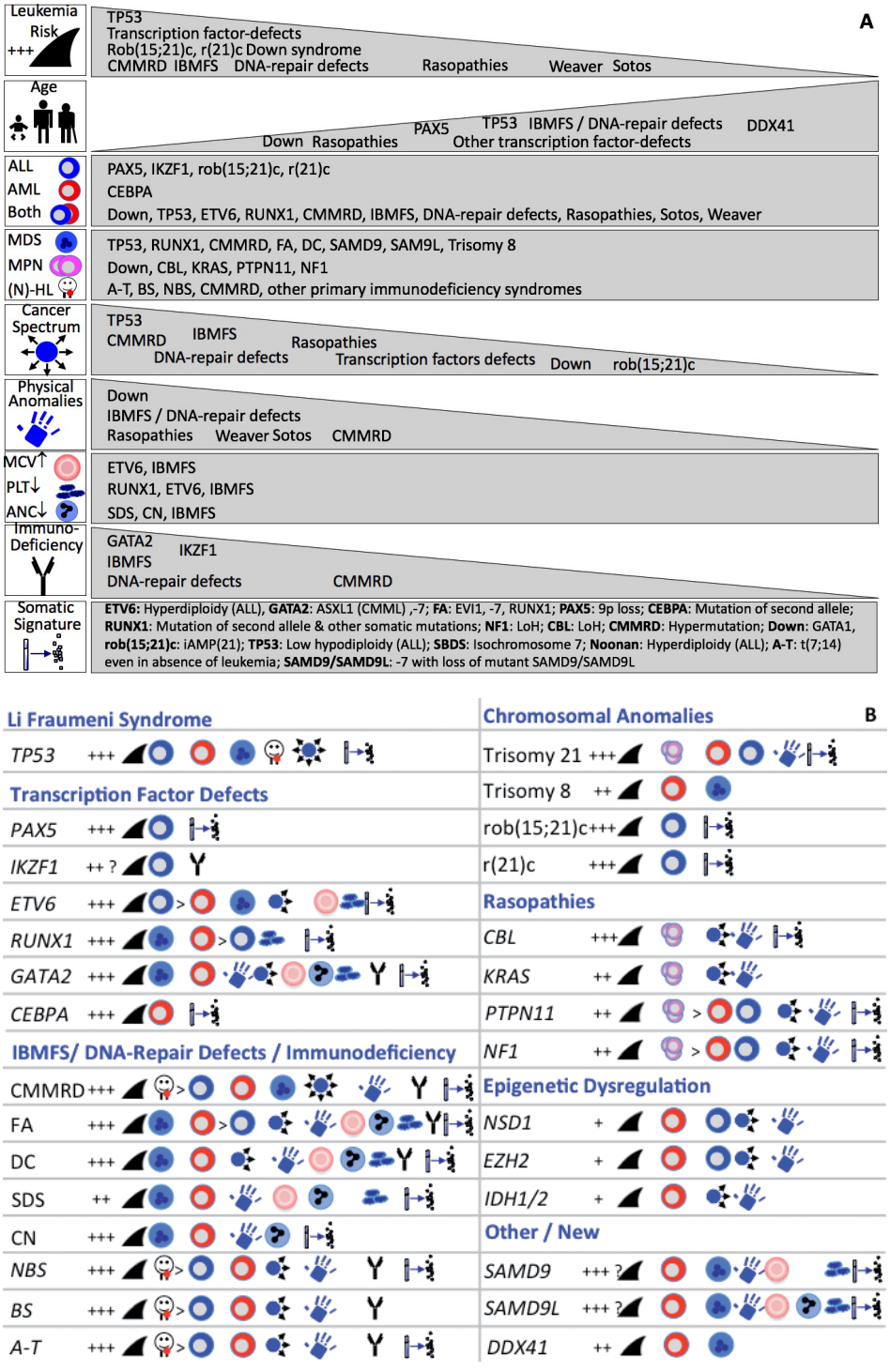

Figure 1. Syndromes predisposing to hematologic malignancies differ regarding different clinical and biological features. A, Overview, and B, more detailed information given for different syndromes.

autosomal recessive: *CTC1, NHP2, NOP10, PARN*, *WRAP53*; dominant or recessive: *ACD, RTEL1*, *TERT*). Patients with FA and DC physical occasionally lack obvious physical anomalies. Both conditions are associated with bone marrow failure, predisposition to MDS/AML and solid tumors (mainly squamous cell carcinoma). An increased MDS/AML risk is also observed in patients with Shwachman-Diamond syndrome (autosomal recessive, *SBDS*) and congenital neutropenia (autosomal dominant: *ELANE*, *GFI1*, autosomal recessive: *HAX1*, *G6PC3*, *VPS45A*, *CSF3R*).[9-12]

Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder caused by *NBN* (nibrin) mutations. Nibrin belongs to the MRE11/RAD50 double stranded break repair complex. Patients show immunodeficiency and a 'bird-like' face. T- and B-NHL are the most common cancer types. Bloom's syndrome (BS) is an autosomal recessive disorder caused by *BLM* mutations (BLM is a RECQ family member DNA helicase). BS patients have severe anomalies and develop a range of neoplasms, including lymphoma and leukemia. Ataxia telangiectasia (A-T) is an autosomal recessive condition caused by *ATM* mutations (ataxia-telangiectasia mutated, cell cycle checkpoint kinase and regulator of TP53, BRCA1, CHEK2, and NBN, important in response to DNA damage). Patients show progressive cerebellar ataxia, conjunctival telangiectasias, immunodeficiency, and high lymphoma and leukemia risk.[13-15]

Chromosomal anomalies

An increased leukemia risk is observed in individuals with Down syndrome (DS) and the rare constitutional trisomy 8 mosaicism (CT8M). Individuals with the constitutional Robertsonian translocation rob(15;21)(q10;q10)c, have a 2,700-fold increased risk of developing ALL. Individuals with constitutional ring chromosomes involving chromosome 21, r(21)c, are also predisposed ALL.[16]

Rasopathies

Patients with *CBL*-syndrome (*CBL*), Noonan syndrome (*PTPN11*, *KRAS*) and Neurofibromatosis 1 (*NF1*) are at increased risk of leukemia, especially juvenile myelomonocytic leukemia. In patients with *CBL*-syndrome the clonal disease may take a benign course. In patients with Noonan syndrome due to a germline mutation of *PTPN11* or *KRAS* a JMML-like picture can be caused by a transient polyclonal proliferative disorder.[17]

Epigenetic dysregulation

Rare cases of leukemia have been described in patients with Weaver (*EZH2*), Sotos (*NSD1*) and Ollier disease/Maffuci syndrome (*IDH1/2* mosaic mutations), suggesting that mutations of these genes represent moderate leukemia risk alleles.[18]

Recently discovered leukemia predisposition syndromes

Ataxia-pancytopenia syndrome (APS) and myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenotypes and enteropathy (MIRAGE) syndrome are caused by activating mutations of *SAMD9L* and *SAMD9* (involved in endosome fusion), respectively. Interestingly, -7 occuring in these rare patients leads to loss of the mutant allele on 7q21. Germline mutations in the DEAD/H-box helicase gene *DDX41* have been recenty identified in adult familial AML.[19,20]

Future perspectives

As new leukemia and lymphoma predisposing genes are continuously being discovered it is important to precisely study the natural history and phenotypic spectrum of each syndrome as well as the implications for cancer prevention and therapy. This is particularly important because some of the conditions discussed in this overview may be associated with significant side effects such as therapy related cancers in patients with LFS. Increasing awareness for these conditions, early diagnosis and - where appropriate - enrolment in surveillance programs and clinical therapeutic trials may improve the lives of affected individuals in the future. Studying these rare conditions has broad implications for cancer biology in general.

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Hereditary hematological disorders - Section 1

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Diagnosis of inherited bone marrow failure and myelodysplastic syndromes

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Take-home messages

- Diagnosis of inherited bone marrow failure (BMF) and inherited myelodysplastic syndromes (MDS) informs surveillance strategies and treatment decisions
- Classical clinical stigmata of these inherited syndromes may be absent
- Understand the indications and caveats of genetic screening strategies for the diagnosis of patients with bone marrow failure.

Introduction

Accurate and timely diagnosis of inherited bone marrow failure syndromes (BMF) and inherited myelodysplastic syndromes (MDS) is essential to guide clinical management. The inherited BMF/MDS syndromes are characterized by an increased risk of progression to leukemia, typically acute myelogenous leukemia.Early diagnosis and surveillance allows initiation of a hematopoietic stem cell transplant (HSCT) prior to the onset of leukemia thus avoiding the need for intensive leukemia-directed therapies for remission induction prior to transplant and reduces the risk of subsequent leukemia relapse or refractory disease. Many of the BMF/MDS syndromes are associated with an increased risk of treatment-related toxicities which may arise from impaired DNA repair, hyperactive stress responses, or organ co-morbidities.Early diagnosis therefore allows tailoring of transplant with reduced intensity conditioning regimens to avoid excessive toxicity. Diagnosis of an inherited BMF/MDS disorder also informs treatment of marrow failure since these syndromes respond poorly or transiently to immunosuppressive therapies used for acquired aplastic anemia.Diagnosis of a genetic BMF/MDS disorder also allows testing of family members to avoid inadvertently choosing a related stem cell donor afflicted with the same disorder.The identification of increasing numbers of genetic BMF/MDS disorders together with the availability of multiplexed genetic testing has expanded our diagnostic approach for BMF and MDS.Recent advances in the diagnostic evaluation for inherited BMF/MDS will be discussed.The benefits and caveats of genetic testing will be explored.

Current state of the art

Distinguishing inherited from acquired bone marrow failure/myelodysplastic syndrome is often challenging. Clinical history and the physical exam provide important clues to the diagnosis of an underlying genetic BMF/MDS disorder. Many of the BMF/MDS disorders initially come to medical attention with characteristic stigmata such as congenital anomalies, dysmorphic features, short stature, poor growth or additional suggestive clinical features.Unexplained red cell macrocytosis or elevated fetal hemoglobin may hint at a genetic BMF disorder. Family history provides another important clue to an underlying genetic BMF/MDS disorder. Suspicion is aroused by a family history of unexplained cytopenias, red cell macrocytosis, cancers presenting in multiple family members, cancers presenting at an unusually young age, excessive toxicity with chemotherapy/radiation, poor stem cell yield after marrow harvest or stem cell mobilization[1], or other characteristic stigmata of inherited BMF/MDS syndromes.See presentation by Dr. Kratz for additional discussion of the clinical features of inherited BMF/MDS syndromes which should arouse clinical suspicion and prompt diagnostic testing.

In the past, only those patients suspected to have a genetic disorder based on clinical stigmata or family history were referred for directed testing of the suspected gene. However, the clinical phenotypes of these disorders are now recognized to vary widely and a significant subset of patients may lack the clinical findings classically associated with these disorders. Indeed, BMF or MDS might be the sole presenting finding for these genetic disorders. Many of these syndromes share overlapping features leading to erroneous diagnosis. Accurate diagnosis is critical because many syndromes are associated with specific treatment considerations, additional organ sys-

tem co-morbidities, or solid tumor risks that affect medical management. The family history may fail to flag an inherited disorder due to a *de novo* constitutional mutation arising in the proband, parental gonadal mosaicism, incomplete penetrance or variable expressivity, or latency to cancer development which has yet to be manifested in family members. A comprehensive and current history of all family members is often unavailable.

Laboratory screening is also helpful in the assessment of BMF/MDS. Increased chromosomal breakage with mitomycin C or diepoxybutane is the diagnostic hallmark of Fanconi anemia.[2] Shortened telomere lengths in multiple lymphocyte subsets raises suspicion for a telomere biology disorder.[3] Low serum trypsinogen or pancreatic isoamylase for age is characteristic of Shwachman Diamond syndrome.[4] Immunologic abnormalities may be seen with *GATA2* disorders, dyskeratosis congenita, or Shwachman Diamond syndrome.[5] Monocytopenia may be seen with GATA2 disorders.[5] Bone marrow clonal cytogenetic abnormalities involving deletion of 20q or isochromosome 7 commonly arise in Shwachman Diamond syndrome.[6,7]

To assess for cryptic presentations of genetic BMF/MDS disorders, a cohort of 71 pediatric and young adult patients with BMF or MDS who remained diagnostically undefined after an initial medical and laboratory evaluation were screened for mutations in 85 BMF/MDS genes.[8] Thirty-two patients, including 6 of the 13 adults, had a positive family history.Causative germline mutations were identified in eight out of these 71 patients (11%) with idiopathic BMF/MDS.[8] All eight of these patients lacked classical clinical stigmata or laboratory findings of these syndromes and only four had a family history suggestive of inherited disease. A subsequent retrospective targeted BMF/MDS genetic screen of samples banked from 98 children and young adults transplanted for aplastic anemia or MDS identified causative mutations in 5.1% (5/98) of aplastic anemia patients and 13.6% (15/110) of MDS patients.[9] Family history or physical examination failed to reliably predict the presence of germline BMF/MDS mutations. Similar findings were reported by Ghemlas *et al*., who identified causative genes in 15 of 83 patients (18%) with idiopathic unclassifiable bone marrow failure screened with targeted sequencing.[10] A study of patients with inherited bone marrow failure from Japan identified causative mutations in 53 out of 121 (44%) patients screened by targeted sequencing and 68 out of 250 patients (27%) screened by whole exome sequencing.[11] The initial clinical diagnosis for a subset of patients was re-classified to a different disorder after genetic testing.[8,10,11] These studies demonstrated that although any single specific BMF/MDS genetic disorder is rare, genetic BMF/MDS disorders in aggregate affect a significant subset of patients presenting with BMF/MDS.

Referral to a center with expertise in the diagnosis of inherited BMF/MDS syndromes is recommended. Navigation of the rapidly growing number of available genetic testing options requires detailed knowledge of the limitations of the specific test ordered.[12,13] Gene panels vary widely with respect to genes included, coverage of a specific gene, detection of copy number

Table 1. Diagnostic evaluation for inherited bone marrow failure (BMF)/MDS.*

Personal history
Cytopenias
Short stature
Congenital anomalies
Other features of inherited BMF/MDS syndromes[12,17]
Excessive treatment-related morbidity (TRM) with cancer treatment/hematopoietic stem cell transplant (HSCT)
Family history
Cytopenias
Congenital anomalies
Other features of inherited BMF/MDS syndromes[12, 17]
Cancers at atypically young age
Multiple first or second-degree relatives with malignancy
Excessive TRM with cancer treatment/HSCT
Physical exam/imaging studies
Short stature
Failure to thrive
Dysmorphologies
Congenital anomalies
Other features of inherited BMF/MDS syndromes[12, 17]
Laboratory testing
Cytopenias
Elevated red cell MCV (mean cell volume)
Elevated fetal hemoglobin
Low Trypsinogen, Pancreatic isoamylase
Elevated erythrocyte adenosine deaminase
Low immunoglobulin levels
Abnormal lymphocyte subsets (often B cell lymphopenia)
Other clinically directed testing
Functional testing
Telomere length
Chromosomal breakage for Fanconi anemia
Germline genetic testing
Single gene Sanger sequencing
Targeted gene panels
Whole exome sequencing

**This is a general schematic outline that may be tailored as clinically indicated.*

variants, coverage of regulatory non-coding regions. Whole exome sequencing may fail to cover specific regions of interest. Importantly, the inability to identify a causative gene mutation does not rule out an inherited marrow failure disorder. Patients may be diagnosed with an inherited marrow failure disorder based on clinical diagnostic criteria for the known syndromes or based on a family history of a familial BMF/MDS disorder. Therefore, a diagnostic approach considering both clinical and genetic diagnostic criteria is essential.

Future perspective

While genomic testing is a powerful addition to the diagnostic armamentarium for inherited BMF/MDS, this remarkable advance also brings additional challenges.[12-16] Pathologic damaging mutations must be distinguished from benign variants or polymorphisms.Variants reported in the literature may lack rigorous assessment of pathogenicity. Development of functional assays to test variants and access to such functional analysis as part of clinical testing would complement the rapid advances in genetic testing.There is an urgent need for centralized database(s) of all identified BMF/MDS genes and their variants with expert annotation regarding the evidence for disease causation. Research is needed to investigate the significant population of patients with familial BMF/MDS who remain genetically undefined. Referral to a center with expertise in the diagnostic evaluation and medical management of these complex patients is recommended.

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These papers provide evidence to support broad genomic testing to identify cryptic presentations of inherited BMF/MDS and show that constitutional genetic causes are found in a significant subset of patients with BMF/MDS.

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Hereditary hematological disorders - Section 3

The 100,000 Genomes Project

Willem H. Ouwehand on behalf of the NIHR BioResource – Rare Diseases

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Take-home messages

- The NHS 100,000 Genomes Project shows that the clinical application of whole genome sequencing for rare inherited hematological diseases is feasible and scalable.
- Clinical and laboratory phenotype information from patients with inherited hematological diseases should be coded with Human Phenotype Ontology (HPO) terms.
- Patients with inherited hematological diseases and their close relatives should be invited to consent on the wide sharing of their phenotype and genotype data via 'safe haven' models.

The 100,000 Genomes Project

The reduction in sequencing costs has enabled initiatives like the 1000 Genomes,[1] UK10K[2] and more recently the 100,000 Genomes Projects (100KGP).[3] The 100KGP aims to achieve analysis by whole genome sequencing (WGS) of the DNA samples of 100,000 NHS patients (Figure 1). The key objective is to establish WGS as standard care in the domains of infection, cancer and rare diseases. The rare diseases element commenced in 2013 and so far samples from just over 36,000 individuals have been analyzed by WGS. Sequencing services are provided by Illumina Cambridge Ltd and are to clinically accredited standard. For the pilot phase for rare diseases, which comprised the first 13,000 DNA samples the sequencing results were transferred to the High Performance Compute Service at the University of Cambridge. For the 100KGP main phase a dedicated data center has been commissioned by Genomics England Ltd (GEL). GEL is a not-for-profit organization entirely owned by the Department of Health and tasked to coordinate the delivery of the 100KGP.

Rare diseases

Twelve rare disease projects were initiated and patients and close relatives were enrolled using a single NIHR BioResource participant information and consent leaflet. The allocated WGS capacity ranged from 1,250 samples/project for five large projects to hundreds of samples for each of the remaining projects. The projects named Bleeding, Thrombotic and Platelet Disorders (BPD), Primary Immune Disorders (PID) and Stem Cell and Myeloid Disorders (SMD) are relevant to the immunology, hematology and hemostasis communities. For these projects clinicians were asked to only enroll patients with molecularly unexplained rare diseases with a high likelihood of being inherited. The sequencing of 13,000 DNA samples for the pilot projects was completed in 2016 and analysis has commenced.

The Human Phenotype Ontology system

Clinical and laboratory phenotype data have been captured using Human Phenotype Ontology (HPO) terms. The HPO is an open source project for phenotypic annotation of genetic disorders. More than 10,000 terms in the HPO are connected via a hierarchy of is-a relationships. Appending of HPO terms allows automated grouping of patients according to phenotypic similarities.[4,5]

Pathogenic and likely pathogenic variants in known genes

Experts in statistical genomics and bioinformatics work with members of the clinical care teams to analyze the phenotyping and genotyping results. There are 264 known genes for BPD, PID and SMD. In the first round of analysis these known genes are reviewed for pathogenic or likely pathogenic variants by a multi-disciplinary team (MDT). So far the analysis has focused on the 65 MB of coding space or exome. As expected for patient with unexplained disorders, causal variants were only identified in <15% of cases and for these samples research reports were issued to

the referring clinician. To address the important question which level of diagnostic sensitivity can be achieved, one of the rare disease pilot projects focused on retinal disease and relaxed eligibility criteria were applied. All patients with visual impairment with a high likelihood of being inherited were enrolled in a cohort of 722 individuals. Likely pathogenic and pathogenic variants were identified for 404/722 (56%) individuals.[6]

Discovery of novel genes

Samples from patients without causal variants are entered in a second round analysis to identify possible new genes. This analysis relies on the development and application of new statistical methods. Such methods can be applied to automatically cluster patients based on phenotypic similarities and also exploits the richness of information from the Mouse Genome Informatics (MGI) and the Online Mendelian Inheritance in Man databases.[7] These approaches, which are critically dependent on the use of HPO terms have shown to be effective in identifying variants in several novel genes.[8-11] For example the identification of gain-of-function (GOF) variants in *DIAPH1* underlying macrothrombocytopenia and deafness illustrates the usefulness of the new methods.[8] Similarly, the discovery that a GOF variant in the kinase *SRC* results in a Grey Platelet Syndrome-like disorder characterized by bleeding, myelofibrosis and osteoporosis was the result of a more integrated approach to data analysis. In addition our approach of bringing genotype and phenoype information from many patients together in a single database also revealed that macrothrombocytopenia, sometimes accompanied by bleeding can be caused by autosomal dominant acting variants in *GP1BB*, one of the well known genes implicated in inherited platelet disorders.[12] Furthermore,

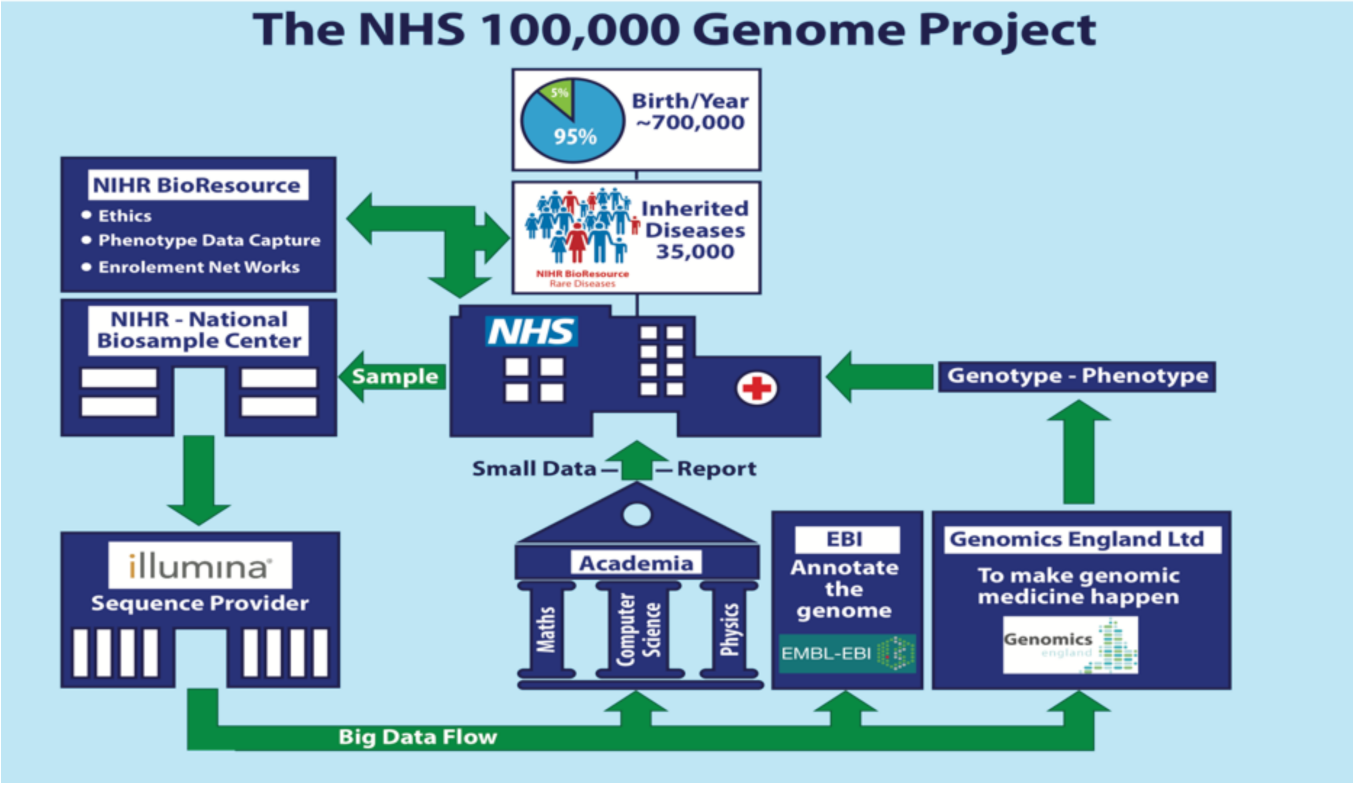

Figure 1. A diagram illustrating the different elements of the 100,000 Genomes Project. There are an estimated 700,000 birth per annum in the United Kingdom and 1 in 20 will experience ill-health during the first decades of life because of an inherited disease. There are an estimated 7,000 inherited diseases of man and the implicated genes have been identified for slightly more than halve of the known diseases

the sequencing of a large number of samples in a single programe has also been helpful in replicating several recent gene discoveries, thereby reducing the risk of erroneous discoveries.[13-15] Another avenue to gene discovery is by combing the results from different genotyping studies. Meyer *et al.* recently illustrated the power of this approach by combining the results from the UK10K Consortium, Deciphering Developmental Disorders study and the NIHR BioResource. They identified 27 unrelated individuals with a childhood-onset dystonia caused by variants in *KMT2B*, the gene encoding the mixed-lineage leukemia protein 4.[16]

Incorporating novel gene findings in diagnostic tests

Historically the cost of Sanger sequencing has prevented the genetic analysis of DNA samples from patients with an assumed inherited disorder. High throughput sequencing (HTS)-based gene panel tests provide an opportunity to analyse a DNA sample at an affordable cost. An international collaboration of BPD experts illustrated that such HTS platforms can achieve excellent sensitivity and specificity.[17-19] Similar HTS panel tests are now available for PID, SMD, pulmonary arterial hypertension and hereditary hemorrhagic telangiectasia. It is expected that by 2022 a WGS test will cost ~€200 rendering HTS gene panel tests obsolete.

Data sharing

The labelling of DNA variants with their level of pathogenicity requires a careful approach because of the risks associated with erroneous genetic diagnosis. So far 1,279 and 635 BPD patient samples have been sequenced by the WGS and HTS tests, respectively. The MDT identified 186 pathogenic and 174 likely pathogenic variants in the 78 known BPD genes, illustrating that nearly halve of the causal variants are novel. To enhance the quality of variant labelling information about genotype and phenotype should be shared through 'safe haven' models. An example of such a safe environment for data sharing is the European Genome-phenome Archive at the European Bioinformatics Institute. Similarly, information of variants should be deposited in freely accessible databases. The ClinVar database, which is being maintained by the National Center for Biotechnology Information provides such an archive on the relationships among DNA variants and phenotypes. The results of the NIHR BioResource rare disease projects will be deposited into ClinVar and EGA.

Conclusions

The 100KGP project commenced in 2013 and so far 36,000 DNA samples have been analyzed. The WGS analysis is now a fully accredited service. Genomic medicine centers have been established at 11 leading academic centers and the sequencing results are reviewed by MDTs with input from clinicians, clinical geneticists and bioinformaticians. The 100KGP has laid the blueprint for the delivery of genomics services in the NHS.

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Immunotherapy in lymphoma

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Introduction

Immunotherapy of malignant lymphoma has fascinated both, basic scientists and clinical researchers for decades. This was particularly true for those lymphomas that were mainly composed of reactive cells such as Hodgkin lymphoma and some of the low grade lymphomas. With the advent of more sophisticated techniques such as single cell analysis and gene expression profiling, our understanding of the microenvironment playing an important role in the pathogenesis of different malignant lymphoma has clearly improved. Particularly, the different immune-checkpoint-pathways have substantially contributed to our better understanding of the interaction of the immune system and the malignant cells. Here, therapeutic targeting of the PD-1 checkpoint resulted in high response rates in malignant lymphoma in a basket phase-IB trial. Particularly classical Hodgkin lymphoma (cHL) patients responded with an overall response rate of 66% in the initial phase I when treated with Nivolumab. Responses were also seen with another anti-PD-1 monoclonal antibody, Pembrolizumab. Both drugs resulted in similar outcomes in cHL patients failing several lines of treatment. The follow-up phase-II trials led to the registration of Nivolumab in the US and Europe; the registration of Pembolizumab is expected in 2017. A number of clinical trials currently evaluate these drugs either alone or in combination in different malignant lymphoma. Clinical trials are also ongoing in which checkpoint inhibitors are being combined with truncated chemotherapy. The combination of immune-checkpoint-inhibition and allogeneic transplant has resulted in some severe side effects such as severe acute GVHD when allo-TX was performed after prior immune-checkpoint-inhibition. Particularly, progressive lymphoma patients treated with allo-TX followed by PD-1 inhibitors showed severe acute GVHD but there appears to be a learning curve. On the other hand, immune-checkpoint-inhibition in malignant lymphoma might lead to less lymphoma patients receiving an allo-transplant.

Learning goals

1. ***Understanding the role of the microenvironment in B-cell lymphoma***
2. ***Current status of PD-1 inhibition in lymphoma***
3. ***Update on auto- and allo-transplant in lymphoma***

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Immunotherapy in lymphoma - Section 1

The role of the microenvironment in the pathogenesis of B-cell lymphomas

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Take-home messages

- The tumor microenvironment plays a critical role in the biology of B-cell malignancies.
- Composition of the tumor microenvironment can impact the clinical course.

Introduction

In the past decades, significant advances in the understanding of the biology of various subtypes of malignant B-cell lymphomas have been made. Numerous studies identified, in the malignant lymphoma cells, different genetic aberrations and dysregulated oncogenic signaling pathways that contribute to the molecular pathogenesis of these entities. However, additionally the importance of the non-malignant cells surrounding the neoplastic B-cells, the so-called tumor microenvironment, became evident. Furthermore, various studies showed that the composition of the microenvironment can impact the clinical course of affected patients further underscoring its importance. Thus, a better understanding of the role of the tumor microenvironment is crucial. Within this review, we summarize the current knowledge of the microenvironment for three B-cell lymphoma subtypes.

Diffuse large B-cell lymphoma

Accounting for roughly 30-40% of all lymphoma cases, diffuse large B-cell lymphoma (DLBCL) represents the most common malignant lymphoma subtype. Gene expression analyses identified two major molecular subtypes, termed activated B-cell like (ABC) and germinal center B-cell like (GCB) DLBCL.[1] Diverse genetic aberrations contributing to either ABC or GCB DLBCL pathogenesis have been unraveled. It has been shown that some of these abnormalities can also influence patients' response to standard of care therapy.[1] However, additionally it became evident that besides genetic features of the lymphoma cells that the microenvironment can dictate prognosis of affected patients. Gene expression profiling identified two signatures called 'stromal-1' and 'stromal-2' that reflect expression patterns of non-malignant cells within the DLBCL microenvironment.[2] The 'stromal-1' signature includes genes that are expressed in normal mesenchymal tissues, many of which encode proteins of the extracellular matrix as well as genes normally expressed in monocytes. This signature was associated with favorable outcome, whereas the so called 'stromal-2' signature that comprised genes expressed in endothelial cells or genes encoding important regulators of angiogenesis, was associated with adverse survival.[2]

However, the specific contribution of individual microenvironmental components on DLBCL prognosis is still not completely understood and data from various studies are even contradictory (Table 1). Especially the role of tumor-associated macrophages and regulatory T-cells (T_{reg}) has been analyzed recently, but could not reveal uniform results. High numbers of macrophages as measured by CD68 staining were found to be a favorable prognostic marker for DLBCLs treated with immunochemotherapy.[3,4] In contrast, high numbers of CD68+ cells indicated poor prognosis for patients treated without rituximab.[4] Furthermore, an increased ratio of CD68/CD163 double positive cells was correlated with adverse survival.[3] Similarly controversial results have been obtained for the prognostic impact of T_{reg}s. Whereas several studies suggested that a high number of T_{reg} cells is correlated with favorable outcome in DLBCL,[3,5,6] one study reported a positive influence of high T_{reg}s on survival only in GCB DLBCLs and a negative prognostic effect in non-GCB DLBCLs.[7] Closely related to the tumor microenvironment is also the ability of tumor cells to evade the immune response of the host. Mechanisms supporting the immune evasion in DLBCL include loss of B2M and CD58 expression to prevent recognition by circulating cytotoxic T-cells and natural killer (NK) cells.[8] Furthermore, the PD-1/PD-L1 pathway can promote immune evasion. In DLBCLs an increased PD-L1 expression on tumor cells was associated with the ABC DLBCL subtype.[9] High expression seems to be due to genetic alterations affecting the *PD-L1*

locus. These alterations are more frequent in ABC DLBCLs leading to an increased expression of PD-L1.[10]

Follicular lymphoma

An important role in the biology has also been established for the tumor microenvironment in follicular lymphoma (FL). Gene expression profiling data revealed two gene expression signatures derived from non-malignant cells that were correlated with prognosis of affected patients.[11] The first signature termed 'immune-response 1 signature' is enriched for genes typically expressed in T-cells and is correlated with favorable outcome. In contrast, the 'immune-response 2 signature' that comprised genes that are expressed in macrophages and dendritic cells, is associated with poor prognosis.[11]

The prognostic impact of individual microenvironmental components has been the focus of several studies (Table 1). For CD8+ cytotoxic T-cells it was shown that increased numbers of cells with high granzyme B expression levels are correlated with a longer progression-free survival, whereas the number of CD8+ cells alone did not correlate with survival.[12] Another study implicated that the numbers of a CD8+ subset with low PD-1 expression correlated with shorter survival.[13]

The impact of macrophages on FL survival has also been controversial. Whereas some studies implicated high numbers of tumor associated macrophages to be correlated with adverse outcome in FL patients, other analyses suggested favorable outcome.[14-16] Potentially, these divergent results are related to patient treatments. PD-1 expression potentially contributing to immune evasion via the PD-1/PD-L1 pathway has also been studied, but again the prognostic implications are not fully elucidated.[17,18]

Classical Hodgkin lymphoma

Classical Hodgkin lymphoma (cHL) is unique with respect to its histopathological appearance, as the malignant Hodgkin Reed-Sternberg (HRS) cells represent only the minority of cells that are surrounded by an extensive number of non-malignant immune cells constituting the tumor microenvironment. As for DLBCL and FL different microenvironmental factors contribute to cHL prognosis (Table 1). Increased numbers of CD68+ macrophages were associated with inferior survival.[19,20] In contrast, high numbers of non-malignant CD20-positive B-cells were correlated with favorable prognosis.[20,21] Similarly, increased numbers of T_{reg} cells pointed towards a superior outcome.[20] In contrast, a low ratio of T_{reg} to cytotoxic T-cells/NK cells correlated with poor survival.[22] The ability of HRS cells to evade the immune response of the host seems to be mediated through different molecular mechanisms. Besides loss of MHCI and MHCII expression due to inactivating *B2M* mutations or translocations involving the *CIITA* gene,[23,24] HRS cells are also characterized by high expression of PD-L1 and/or PD-L2 due to chromosomal amplifications affecting 9p24 resulting in binding to PD-1 positive T-cells and subsequent T-cell exhaustion most likely explaining the high efficacy of checkpoint inhibitors in relapsed/refractory cHL patients.[25-27]

In summary, the microenvironment plays an important role in the pathogenesis of different B-cell malignancies. However, the exact contribution of individual components needs to be addressed and defined more precisely in future studies. Deciphering the exact role of the tumor microenvironment in the biology of these entities might lead to more specific and potentially less toxic treatment regimens.

Table 1. Prognostic impact of microenvironmental factors in B-cell lymphoma.

Lymphoma subtype	Microenvironmental factor	Prognostic impact
DLBCL	Macrophages	High numbers of macrophages associated with favorable outcome when treated with immunochemotherapy[3,4] High numbers of macrophages associated with adverse outcome when treated without rituximab[3,4]
	T_{reg} cells	High numbers of T_{reg} cells correlated with favorable outcome in GCB DLBCL/DLBCL[3,5-7] High numbers of T_{reg} cells correlated with adverse outcome in non-GCB DLBCL[7]
Follicular lymphoma	Cytotoxic T-cells	High numbers of granzyme B+ cells correlated with favorable outcome[12] Numbers of CD8+ cells with low PD-1 expression correlated with shorter survival[13]
	Macrophages	Impact of numbers of macrophages on FL survival controversial; some studies implicated high numbers of tumor associated macrophages to be correlated with adverse outcome in FL patients, other analyses suggested favorable outcome[14-16]
Classical Hodgkin lymphoma	Macrophages	Increased numbers correlated with inferior survival[19,20]
	Non-malignant B-cells	Increased numbers correlated with favorable outcome[20,21]

Immunotherapy in lymphoma - Section 1

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Immunotherapy in lymphoma - Section 2

Immune checkpoint inhibitors

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Take-home messages

- Anti PD1 antibodies are highly active in relapsed Hodgkin lymphoma.
- Immune related flares may occur and should not result in discontinuation of therapy.

Immune checkpoints regulate T-cell activation, maintaining self-tolerance and preventing autoimmunity.[1] Aberrant expression of immune checkpoint ligands by tumor cells can enable them to inhibit T cell activation, evading T-cell mediated antitumour response.[2] Importantly, infiltrating T lymphocytes in the tumour microenvironment frequently express immune checkpoint receptors, such as programmed cell death protein 1 (PD-1) and hepatitis A virus cellular receptor 2 (also known as TIM-3), and are functionally incompetent to mount an immune response against tumor cells.[1,3-6]

Immune checkpoint inhibitors are rapidly gaining momentum as effective agents for the treatment of variety of lymphomas.[7-9] Earlier studies targeting the immune check receptor CTLA-4 using the monoclonal antibody ipilimumab demonstrated a modest clinical activity in patients with relapsed Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), with a reasonable safety profile.[10] More recently, therapeutic targeting of PD-1 demonstrated a significant clinical activity in patients with relapsed HL, and to a less degree, in patients with NHL. The efficacy of the anti-PD-1 antibodies, nivolumab and pembrolizumab, was evaluated in patients with relapsed B-cell lymphoma, T-cell lymphoma, or HL.[11-13]

In patients with relapsed HL that received treatment as part of these phase I trials, both antibodies produced response rates >60%, although the complete response rates were more modest. A phase II study investigating nivolumab in patients with relapsed HL after autologous stem-cell transplantation and brentuximab vedotin produced a response rate of 66%, which led to FDA approval of nivolumab for this indication in May 2016 (Table 1).[14] A similar study using pembrolizumab also produced a high response rate of 72%, and is expected to be approved by regulatory agencies in the near future (Table 1).[15] Interestingly, both agents produced similar results in brentuximab naive patients after failing therapy with stem cell transplant (Table 1).

The high response rate observed in patients with HL might be caused by the fact that Reed-Sternberg cells express high levels of programmed cell death 1 ligand 1 PD-L1, and are surrounded by a large number of T cells in the microenvironment.[6,16,17] However, whether or not other cells in the microenvironment contribute to antitumour activity currently remains unclear. Other studies are currently evaluating the efficacy of various antibodies targeting PD-L1, such as those involving atezolizumab, with promising early results.[18] The safety and efficacy of antibodies targeting other immune checkpoints, including tumour necrosis factor receptor superfamily member 9 (also known as 4-1BB) or varlilumab, are currently being examined in a variety of clinical trials.

Table 1. Results of recent phase II trials of pembrolizumab and nivolumab in patients with relapsed Hodgkin lymphoma.

Drug	Dose/Schedule	N	% ORR	% CR	1st author/Reference
Post autologous stem cell transplant and brentuximab vedotin therapy					
Pembrolizumab	200 mg IV every 3 weeks	69	72%	21%	Moskowitz[15]
Nivolumab	3 mg/kg IV every 2 weeks	80	66%	9%	Younes[26]
Post autologous stem cell transplant, but brentuximab vedotin naiive					
Pembrolizumab	200 mg IV every 3 weeks	60	67%	21%	Moskowitz[15]
Nivolumab	3 mg/kg IV every 2 weeks	63	68%	22%	Timmerman[27]

Immunotherapy in lymphoma - Section 2

A variety of studies, designed to assess the efficacy of anti-PD-1 therapy in combination with other checkpoint inhibitors or immunologically active treatment involving combination therapies, are also currently underway. These include combinations of nivolumab with ipilimumab or lirilumab, which target CTLA-4 and inhibitory KIR receptors, respectively.[2] Furthermore, ongoing clinical trials are assessing the combination of urelumab (targeting 4-1BB) with rituximab or pembrolizumab plus rituximab (NCT02446457). Clinical trials investigating the efficacy of nivolumab in combination with urelumab are also ongoing. Another approach would be to combine an immune-checkpoint inhibitor with a small-molecule inhibitors. BTK-inhibition has specifically been studied as these inhibitors might also target interleukin-2-inducible T-cell kinase (ITK), thereby activating T cells and thus promoting a T_H1 response.[19] Taking advantage of this observation and based preclinical studies using mouse xenograft models, trials investigating the efficacy of PD1 targeted antibodies in combination with ibrutinib are currently being conducted.[19]

Because brentuximab vedotin and PD-1 antibodies produce high response rates in patients with relapsed HL, with excellent safety profiles, they are likely to be combined at full doses.[7,20] The combination of nivolumab plus brentuximab vedotin is currently being evaluated in patients with relapsed and refractory HL who are eligible for stem cell transplant.[21] Preliminary results from the first 42 patients treated on the study demonstrated a high response rate of 90%, with a complete response (CR) rate of 62%.

In B-cell NHL, response rates were lower than those observed HL, ranging between 30%-40%, but the number of patients with B-cell NHL treated with immune checkpoint inhibitors remain relatively small.[22] However, in subsets of B-cell NHL where PDL1/PDL2 are overexpressed, the response rates are usually higher. In these cases, 9p24.1/ PD-L1(CD274)/ PD-L2 (PDCD1LG2) copy number alterations and additional translocations are typically observed. These genetic alterations are associated with increased expression of the programmed cell death protein 1 (PD-1) ligands, PD-L1 and PD-L2.[23] For example, Zinzani et al, reported an overall response rate of 44% in 9 patients with primary mediastinal large cell lymphoma.[13] Similarly, 5 patients with relapsed testicular lymphoma or primary central nervous system lymphoma were treated with single agent nivolumab, and all achieved a major clinical response.[24] Finally, the number of patients with relapsed cutaneous T cell lymphoma is also relatively small, with a response rate of 38% achieved with pembrolizumab.[25]

In conclusion, immune checkpoint inhibitors are among the most active agents for the treatment of HL.[26] Accordingly, single agent nivolumab was recently approved in these patients.[27] In NHL, immune checkpoint inhibitors have a modest activity, and therefore are being actively developed in combination with other agents.[8,16]

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Immunotherapy in lymphoma - Section 2

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Immunotherapy in lymphoma - Section 3

Is transplantation in lymphoma still needed in the era of immunotherapy?

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Take-home messages

- The introduction of check point inhibitors in the treatment landscape of patients with lymphomas might potentially change the profile of patients being candidates for this procedure; number of patients treated with check point inhibitors is still low and follow up limited.
- Allotransplant related toxicity seems to be modified / increased by the prior use of check point inhibitors in these patients
- Transplant related outcomes in patients pre-treated with check point inhibitors do not seem to be worse than those of historical controls.

Introduction

Hematopoietic stem cell transplantation (HSCT), autologous or allogeneic, is used with increasing frequency in Europe where in 2014 over 40 000 transplants were reported for the first time.[1-6] Transplant-related mortality remains high between 10 and 20% in allogeneic HSCT and although much lower, ~1% for autologous HSCT, high-dose chemotherapy is toxic and demanding for patients. Progress has been made over the years reducing non-relapse mortality (NRM) by ~50% with the introduction of reduced intensity conditioning (RIC) protocols, better HLA typing and donor selection as well as better anti infection compounds and supportive care, but toxicity in HSCT is still a challenge.[7,8] Auto-HSCT remains the standard of care for patients with chemosensitive relapse of diffuse large B cell lymphoma (DLBCL) in the rituximab era.[9-11] For follicular lymphoma patients in first chemosensitive relapse, high-dose chemotherapy followed by high-dose chemotherapy is often regarded as treatment of choice.[9-12] For Hodgkin lymphoma in first chemosensitive relapse or refractory to first-line therapy, HDCT is also standard as shown by two prospective clinical trials.[13,14] Allo-HSCT can provide long-term disease control in up to 40% of patients with DLBCL who have failed auto-HSCT, in particular if performed in chemosensitive disease.[15,16] In FL patients, allo-HSCT is reserved as a potentially curative option for those patients who have failed auto-HSCT or multiple therapy lines, or who have become refractory.[11,12,17] Prospective phase II trials as well as retrospective cohort comparisons and registry analyses suggest that allo-HSCT can prolong survival in selected patients when compared with the limited non-transplant options in HL failing auto-HSCT but responding to salvage therapy.[18-20]

Drug development is accelerating and many new drugs have been developed and marketed for hematologic malignancies in the past few years. Some of the more targeted drugs have limited toxicity and it is of interest to examine whether these have changed the use of HSCT for selected indications. At the same time, accessibility to targeted drugs is an issue in some countries. A specific very-effective drug may replace HSCT and lead to decreased use of this technology, whereas another drug may enhance HSCT use and function as a 'bridge to transplant'.

Amongst many others, antibodies targeting programmed death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein-4 (CTLAP-4) are being investigated in lymphoid malignancies with varying levels of activity and an interesting toxicity profile. Anti-PD-1 antibodies such as nivolumab and pembrolizumab show encouraging response rates particularly in classical HL. Results in FL and DLBCL so far are less impressive. Results of a phase II trial in relapsed/refractory classical HL patients who had relapsed after auto-HSCT and brentuximab vedotin, showed an overall response rate (ORR) of 66% based on central review and of 72% based on investigator evaluation after a median follow-up of 8.9 months.[21] As the first immune checkpoint inhibitor in lymphoma, nivolumab was approved for the treatment of relapsed or refractory classical Hodgkin lymphoma by both, the Food and Drug Administration and the European Medical Agency in 2016.

Immunotherapy in lymphoma - Section 3

The possibility to combine both, autologous and allogeneic stem cell transplantation with checkpoint inhibitors is of potential clinical interest. This can be done in different settings namely post auto-HSCT, before allogeneic transplant and after the allogenic procedure. Check point inhibitors function by amplyfing effector T cell responses, and therefore combined therapy with allo-HSCT may have a synergistic anti-tumor effect (GvT), but may also result in increased toxicity with increased graft versus host disease (GvHD).

The post auto-HSCT setting is an attractive one for immune-directed therapy because of several reasons: the existence of a state of minimal residual disease and additionally, the conditioning regimen delivered alters the immune system in a way that may make check point inhibitors more effective. A phase II study of patients with DLBCL evaluated the potential benefits of adding three doses of pidilimumab one to three months after auto-HSCT. Progression free survival (PFS) at 18 months was 72%, which compared favorably to 52% seen in the control group. PFS for patients with PET positive disease before transplantation was 72% and patients with active disease after transplant had a response rate of 51%.[22] These early results have prompted the development of several prospective ongoing clinical trials in HL and DLBCL in this setting.

In the pre-allogeneic setting, most information comes from relapsed HL patients. Some potential candidates for an allogeneic procedure have previously received PD-1 inhibitors, most frequently nivolumab. The immunomodulatory effects and long half-life of check point inhibitors may alter the outcomes and toxicity profile of allo-HSCT in these patients. Residual blockade of inhibitory checkpoints at the time of transplant may result in a potentially enhanced GvT effect, but also increased immunological side effects such as GVHD. Animal models demonstrate that PD-1 blockade after allo-HSCT may augment GvT responses,[23] but could also result in higher rates of acute GVHD and higher mortality related to GHVD.[24] Clinical experience in this setting is still limited and comes basically from a retrospective multicer analysis including 39 patients who received pembrolizumab or nivolumab for the treatment of refractory / relapsed HL or NHL and subsequently underwent allo-HSCT.[25] Clinical characteristics are depicted in Table 1. With

Table 1. Clinical characteristics of lymphoma patients receiving allo-HSCT after check point inhibitors exposure*.

Patient characteristics	N = 39 (%)
Gender Male / Female	20 (51%) / 19 (49%)
Age at allo-HSCT in years (median, range)	34 (21 - 67)
Histological diagnosis HL / NHL	31 (79%) / 8 (29%)
Prior lines of therapy (median, range)	4 (2 - 8)
Prior auto-HSCT	32 (82%)
PD-1 inhibitor received Nivolumab / Pembrolizumab	28 (72%) / 11 (28%)
Cycles of PD-1 inhibitor received (median, range)	8 (3 - 27)
Time interval between last PD-1 treatment and allo-HSCT in days (median, range)	62 (7 - 260)
Source of stem cells Peripheral blood / Bone Marrow	28 (72%) / 11 (28%)
Donor type MRD / MUD / Haplo / mmURD	9 (23%) / 12 (31%) / 14 (36%) / 4 (10%)
Disease status before allo-HSCT CR / PR / SD / PD	25 (64%) / 11 (28%) 2 (5%) / 1 (3%)
Conditioning regimen RIC / MAC	38 (97%) / 1 (3%)

Allo-HSCT. Allogeneic stem cell transplantation; HL. Hodgkin's lymphoma; NHL. Non-Hodgkin's lymphoma; MRD. Matched related donor; MUD. Matched unrelated donor; Haplo. Haploidentical donor; mmURD. Mismatched unrelated donor; CR. Complete remission; PR. Partial remission; SD. Stable disease; PD. Progressive disease; RIC. Reduced intensitity conditioning; MAC. Myeloablative conditioning. Adapted from Merryman et al. Blood 2017;9:1380-88; with permission.

a median follow-up for survivors of 12 months, 1-year overall survival and PFS were of 89% and 76%, respectively, while the 1-year cumulative incidence of relapse and NRM were 14% and 11%, respectively. One-year cumulative incidence of grade 2-4, 3-4 and grade 4 acute GVHD were 44%, 23% and 13%, respectively. 1-year cumulative incidence of cGVHD was 41%. Three patients (8% of the series) developed severe hepatic sinusoidal obstruction syndrome despite having received a RIC protocol with one fatality 51 days after transplant. In addition, 7 patients (18%) developed a prolonged febrile syndrome beginning one to seven weeks after transplant. In terms of predictors of survival, non-relapse mortality and GVHD, patients receiving 8 or more doses of PD-1 inhibitors (the median number of doses in this group of patients) had an improved 1-year PFS in comparison to those receiving less doses; there were no differences between both groups of patients regarding overall survival and NRM. Time interval between the last dose of PD-1 inhibitors and allo-HSCT did not significantly impact outcome. Moreover, transplant-related outcomes were not significantly modified by donor and graft characteristics. Although there are still many questions regarding the role and appropppriateness of allo-HSCT after PD-1 blockade, the results of this retrospective study might suggest that this approach is feasible in adequately selected patients and may be associated with increased immune toxicity but also good disease control.

The largest experience presented today using nivolumab for relapse after transplant includes 20 patients with HL.[26] Nivolumab was started at a median time of 23 months after allo-HSCT and patients could not have a prior history of grade 4 aGvHD or extensive chronic GvHD as well as no need for immunosuppressive therapy for the last 4 weeks. 1-yr PFS and 1-yr OS in this series were 58.2% and 78.4%, respectively. Clearly, larger prospective experience is needed to better describe the safety and efficacy of check-point inhibitors after allo-SCT.

In summary, these preliminary findings suggest that it is possible to combine HSCT with check point inhibitors in the relapsed / refractory lymphoma landscape. Nevertheless, we are still left with more questions than answers; these questions need to be explored through well designed prospective clinical trials in order to better define biomarkers that may predict toxicity in this setting and the best way to combine both strategies in order to maximize effectiveness and mitigate toxicity. On the other hand, the incorporation of these novel agents in lymphoma therapy may eventually overcome the actual HSCT indications in these diseases.

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Multiple myeloma

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Introduction

Multiple myeloma (MM) is a very heterogeneous disease, clinically, biologically, and genetically. Moreover, MM is one of the hematological neoplasia with more clinical advances in recent years with a significant improvement for patient survival. These advances have been possible due to a better understanding of the landscape of the disease, the advent of new drugs with original mechanisms along with new strategies of early combined treatment, sequential, intensified and maintenance therapies and new tools for monitoring evolution. In this education session, the participating experts will review these aspects. In first place Dr. N. Munshi and colleague, from Dana Farber Cancer Centre (USA), will review the immunopathology of multiple myeloma. Multiple myeloma origin is related to antigen-driven processes. The myeloma cells have bidirectional and major interactions with the bone marrow microenvironment enhancing growth, survival and drug resistance. The targeting of the immune system could provide new and very effective therapy strategies for multiple myeloma. In the second talk Dr. H. Avet Loiseau from the University Cancer Center of Toulouse (France) will review the genetic classification of myeloma for prognosis and treatment selection. Genetic analyses are mandatory at the time of diagnosis, and probably also at relapse to define the prognosis. The mutational landscape in MM, which is mainly based on whole exome sequencing, has confirmed the genetic heterogeneity of MM, with no specific common mutation. The genetic profile information could be used to propose specific drug targeted combinations although this is still a matter of debate. And finally, Dr. J San Miguel from the Clínica Universidad de Navarra (Spain) and colleagues from the Spanish Myeloma Group, will review the new treatment approaches in myeloma in 2017. The outcome for MM patients has significantly improved in the last 15 years, mainly due to the use of proteasome inhibitors (bortezomib, carfilzomib, ixazomib) and immunomodulatory agents (thalidomide, lenalidomide, pomalidomide), and more recently, monoclonal antibodies (daratumomab, elotuzumab) and other novel drugs with a singular mechanism of action as HDAC inhibitors (panobinostat). The combination of a MoAb plus a triplet based on a PI-IMiD-Dex could be the future standard for induction. Intensification with autologous stem cell transplantation (ASCT) is still the standard of care to enhance response rate and prolong PFS and OS. The options for treatment at relapse have also improved with immunotherapy strategies with check-point inhibitors as pembrolizumab, the use of anti-BCMA CAR-T cells and a large list of new promising investigational drugs. Moreover, the introduction of new criteria for early diagnosis with the option of early intervention are opening new therapeutic avenues. The new IMWG response criteria, with the concept of minimal residual disease, should contribute to individualized treatment based on highly sensitive methods for monitoring treatment efficacy. The treatment goal for multiple myeloma should be to find a balance between efficacy, toxicity and cost, with the ultimate aim of achieving a cure for the disease.

Learning goals

1. ***To understand the bidirectional interaction of myeloma cells with the bone marrow microenvironment and with the immune system and to highlight their therapeutic implications.***
2. ***To review the cytogenetics and molecular landscape of multiple myeloma and to know the main genetics tests required at diagnosis and at relapse to identify patients with high risk.***
3. ***To understand the advances observed during recent years due to the novel drugs including immunotherapy and their favourable clinical impact on survival, to learn about new biomarkers, to decide early treatment and new integrated tools for minimal residual disease monitoring.***
4. ***To understand the best option therapy for upfront myeloma patients candidates and non-candidates for autologous hematopoietic transplant including the role of consolidation and maintenance therapies, and the different options of rescue with new drugs for refractory/relapse patients.***

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Multiple myeloma - Section 1

Immunopathology of multiple myeloma

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Take-home messages

- Multiple myeloma origin is related to antigen-driven processes.
- Multiple myeloma cells have bidirectional and major interactions with the bone marrow microenvironment enhancing growth, survival and drug resistance. These interactions lead to various alterations of the immune system.
- Targeting the immune system in multiple myeloma provides new very effective therapeutic strategies.

Abstract

Multiple myeloma (MM) is defined by the malignant proliferation of plasma cells, a major component of the immune system. MM is a heterogeneous disease featured by a multistep progression from monoclonal gammopathy of undetermined significance (MGUS) to symptomatic disease, with distinct molecular subgroups and clinical outcomes. The immune system related characteristics of the plasma cells determine specific features involved in myelomagenesis and progression. The mechanisms contributing to the plasma cell differentiation and the multiple interactions of the plasma cells with other immune cells are crucial in both myeloma cell growth and survival as well as development of drug resistance. In this review, we discuss the immunopathology of MM, from the early driving events to myeloma progression and its therapeutic implications.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy with genomic and clinical heterogeneity.[1,2] The disease process is modulated by bidirectional interaction between MM cells and accessory cells in the bone marrow (BM) microenvironment which not only affect growth and survival of MM cells, but also development of drug resistance. As MM cells are of immune origin and in the vast majority of cases secrete clonal immunoglobulin and/or light chains,[3] its interaction with components of the BM immune microenvironment[4,5] leads to various alterations of the immune system. Some of the changes inhibit anti-MM immune responses as well as promote MM growth and survival. With the advent of new immunotherapy including monoclonal antibodies targeting the myeloma cells or the immune check-point inhibitors, or engineered T cells including chimeric antigen receptor (CAR) T cells, targeting the immune system is becoming a new treatment paradigm. Moreover, the effect of immunonomodulatory agents (Imids) on the immune system, especially increase in T and NK cells cytotoxicity including the antibody-dependent cell-mediated cytotoxicity, has greatly contributed to develop this therapeutic field.[6-8] Thus, the combination of Imids with monoclonal antibodies (anti-CD38 or anti-SLAMF7[9,10] or check-point inhibitors -anti PD-1) has already provided promising results. Monoclonal antibodies targeting MM cells are effective and will probably constitute next gold standard therapy.[11] Here, we review the main features of the immunopathology of MM and highlight its therapeutic implications. Therefore, understanding and improving immune function has application for both extending anti-MM responses as well as decrease susceptibility to infections observed in MM.

Origin of the MM cell – An antigen-driven process and myelomagenesis

MM is defined by presence of IgH translocations which are recurrent and considered to be generated during somatic hypermutations, VDJ recombination and class switch recombination.[12] Moreover, these distinct breakpoints suggest that the initial driver events occur at different stages of the B cell development and are related to AID/APOBEC activity as suggested by whole exome sequencing.[13,14] Several observations and studies have highlighted the role of an antigen-driven immune stimulation to trigger the development of MM. More recent reports of an anti-lysolipids activity in Gaucher disease, a lysosomal disease known to be associated with monoclonal gammopathies[15] and a similar anti-lysolipid activity in a subset of patients with monoclonal gammopathies without Gaucher

disease[15] suggest that the eradication of the causal and persistent antigen and/or the inhibition of the B cell receptor signaling could be an effective strategy in early stage MM. However, an antigen-driven mechanism hasn't been established for all types of myeloma and targeting B-cell signaling could be an effective strategy in only some myeloma subgroups.

Myeloma cells and the immune system

Dendritic cells (DC)

DCs play an important role in antigen presentation and interactions with other immune cells. In MM, 2 major subtypes of DCs, monocyte-derived (mDC) and plasmacytoid (pDCs),[16] have been shown to accumulate in the bone marrow (BM). Increased dysfunctional, mainly immature pDCs, expressing PD-L1, contribute to MM cells growth, survival, chemotaxis, and drug resistance as well as suppressed T cell responses. Targeting pDC using oligonucleotides or a TLR9 agonist is effective in impacting MM cells survival and restoring immune functions.[17,18]

T cells and T regulators

In MM, dysregulation of various T cell subsets have been observed. The circulating CD4/CD8 ratio, the Th1/Th2 ratio are decreased. More importantly, T regulator cell dysfunction and suppressed T cell responses have been reported in all stages of MM. This is driven by effect of cytokines, the interacting immune microenvironment in the BM and an abnormal antigen presentation by dendritic cells and other antigen presentation cells.[19-21] γδ T cells have been shown to have a cytotoxic activity against myeloma cells enhanced by MHC class I polypeptide-related sequence (MICA) expression on plasma cell surface and the use of bipohosphonates but its role remains uncertain.[22,23]

B cell

Several studies have shown that the total number of B cells is decreased in MM. In particular, the naïve and transitional B cells subsets are decreased in contrast with memory B cells that are increased in MM patients as compared with MGUS and healthy donors.[24,25] The modifications of the B cells subsets are important to understand the humoral immune deficiency that characterize MM patients and generate an impor-

Table 1. Main immunological abnormalities and therapeutic options in multiple myeloma.

Cellular Component	Observed Abnormalities in MM	Consequences	Therapeutic implication / potential therapeutic options
B Cells	Low level of naïve and transitional B cells	Humoral immunity deficiency and predisposition to infections	Early vaccination Antibiotic prophylaxis
T cells	Low levels of T cells Dysfunctional T cells	Reduced anti-myeloma cytotoxic activity	Imids Check-point inhibitors Activation of γδ T cells with bisphosphonates
T regulatory cells	Dysfunctional T reg	Contribute to immune evasion	Imids
T helper 17 cells	High level of TH17 and IL-17	Promotes MM cells survival and growth, T cell dysfunction and bone disease	Anti-IL17 antibody
Macrophages	High level of TAM featured by M2 phenotype	Enhance MM survival and chemoresistance	Reprogramming macrophages
Myeloid Derived uppressor cells	MDSC	Inhibit T and NK cells anti-myeloma responses	Check-point inhibitors
Natural Killer T cells	Dysfunctional NKT cells	Reduced anti-myeloma activity	Imids Check-point inhibitors
Natural Killer cells	Dysfunctional NK cells	Reduced anti-myeloma activity	Imids Check-point inhibitors
Dendritic cells	Increased number of dendritic cells (mDC and pDC)	Increase with the stage of the disease Enhance proliferation and growth of MM cells Contribute to immune evasion	Direct targeting using anti-IL3R immunoconjugate, TLR9 activator
Immune checkpoint	High PD1 expression on T and NK cells	Contribute to immune evasion	Check-point inhibitors
Immune checkpoint	High PDL1 expression on MM cells, pDCs, mDCs, MDSC	Contribute to immune evasion	Check-point inhibitors

TAM: tumor-associated macrophages, MDSC: Myeloid Derived suppressor cells, mDC: monocyte derived dendritic cells, pDC: plasmacytoid dendritic cells, PD1: Program-death 1, PDL1: Program-death ligand 1.

tant cause of death and morbidity.[26] No clear mechanisms have been demonstrated so far.

Myeloid Derived suppressor cells (MDSC)

MDSCs correspond to a heterogeneous group of myeloid cells able to suppress T cells, natural killer T (NKT), and natural killer (NK) cell antitumoral activity. MDSCs are significantly increased in the blood and in the bone marrow of MM patients, enhance MM cells proliferation and suppress T-cell–mediated immune responses. Reciprocally, MM cells induce MDSCs growth.[27,28] Imids and bortezomib, 2 of the most active MM drugs modify MDSCs phenotype suggesting that targeting MDSCs may represent a novel therapeutic strategy.[29]

T Helper 17

TH17 cells are a subset of T helper cells that contribute to inflammatory and auto-immune response regulation. In the context of high interleukin (IL)-6 and TGF beta, TH17 cells are increased in MM blood and bone marrow and secrete high levels of IL-17 in the blood which promotes myeloma cell growth and proliferation through IL-17 receptor that is expressed in MM cells.[30,31] IL-17 also induces suppression of Th1 responses and induces bone disease by activation of osteoclast function. Targeting IL17 with a specific antibody inhibit MM cells proliferation and osteoclast differentiation.[32]

NK and NKT cells

NK cells play an important role in immune surveillance and depletion of NK cells in mouse models highlighted their importance for myeloma progression.[33] NK cells play an important role in mediating antibody dependent cell cytotoxicity (ADCC). These activities are exploited by myeloma therapeutics including Imids as well as the recently approved antibodies daratumumab and elotuzumab. NKT cells are immune cells that recognize foreign and self (glyco)sphingolipid antigens when presented by the CD1d molecule. In addition to a direct cytotoxicity, NKT cells produce various type of cytokines that regulate and modulate other immune cell activities. The evaluation of NKT cells in MM has pointed out a marked deficiency of ligand-dependent interferon-production mainly in the context of disease progression, with variable CD1d level of expression and glycolipid presentation by the MM cells. The use of dendritic cells with α-galactosylceramide (a NKT ligand) is able to reverse this deficiency.[34] Several studies have shown that Imids increase NKT population and enhance its activity suggesting that therapy targeting NKT cells is effective in MM.[35,36]

Immune check-points

The anti-myeloma activity of immune cells is impacted by its ability to identify the MM cells as a target and by the co-stimulatory signaling. The programmed-death 1 (PD1)/programmed-death ligand 1 (PDL1) axis has been particularly evaluated in MM. High expression of PDL1 in MM cells and the BM microenvironment cells such as pDCs and MDSCs has been shown to decrease T and NK cytotoxicity. Although single agent therapy targeting PD-1 in relapsed/refractory MM has not shown significant activity,[37] the combination of checkpoint inhibitors with Imids has shown remarkable activity and is being evaluated in phase III studies. In addition to targeting PDL-1, other co-stimulatory axes are currently under investigation (CD137, LAG3, CTLA4 or TIM for example).[38-40]

Future perspectives

The improved understanding of immunopathology of myeloma has provided number of avenues for possible preventive as well as effective therapeutic strategies. The understanding of possible role of an antigen-driven immune stimulation via lysolipids activity may allow directed approaches in the early stages of the disease. While characterization of T and B cell function and the role of other immune microenvironmental cells has allowed for development of strategies using combination of Imids with antibodies targeting MM cells and/or immune checkpoint inhibitors. In addition, recent progress in adoptive transfer of cellular components, especially of activated lymphocytes[41] or as chimerical antigen receptor T (CAR-T) cells targeting either directly myeloma (BCMA)[42,43] or other B cells compartment,[44] have provided remarkable excitement for possible future curative strategies. Vaccine therapy using patients' dendritic cells are also undergoing clinical evaluation at early stage of the disease or as a consolidation therapy.[45]
In conclusion, multiple and complex immunological mechanisms contribute to myeloma genesis and myeloma progression. Their understanding provides a rationale to develop and evaluate new effective strategies in MM.

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Multiple myeloma - Section 1

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Multiple myeloma - Section 1

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Multiple myeloma - Section 2

Genetic classification of myeloma for prognostication and treatment selection

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Take-home messages

- Genetic analyses at the time of diagnosis, and probably first relapse, are mandatory in MM to define the prognosis.
- Genetic abnormalities in MM are used to predict prognosis and currently, most of the prognostic genetic changes identify patients with high risk
- The mutational landscape in MM, mainly based on whole exome sequencing, have confirmed the genetic heterogeneity of MM, with no specific common mutation
- The genetic profile information could be used to propose specific drug targeted combinations although this is still a matter of debate.

Introduction

Multiple myeloma (MM) is a very heterogeneous disease, clinically, biologically, and genetically. In contrast to non-Hodgkin's lymphomas, in which genetic and immunophenotypic characteristics define clear sub-entities, we so far failed to define different diseases in MM. Several attempts have been proposed, the most recognized classification is based on genetic abnormalities. However, such classifications do not clearly identify subgroups of patients with a different biology and outcome.

State of the art

In MM, genetic abnormalities have been mainly used to predict prognosis (Table 1). Currently, most of the prognostic genetic changes identify patients with high risk, i.e., short progression free survival (PFS) and overall survival (OS). The first and most important abnormalities are the loss of part of the short arm of chromosome 17, known as del(17p), and the translocation t(4;14) both identifying a high risk subgroup of about 20% of the patients. More recently, other abnormalities have been also described to be associated with a poor outcome, loss of the 1p32 region, and to a lesser degree, gains of 1q. In contrast, almost no good risk abnormalities have been identified, except hyperdiploidy, which represents probably a heterogeneous subgroup.

Very recently, several publications reported the mutational landscape in MM. Mainly based on whole exome sequencing, these studies confirmed the genetic heterogeneity of MM, with no specific common mutation, two mutations seen in ~ 20% of the patients (KRAS and NRAS), and the others observed in less than 10% of the patients. These mutations did not enable the definition of specific subclasses. Of note, none of these mutations display a specific poor or good outcome.

Could we use these abnormalities to design specific treatment approaches? This question has been addressed by several trials, focusing on the outcome of patients with high risk features, however, data are not clear-cut. If the combination of lenalidomide with dexamethasone (len-dex) is clearly not the best choice for high risk patients, the association of a third drug seems to improve their outcome at the time of relapse. This has been first suggested in the ASPIRE trial (len-dex +/- carfilzomib). In the experimental arm, high risk patients (del(17p) and/or t(4;14)) presented a much longer PFS. This has been confirmed in the TOURMALINE 1 trial (len-dex +/- ixazomib). Similar data have been described with monoclonal antibodies, first in the ELOQUENT 2 trial (len-dex +/- elotuzumab), and recently in the POLLUX trial (len-dex +/- daratumumab). However, all these trials did not define the high risk in the same way, especially in the cutoff for del(17p) assessment. Furthermore, all these trials were dedicated for relapsed patients, and no data is currently available in the frontline setting.

Finally, could we use the mutational analyses to propose targeted therapies, as currently performed for solid tumors? Few

mutations are really 'drugable'. Only one report described the use of vemurafenib (a BRAF inhibitor) in a patient with relapsed MM and a specific V600E BRAF mutation. This patient responded dramatically. But this is a single case report, and this mutation is present in only 3-5% of the patients.

Future perspectives and conclusions

Genetic analyses at the time of diagnosis, and probably first relapse, are mandatory in MM to define the prognosis. Whether this information can be used to propose specific drug combinations is a matter of debate. Current data are suggesting that high risk patients may benefit from triplet combinations. The future of targeted therapies in MM is undefined, but probably rather obscure due to the low mutational profile and clonal heterogeneous evolution observed in most MM patients.

Table 1. Main genetic abnormalities with poor prognosis in MM.

Deletion del(17p)
Translocation t(4;14)
Loss of 1p32 region
Gains of 1q
Non hyperdiploidy

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Multiple myeloma - Section 3

New approaches to myeloma treatment in 2017

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Take-home messages

- Better tools for diagnosis and monitoring treatment efficacy are being implemented.
- Early treatment and the use of more efficient drugs upfront prolong survival.
- The treatment goal is to find the best possible balance between efficacy, toxicity and cost, particularly at the time of relapse.

Introduction

The treatment goal for multiple myeloma should be to find a balance between efficacy, toxicity and cost, with the ultimate aim of achieving a cure for the disease. The outcome for multiple myeloma (MM) patients has significantly improved in the last 15 years, mainly due to the use of proteasome inhibitors (bortezomib, carfilzomib, ixazomib) and immunomodulatory agents (thalidomide, lenalidomide, pomalidomide), and more recently, monoclonal antibodies (daratumumab, elotuzumab) and other novel drugs with a singular mechanism of action. Moreover, the introduction of new criteria for early diagnosis of symptomatic MM and the possibility of early intervention are opening new therapeutic avenues. The new response criteria, particularly the concept of minimal residual disease, should contribute to individualized treatment based on highly sensitive methods for monitoring treatment efficacy.

Smouldering and early myeloma

The Spanish group has shown that early intervention in smouldering multiple myeloma (SMM) is associated with a highly significant prolongation of time to progression (TTP) (hazard ratio, HR: 0.24) and overall survival (OS) (HR: 0.43).[1] These results, along with the availability of more sensitive diagnostic tools, have prompted a revision of the criteria for diagnosing early myeloma requiring immediate treatment: patients without CRAB symptoms, but with >1 focal lesions detected by MRI or 60% plasma cells (PCs) in bone marrow (BM) or a free light chain (FLC) ratio >100.[2]

Treatment of newly diagnosed transplant candidate patients

Currently, treatment of young patients usually includes 3-6 cycles of induction therapy, intensification with autologous stem cell transplantation (SCT) and the possibility of consolidation and maintenance therapy.
Using induction with bortezomib (Bz)-based triplet combinations, either with alkylators or immunomodulatory drugs (IMiDs), >90% of patients respond including 20-30% complete responses (CR), and around 10% minimal residual disease (MRD) negative cases.[3,4] Preliminary data with new proteasome inhibitors (PI) such as carfilzomib (K) and ixazomib (Ixz) in combination with len-dex (Rd) (lenalidomide [R] with low-dose dexamethasone [d]) also shows a high level of activity. The former is probably the more potent triplet in terms of depth of response, while the latter is very attractive due to its oral formulation. The efficacy of these induction triplets will probably be enhanced by the addition of CD38 monoclonal antibodies (MoAb); accordingly, we foresee the combination of a MoAb plus a triplet based on a PI-IMiD-Dex as the future standard for induction. Intensification with autologous stem cell transplantation (ASCT) is still the standard of care, since it enhances the response rates obtained with these new induction regimens.[5,6] Four randomized trials comparing early and

late ASCT have demonstrated the benefit in progression-free survival (PFS) of early ASCT, although not yet in OS. Two European trials have shown that tandem ASCT is superior to single ASCT, although this was not reproduced in the US STaMINA trial. The role of consolidation therapy is also controversial, while maintenance treatment with lenalidomide (until progression or at least for 1-2 years) is associated with a marked prolongation of PFS (median prolongation of 18 months), and an estimated 2.5-year increase in median OS, according to a meta-analysis.[7] Many aspects of maintenance treatment remain to be clarified, such as the optimal duration, the long term toxicity, the benefits for specific cohorts, and the effects of adding corticosteroids, oral PI (ixazomib) and MoAb. Allogeneic transplant should not be recommended for newly diagnosed patients outside clinical trials.[8] The high efficacy of these treatment strategies has revealed the need for more sensitive techniques (MRD) to evaluate the depth of response both outside the bone marrow (BM) (e.g., using imaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography) and inside the BM (e.g., using immunophenotyping by multiparametric flow cytometry, and molecular analysis by next-generation sequencing). Accordingly, new, revised response criteria have recently been implemented and should also help to avoid over- and under-treatment, and may become a surrogate biomarker for OS and an operational cure.[9]

Treatment of newly diagnosed elderly and non-transplant candidate patients

Six randomized trials have compared thalidomide (T) + melphalan and prednisone (MP) (MPT) with MP alone, showing a median of 6-month prolongation of PFS and OS and it was approved as a standard of care.[10] Administering Rd until progression has become a new standard for elderly MM patients, based on its superiority over MPT in terms of PFS (26.0 *vs.* 21.9 months) and OS (59 *vs.* 49 months).[11] Btz in combination with MP (BzMP) for 9 cycles was associated with a longer TTP (24.0 *vs.* 16.6 months) and one-year prolongation of OS (56 *vs.* 43 months) compared with MP, and has been approved as another standard of care.[12] The Spanish group has combined BzMP (9 cycles) followed by Rd (9 cycles) obtaining a PFS of approximately 3-years. Carfilzomib in combination with MP has shown similar efficacy to BzMP in terms of PFS (22.3 *vs.* 22.1 months) and OS (although the latter data are not yet mature). Investigations of carfilzomib and ixazomib in combination with Rd are yielding encouraging results, particularly for the former combination.

Options for treatment at relapse

Figure 1 summarizes therapeutic options at relapse. The second-generation proteasome inhibitor, carfilzomib in combination with low-dose dexamethasone (Kd) has twice the PFS as bortezomib-dex (btz-dex) (HR: 0.53) and the triplet carfilzomib+len-dex (KRd) is also significantly superior to Rd in terms of PFS (HR: 0.69) and OS (HR: 0.79).[13] Carfilzomib is associated with a very low incidence of peripheral neuropathy but higher cardiovascular toxicity. The oral protease inhibitor, ixazomib has a very good safety profile and, in combination with Rd (IRd), also yielded a longer PFS than Rd (HR: 0.74) but with no significant difference in OS.[14] Pomalidomide, a third-generation IMiD, in combination with low-dose dexamethasone has been approved for treatment of double-refractory patients,[15] and the efficacy can be increased by adding cyclophosphamide or bortezomib.

The use of MoAbs represents a major step forward in MM treatment. Elotuzumab (anti-SLAMF7) has no activity as a single agent but in combination with Rd is significantly superior to Rd alone in terms of PFS (HR: 0.73) and OS (HR: 0.72).[16] The results are even more promising with anti-CD38 (daratumumab, isatuximab, MOR202), since they already demonstrate activity in monotherapy, with an approximately 30% response rate in double-refractory patients. Impressive results have been reported for daratumumab in combination with Rd, with 43% complete response (CR) (including 10% MRD-cases at 10^{-6}) in relapsing patients and a 63% reduction in risk of progression or death compared with Rd (HR: 0.36).[17] Similarly, Daratumumab in combination with btz-dex is also highly superior to btz-dex alone (CR: 20 *vs.* 9%; HR: 0.39 for PFS).[18]

Other immunotherapeutic strategies are being investigated. Anti-BCMA conjugated with monomethyl auristatin-F has produced a clinical benefit in 25% of patients. CD19-CART and BCMA-CART have been tested, and the second one has shown 4 out of 12 partial responses (PRs) in highly refractory patients. The anti-PD-1 drug, pembrolizumab, in combination with lenalidomide or pomalidomide plus dexamethasone gave 36-55% responses in double-refractory patients.

Panobinostat (a hystone deacetylase inhibitor [HDAC]) has been approved for the use in combination with btz-dex for patients who have received at least two lines including btz and len.[19] More selective HDAC inhibitors (HDAC6, acetylon) with improved tolerability are under investigation. Filanesib (a kinesin spindle protein inhibitor) plus dexamethasone has shown ≥22% PR in double-refractory patients. Selinexor (exportin-1 inhibitor) plus dex yielded 20% overall response

rate (ORR) in pentarefractory patients and is synergistic with proteasome inhibitors. The BCL-2 inhibitor venetoclax has shown an ORR of 21%, with 12 of 14 responding patients harbouring t(11;14), and it is also being investigated in combination with Btz-dex.

Future perspectives

Myeloma should no longer be considered as a single entity. This, in conjunction with new monitoring tools, will contribute to treatment individualization. The combination of a MoAb plus a triplet based on PI-IMiD-Dex may become the future upfront standard. Immunotherapy will play an important role in achieving our ultimate goal of curing MM.

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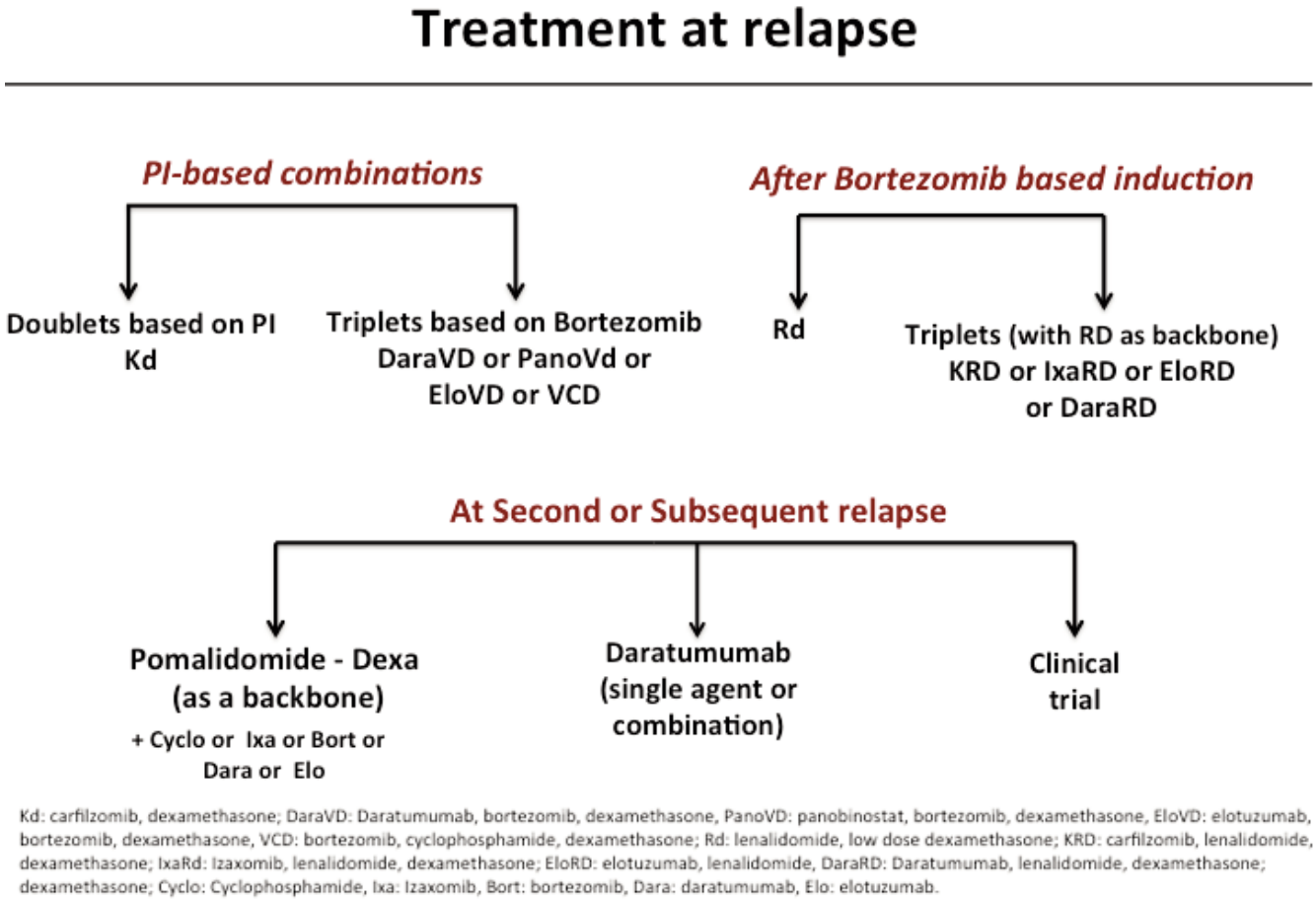

Figure 1. Proposal of therapeutic options at relapse in MM in 2017. The figure summarizes potential therapies for the treatment of relapse patients depending on the sensitivity or refractoriness status to the prior lines of treatment.

Multiple myeloma - Section 3

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Myelodysplastic syndromes

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Introduction

The last years have seen breakthroughs in the pathophysiology of myelodysplastic syndromes (MDS), especially regarding somatic mutations observed in most MDS cases, and the frequent association between MDS and immune abnormalities. While treatment has improved, allogeneic SCT remains the only potentially curtative approach in MDS. J. Jansen describes clonal progression in MDS, with the occurrence of new cytogenetic abnormalities or somatic mutations, driving more aggressive and dominant subclones that may ultimately lead to AML progression. Conversely, some subclones may be particularly sensitive to a given treatment, with disease regression to earlier MDS stage. The author also reports on the progression from clonal hematopoiesis of indeterminate potential (CHIP) to MDS. G. Mufti reports on the immune abnormalities and immune disorders observed in MDS, a situation where it is often unclear if the former induces the latter or vice versa. Recent reports suggest in particular that an inflammatory microenvironment could contribute to the induction of MDS, while genetic abnormalities of MDS cells, and/or the fact that cells of the immune system may be part of the MDS clone could contribute to the development of immune abnormalities. Finally, M. della Porta reviews indications for allogeneic SCT in MDS. While transplant used to be restricted to higher risk MDS according to the classical IPSS, the advent of revised IPSS, the assessment of the somatic mutational profile (most mutations have a poor prognosis) and the impact of severe cytopenias is extending the indications to some IPSS lower risk MDS patients. Prospective studies are however required to validate those new indications.

Learning goals

1. ***Somatic mutations are seen in most MDS, and their number increases during evolution, creating subclones that may compete with each other.***
2. ***Immune disorders are frequent in MDS, and it is not always clear if they are the cause or the consequence of MDS.***
3. ***Allogeneic SCT remains the only curative treatment of MDS. Its indications tend to extend to several types of lower MDS, based in particular on the revised IPSS, the presence of somatic mutations or the importance of cytopenias.***

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Myelodysplastic syndromes - Section 1

Clonal evolution in myelodysplastic syndromes

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Take-home messages

- Many mutations involved in the pathogenesis of MDS have been identified. These mutations may be used for diagnostic and prognostic purposes and in some selected cases, may contribute to therapeutic decisions.
- Accumulation of genetic mutations leads to the evolution of genetically distinct MDS subclones that may co-exist, or outcompete each other.
- Treatment may create an evolutionary bottleneck and alter the clonal composition of the disease.

Introduction

By the application of whole genome and exome sequencing technology, many genetic mutations underlying the pathogenesis of myelodysplastic syndromes (MDS) have been identified. Most of the recurrently affected genes can be classified in a limited number of biological categories, including transcription factors, signal transduction proteins, epigenetic modifiers, proteins involved in RNA splicing and proteins of the cohesin complex.[1-3] Different patients carry different combinations of mutations, which correlates with the heterogeneity that is seen in MDS. The prognostic value of these mutations is investigated in clinical trials and registries, and in the various risk scoring systems, molecular aberrations are becoming increasingly important. However, the genetic aberrations that are present in a given MDS patient are not stable over time. During the course of the disease, the genetic composition of the MDS cells may evolve due to the acquisition of additional mutations (for an example, see Figure 1). This may lead to altered biological behavior of the MDS cells, including their sensitivity to specific forms of therapy. The assessment of the genetic composition, and the genetic evolutionary patterns will be increasingly important for proper clinical decision making.

Current state of the art

Oncogenesis is a multistep evolutionary process and the successive acquisition of several mutations that confer a selective advantage may result in progression of the disease.[4-6] During life, all cells may acquire genetic mutations, caused by different mechanisms such as irradiation, chemical exposure and DNA-copy errors during cell division. Most of these mutations are non-pathogenic, but when a mutation occurs in a gene that is involved in maturation and growth regulation, this may result in a proliferative advantage and the expansion of a clone of cells carrying this mutation. In addition to this pathogenic mutation (driver mutation), the cells will also carry along any pre-existing non-pathogenic mutation (passenger mutations). The subsequent acquisition of additional mutations may result in a further growth advantage. Recent studies indicate that clonal expansion of hematopoietic cells occurs more often than initially thought.[7-9] In many healthy individuals, expanded clones of hematopoietic cells can be found that carry somatically acquired mutations in genes that have been implicated in hemato-oncological diseases. In most of these cases, only one mutation is found to be present and apparently, these cells do not (yet) carry enough mutations to be completely transformed resulting in clinical symptoms. The incidence of such clonal processes in healthy individuals (termed CHIP, clonal hematopoiesis of indeterminant potential) increases with age, and depending on the sensitivity at which mutations are screened for, above the age of 70 as much as 10% or more of the general population may carry significantly large, clonal hematopoietic populations. When more pathogenic mutations are acquired, the cells may become more malignant. In MDS, mutations in epigenetic regulators including TET2, ASXL1, IDH1/2, EZH2 and DNMT3A are often found, as well as mutations in genes that code for proteins involved in RNA splicing, including SF3B1, SRSF2, U2AF1 and ZRSR2. These mutations are not specific for MDS, but especially the splicing factor mutations are more often found in MDS compared to several other myeloid malignancies. In the case of

Myelodysplastic syndromes - Section 1

MDS with ring sideroblasts, a very high correlation has been found with the occurrence of SF3B1 mutations. Some mutations are often found together within a certain patient, whereas other mutations are mutually exclusive. Mutations in genes that are involved in the same biological process are not likely to enhance each other's transforming effect; cells that acquire an additional mutation that affects an already activated pathway have no extra growth advantage and are therefore not clonally expanded. In contrast, mutations that affect genes from different biological processes may have an additive or synergistic effect on malignant transformation, and a combination of such mutations may confer a further growth advantage, causing the cells that harbor both types of mutations to clonally expand. In MDS both linear and branching patterns of evolution have been described. Linear evolution is characterized by the successive appearance of dominant clones that overgrow their ancestral clone after the acquisition of additional mutations. Branching evolution is characterized by the emergence of different subclones from one common ancestral clone, leading to the co-existence of related (sub)clones that contain a partially overlapping set of mutations.[10,11] The genetic evolution which may take place over time within a specific patient may result in a change of disease characteristics and progression towards a more aggressive disease, including the development of acute myeloid leukemia. Using exome- and targeted deep-sequencing, clonal evolution can be monitored. The order in which specific mutations are acquired in time differs between patients, but in general, mutations in genes involved in RNA splicing and DNA methylation appear to occur early, whereas mutations in genes involved in chromatin modification and signal transduction tend to occur later. Recent studies demonstrate that therapy can influence clonal evolution by suppressing or selecting particular subclones, while being ineffective towards others.[12-15] For example, treatment with lenalidomide leads to an efficient suppression of malignant cells harbouring a deletion of chromosome 5q. This response may however be lost when an additional *TP53* mutation is acquired and TP53 mutated subclones may emerge dur-

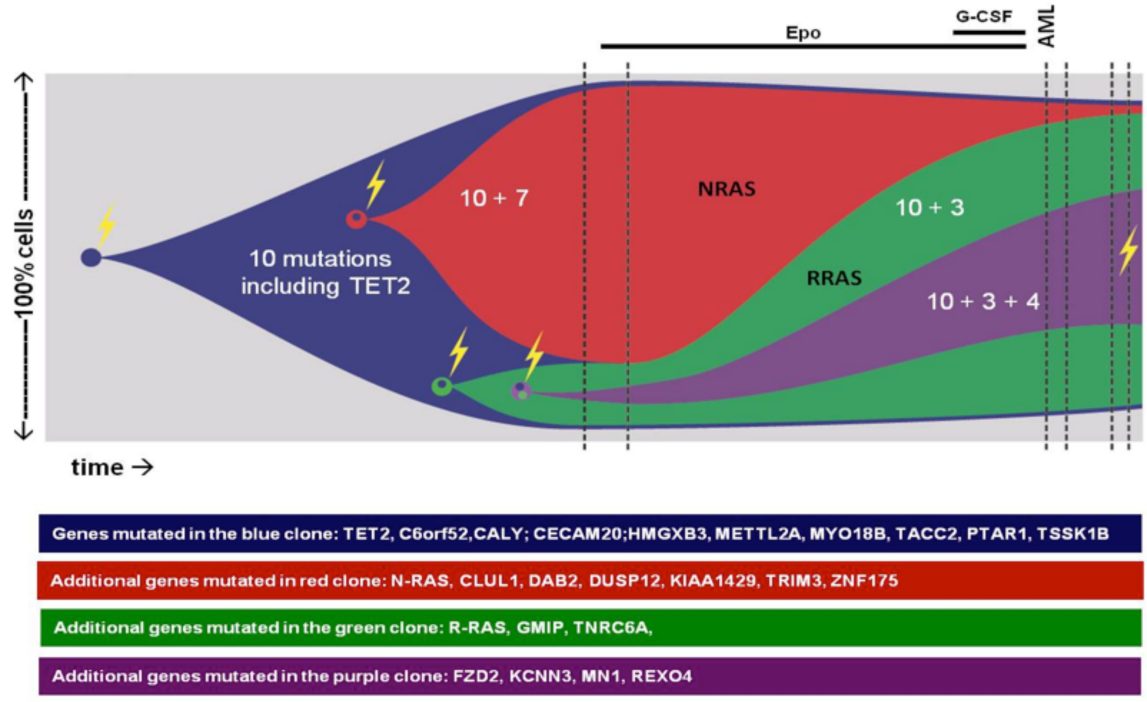

Figure 1. Clonal evolution pattern in a MDS patient over the course of several years. Initially, a clone of cells (blue clone) developed that contained 10 different mutations (passenger plus driver mutations). Subsequently, a branched evolution pattern was observed; from the blue clone, two daughter clones developed that carried an extra 7 (red clone) or 3 (green clone) mutations. Within the green clone another subclone developed (purple) containing yet another 4 mutations. Eventually, the disease progressed into AML. (EPO=erythropoietin, G-CSF= granulocyte colony stimulating factor, AML=acute myeloid leukemia)

ing treatment. In addition to differences between patients, also intra-tumoral differences may be present. Often, genetically different subclones with a different set of mutations are present simultaneously, even before treatment. As the genetic diversity amongst these co-existing subclones may result in a different response to therapy, the genetically complex cases with various subclones may be more difficult to treat. Some subclones may be resistant to one form of therapy, while others are resistant to other treatment modalities, necessitating a multimodal treatment approach. Finally, therapy can create an evolutionary bottleneck, potentially expediting or altering the evolutionary process of the disease. If treatment reduces the hematopoietic compartment but fails to eradicate all the MDS cells from the different subclones, preferential repopulation of the bone marrow may occur by resistant subclones that are able to outcompete the others, including the healthy cells.

Future perspectives

Comprehensive genetic analyses may give insight in subclonal composition and clonal evolution patterns in MDS. Clinically, this information may be used for prognostication at diagnosis, to predict response to specific forms of therapy, to follow and predict disease progression and to monitor the effectiveness of therapy. Sensitivity and specificity of the techniques that are used to simultaneously screen for many different mutations can still be improved. Depending on the sequence context, not all mutations can easily be called reliably when they are present in a small fraction of the cells, and better techniques and bioinformatic algorithms are still under development. Implementation in clinical practice will be enhanced by the decreasing cost of next generation sequencing technology, but most importantly, more extensive studies are still necessary to further establish the true predictive value of the various combinations of mutations, both with and without therapy.

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Myelodysplastic syndromes - Section 2

The role of immune response in myelodysplastic syndromes pathophysiology

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Take-home messages

- The role of immune system in MDS pathophysiology and genomic instability.
- The importance of immune-signature "switch" in disease progression.

Introduction

Myelodysplastic syndromes (MDS) are part of a larger group of diseases known as bone marrow failure syndromes (BMFs). BMFs are ranging from mainly autoimmune aplastic anemia (AA)[1] to MDS, which characterized by ineffective hematopoiesis and increased risk of transformation to acute myeloid leukemia (AML).[2,3] Like many other malignancies, a combination of environmental and genetic factors as well as immune dysregulation contribute in MDS pathophysiology. Nevertheless, the sequence of events that lead to dysplasia and subsequent malignant transformation as well as the interaction/role of other contributing factors are not fully understood in MDS.

The role of immune system in MDS pathophysiology

Despite the established role of chronic inflammation in the pathogenesis of many malignancies, its potential role in MDS remains less clear. Until recent years, autoimmune diseases (AID) were considered as coincidence rather than as a predisposing factor for MDS. However, in a comprehensive study on more than 10,000 AML and MDS patients and 43,000 healthy donors, it has been shown that a history of any infectious disease three or more years before AML/MDS, was associated with around 1.5 times increase in risk of both diseases. A previous history of any autoimmune disease was also associated with a 1.7-fold increased risk for AML and 2.1-fold increased risk for MDS.[4] On the other hand, AID can be a favorable prognostic factor in patients who have established MDS. In a collaborative study between the Moffitt Cancer Centre and King's College London, 1408 patients with MDS and AID were studied and we demonstrated that the presence of AID independently increased overall survival in MDS, which may be due to initiation of a 'protective' adaptive immune response.[5]

There is also evidence of the presence of "smouldering" inflammation in MDS in the absence of classic autoimmune diseases. It has been shown that augmented levels of pro-inflammatory cytokines (ie. TNF-α, IFN-ϒ and IL-1β) lead to bone marrow apoptosis in MDS[6-9]. Impaired clearance of apoptotic cells in MDS induces HMGB1 and TLR-4 mediated cytokine production and a vicious circle of inflammation and apoptosis[10]. The increased levels of TNF-α and IFN-ϒ lead to overexpression of an immunoinhibitory molecule, B7-H1 (CD274), which can convey a growth advantage to MDS clone[11]. Moreover, treatment of underlying MDS leads to improvement of immune mediated 'para-neoplastic' diseases like Sweets syndrome[12]. In a study on more than 200 MDS patients, we have shown that the *NLRP3* variants are enriched in MDS patients with Sweet's syndrome compared to historical data on the general population, suggesting an inflammasome-mediated chronic inflammation in these patients[13]. One of the major effects of chronic inflammation is an increased proliferation pressure on stem/progenitor cells and subsequent genetic instability and somatic mutations. In recent years, comprehensive mutational profiling has helped identify the presence of somatic mutations in nearly 80-90% of MDS patients, some of which correlate with clinical phenotype, predict outcome and response to therapy, especially hypomethylating agents[14]. These somatic mutations could lead to expression of neo-antigens which some of them are immunogenic and provoke cellular immune response. Using a combination of HLA-typing, somatic mutation analysis and antigen prediction algorithm (NetMHCpan3.0) in a cohort of 109 MDS patients, we have investigated the immunogenicity of known somatic mutations in low and high risk MDS and shown that patients with predicted neoantigens had significantly longer survival compared to the patients without neoantigens.

Cellular immune response in established MDS

Similar to the role of inflammation in the initiation of MDS, cellular immune response in established MDS is multifactorial and follows a stepwise transformation as the disease progresses toward AML. While there is enough evidence to support a relatively effective cellular immune response in low risk MDS, in high risk disease, accumulation of inflammatory derived myeloid suppressor cells (MDSCs)[15] and increase in regulatory T cells (Tregs) switch the effective immune response to a suppressive response. We have shown a significant increase in the number of Tregs in high-risk disease whereas on the low risk disease the main feature was an increase in the number of pro-inflammatory Th17 cells[16,17]. The importance of Th17 and the balance between Tregs and Th17 in the disease progression and bone marrow apoptosis has also been shown in low risk MDS. However, the difference between low and high risk MDS is not limited to $CD4^+$ T cells and similar differences are reported for other immune cells such as natural killer (NK)[18] cells, DCs[19] and MDSCs[15,20]. In summary, inflammatory environment, which is likely to be the result of a combination of chronic infection/autoimmune diseases, age related increase in pro-inflammatory cytokines and activation of inflammasome pathway in myeloid cells, plays a crucial role in MDS pathophysiology. This inflammatory environment has two overlapping effects; first is to induce apoptosis in stem/progenitor cells and subsequently increase proliferation pressure on these cells and secondly induces an auto-reactive adaptive immune response against stem/progenitor cells due to epitope spreading following apoptosis. The

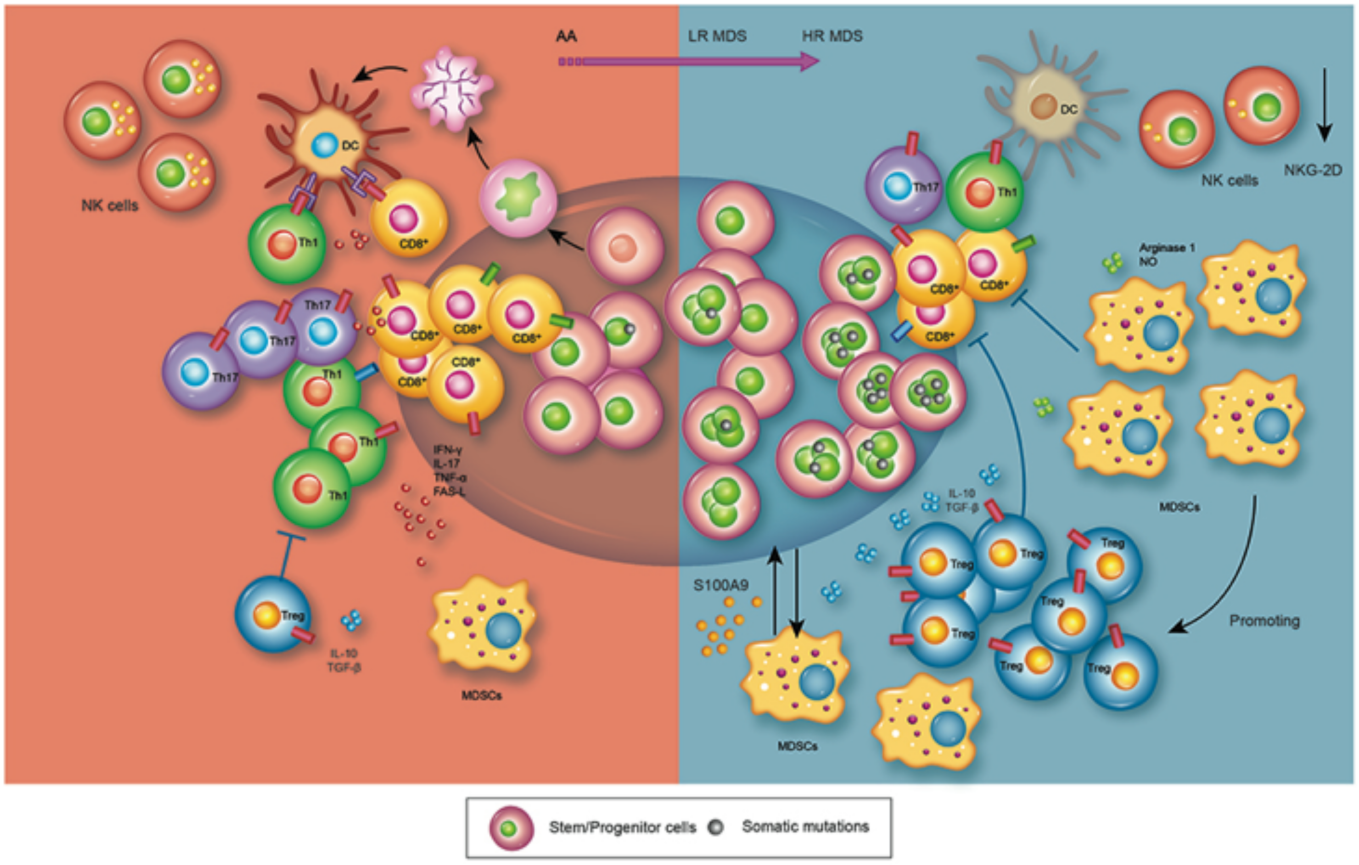

Figure 1. Immune response in MDS: The immune-signature of bone marrow failure syndromes varies and ranging from a pro-inflammatory response in AA and low-risk MDS to a more immunosuppressive environment in high risk MDS. While pro-inflammatory T cells such as Th1 and Th17 cells are more prominent in AA and low risk MDS, the suppressive immune cells such as Tregs and MDSCs play the dominant role in high risk MDS.

expansion of auto-reactive T cells would contribute further in the aforementioned proliferation pressure. Age related inefficient DNA repair mechanisms, genetic background such as detoxification genes' polymorphism and possibly HLA type as well as environmental factors are additional contributing factors into this vicious circle of inflammation-apoptosis-immune response. Adding proliferation pressure to the aged and already genetically unstable progenitor cells substantially increases the chance for acquiring additional mutations (early initiation mutations) and development of dysplasia (critical stage). At this stage while the adaptive/innate immune-surveillance (good inflammation) to some extent keep the dysplastic clone under check, the smouldering myeloid related inflammation fuels the proliferation pressure, which facilitate immune-selection and growth advantage of dysplastic clone(s). Simultaneously the inflammatory environment promotes the expansion of MDSCs, which contribute to the expansion of dysplastic clone and suppress the effective immune-surveillance both directly and indirectly through expansion of Tregs. As disease progresses, subsequent co-operating mutations appear and give growth advantage to dysplastic clones and further genetic instability. At this stage, a combination of MDSCs/ Tregs expansion and reduction in the number and function of effector immune cells and APCs lead to an ineffective immune-surveillance and immune-subversion similar to other malignancies and expedite disease progression to AML (Figure 1). It is anticipated that with advances in the immunology of MDS/AML, therapeutic strategies will evolve that will specifically affect distinctive immunological pathways so as to increase protective immunity against the early as well as evolving dysplastic/leukemic clones, the advance of immune check point inhibitors is hopefully the start of this journey.

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The role of MDSCs in MDS pathophysiology

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Myelodysplastic syndromes - Section 3

Indications for transplantation in myelodysplastic syndromes

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Take-home messages

- Selection of MDS patients candidate to allogeneic transplantation should be based on both disease and patient-related factors
- Revised IPSS (IPSS-R) is expected to improve the choice of optimal timing of transplantation in early disease stages
- The use of hypomethylating agents is of increasing interest as part of a comprehensive strategy to prevent relapse after transplantation in high risk patients
- Somatic mutations may provide more accurate risk stratification of individual patients and further refine transplantation decision-making in MDS

Introduction

Despite improved understanding of the molecular pathogenesis of myelodysplastic syndromes (MDS) currently available therapies lead to prolongation of life and no cure.[1] Therefore, allogeneic hematopoietic stem cell transplantation (HSCT) is increasingly used as a curative treatment option. This increase in HSCT activity can be attributed largely to the introduction of reduced-intensity regimens that have extended the indication for HSCT to older patients with comorbidities or reduced fitness.[2] Despite its curative potential, because of the inherent complications of the transplantation leading to treatment-related mortality and the risk of relapse, a careful calculation of the benefit for each patient is mandatory, taking into account disease status, comorbidities and effective non-transplant therapies.[1,3]

Current status of the art

Which tools are available for transplantation decision making?

Since MDS range from indolent conditions to subtypes analogous to acute myeloid leukemia (AML) a risk-adapted treatment strategy is mandatory. Prognostic factors may be subdivided into those related to the patient's general health condition and those related to the characteristics of the MDS clone.[1] The definition of disease-related risk in MDS is based on the use of International Prognostic Scoring System (IPSS).[4] A number of studies have shown that advanced disease risk at transplantation is associated with inferior survival, and cytogenetic abnormalities (i.e., complex/monosomal karyotype) have been found to be predictive of high risk of disease relapse.[2] Recently a revised version of IPSS (IPSS-R) was proposed, including five cytogenetic risk groups together with refined categories for marrow blasts and cytopenias.[5] In patients receiving HSCT, IPSS-R score significantly improves the prediction of patient prognosis with respect to IPSS.[6] The implementation of IPSS-R is expected to result in a more effective selection of candidates to HSCT among patients with early disease stage.

Different patient-related factors may affect clinical outcome and decision-making in MDS. The majority of trials in patients treated with HSCT consider a patient's age as a major prognostic factor for non-relapse mortality (NRM). However, these results were obtained mainly after standard myeloablative conditioning. Two recent large studies address the specific issue of elderly MDS receiving reduced intensity conditioning regimens (RIC).[7,8] There was only a trend for a higher incidence of relapse and NRM in the group >60 vs. <60 years of age, which was not statistically significant. Because patient's age per se is not a major risk factor, other factors, such as comorbidities are taken into account. Sorror et al. found that comorbidity predicts posttransplantation outcome in MDS, and they developed the Hematopoietic Cell Transplantation Comorbidity Index (HCT-CI) to estimate the individual risk of NRM after HSCT.[9] Accounting for both disease- and patient-related factors considerably improves risk stratification than considering IPSS alone.

Myelodysplastic syndromes - Section 3

What is the optimal timing of transplantation?

Although transplantation early after diagnosis is associated with the most favourable post-transplantation outcome,[2] it remains unclear whether early transplantation leads to maximal life expectancy for patients with early stage MDS that may experience a long period of stable disease after diagnosis. A previous decision analysis by the IBMTR concluded that life expectancy of patients with low or intermediate-1 IPSS risk was higher when transplantation was delayed but performed before the progression of AML. Conversely, for high-risk MDS transplantation soon after diagnosis conferred the best prognosis.[10] This study has substantially influenced clinical practice. Preliminary data suggest that the clinical implementation of IPSS-R may improve transplant decision making process.[11] Using IPSS-R, the estimated life expectancy was maximized when transplantation was delayed until progression from the very low/low to the intermediate risk, and then decreased (Figure 1). Within the low and intermediate-1 IPSS risk, IPSS-R identified a subgroup of patients (30%) who may benefit from early transplantation. Overall, there was a 2-year gain in life expectancy using the IPSS-R vs. IPSS-based transplant policy.[11]

Should cytoreductive treatment be performed before transplantation in high risk patients?

In patients with advanced disease, disease relapse represents the leading cause of transplant failure. The issue of performing cytoreductive treatment before HSCT to reduce the risk of relapse is a matter of debate. Concerns about AML-like chemotherapy mainly include risk of long-lasting myelosuppression and organ toxicities.[1,3] It should be considered in addition that there is no definitive evidence of a survival benefit associated with administering chemotherapy before HSCT

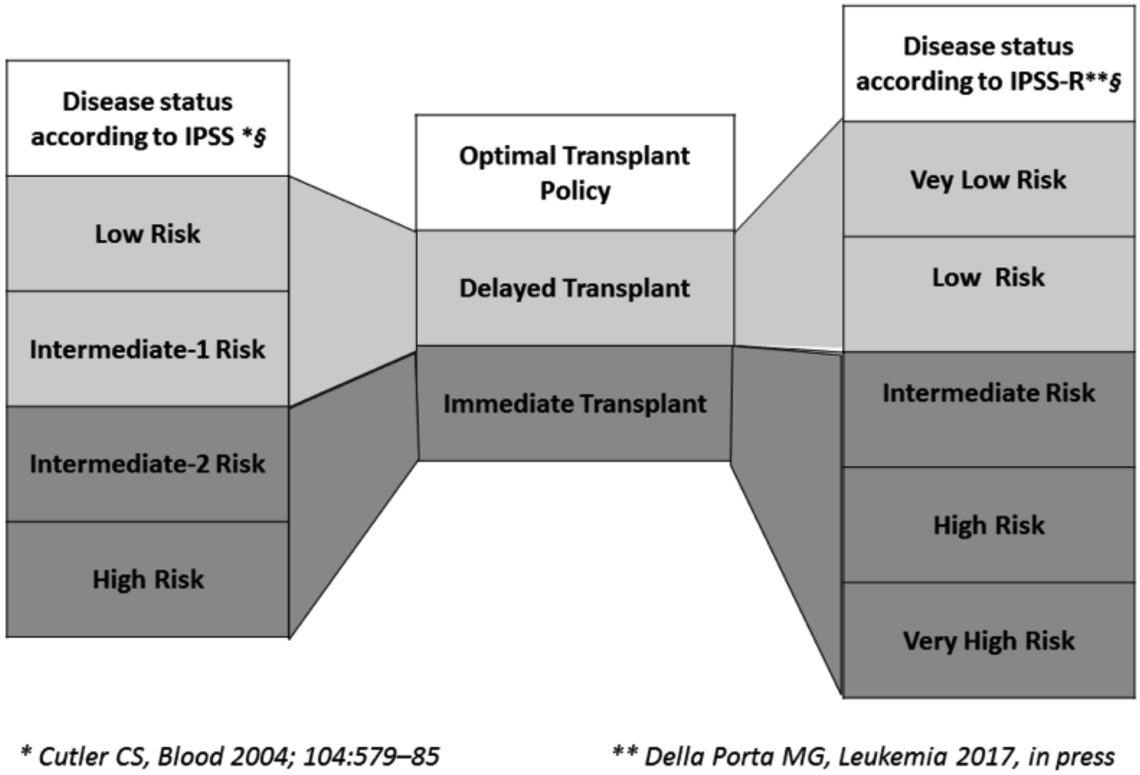

Figure 1. Decision analysis of allogeneic hematopoietic stem cell transplantation for patients with myelodysplastic syndrome stratified according to currently available prognostic scores.

in MDS.[12]

The availability of hypomethylating agents (HMA) has changed the landscape of MDS treatment. Importantly, HMA were found to be effective even in MDS who had unfavourable cytogenetics and/or TP53 mutations, in which chemotherapy is clearly ineffective.[13,14] Although azacitidine and decitabine can induce hematological and cytogenetic responses, these therapies do not appear to eradicate MDS clones, and recent data suggest that even in high risk patients aged >60 years, transplantation (RIC) offers survival benefit with respect to non-transplant procedures.[15] Several studies have evaluated the role of HMA given before transplantation, though very few were conducted prospectively. Overall, these investigations showed similar post-transplantation outcome for patients receiving HMA vs. chemotherapy. Moreover, in some cases an improved survival of patients transplanted in complete remission vs. active disease at the time of HSCT was reported.[16] In the absence of data from prospective trials, the decision to perform a cytoreductive treatment should be made accounting for clinical considerations with respect to each specific patient.

Future perspectives

Mutations in several genes have been reported to influence survival and risk of disease progression in MDS.[17] MDS associated with SF3B1 mutations form a distinct entity with a favourable prognosis, while SRSF2, RUNX1, U2AF1, ASXL1 and TP53 mutations are associated with increased risk of leukemic evolution.[18] The integration of somatic mutations into prognostic scoring systems may provide more accurate risk stratification of individual patients and further refine clinical decision-making in MDS. A recent study in 401 patients who received HSCT for MDS or MDS/AML showed that somatic mutations in ASXL1, RUNX1, or TP53 were associated with unfavorable outcomes and shorter survival.[19] A larger CIBMTR study reported relevant new findings. RAS pathway mutations and JAK2 mutations were associated with a poor outcome after HSCT, independently of TP53 mutations in patients >40 years.[20] Possible interventions in patients with high risk of disease relapse according to genotype may include the anticipation of the transplant procedure in early disease phase, the use of innovative conditioning regimens to increase the probability to eradicate MDS clone, and prophylaxis of disease recurrence after transplantation. These results serve as a proof of concept that the integration of somatic mutations significantly increase the capability to capture prognostic information in MDS patients receiving HSCT, and may provide a basis for improving transplantation decision-making.

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Myeloproliferative neoplasms

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Introduction

The knowledge regarding the pathogenesis and prognosis of the classic Philadelphia chromosome negative myeloproliferative neoplasms (MPN), that include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), has increased tremendously in the last 15 years. Since the discovery of the JAK2V617F mutation in 2005, additional mutations in JAK2 exon 12, as well as in the calreticulin and mpl genes have been identified. In PMF, several additional non-driver mutations coupled with worse prognosis have also been delineated. The WHO classification of MPN:s from 2016 recognize the pathogenetic and clinical importance of these findings. The need for bone marrow assessment in the work-up of MPN patients is emphasized, an accurate diagnosis is an essential first step in the management of MPN. Important therapeutic progress has been made with the increased use of interferon-α in PV, as well as the introduction of JAK2 inhibitors both in PMF and PV. Despite a profound clinical benefit of these agents, further improvement is needed. Many of the above mentioned mutations represent potential targets for therapy, and may include type II JAK inhibitors, PI3 kinase inhibitors, immunotherapy against calreticulin, telomerase inhibition, recombinant human pentraxin-2 among others, or a combinatorial approach utilizing more than one agent.

Learning goals

1. ***To gain knowledge regarding the driver mutations that represent major diagnostic criteria for MPN in the revised 2016 WHO classification, as well as additional mutations associated with worse prognosis in PMF.***
2. ***To be able to use in daily clinical practice JAK2 inhibitors, interferon and other agents that have significantly advanced MPN treatment.***
3. ***To gain an insight into the fact that several avenues of research are being explored with the goal of improving the therapy of especially PMF patients including combination treatments with existing JAK inhibitors, developing mutant specific JAK2 inhibitors, and potentially immunological targeting of mutant CALR, as well as other avenues of treatment.***

EUROPEAN HEMATOLOGY ASSOCIATION

Myeloproliferative neoplasms - Section 1

Molecular genetics in negative myeloproliferative neoplasms

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Take-home messages

- Mutational profiling of MPN should include driver mutations and, in selected cases, additional mutations in genes associated more generally with myeloid malignances; in about 10-15% of the patients, however, no driver mutation is detected ('triple negative').
- Driver mutations represent major diagnostic criteria for MPN in the revised 2016 WHO classification and, therefore, these should be determined in each patient.
- Both driver and additional non-driver mutations are associated with phenotypic traits and, importantly, they may contribute to estimate prognosis.

Introduction

The classic Philadelphia chromosome negative myeloproliferative neoplasms (MPN), that include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), originate from a single hematopoietic stem cell (HSC) that, having somatically acquired one or more mutations, expands and produces a progeny of terminally differentiated myeloid cells of different lineages as well as, at least in some cases, B and NK cells.[1-3] Phenotypically, MPN are very akin in terms of clinical presentation: thrombosis, hemorrhage, burdensome systemic manifestations, splenomegaly, progression to secondary forms of myelofibrosis (in case of PV and ET) and acute leukemia or myelodysplasia, represent the main clinical events occurring over the course of the disease, and may eventually lead to death.[4] On the other hand, the clonal expansion of mutated HSC may manifest with either prevalent multilineage (PV, MF) or unilineage hyperplasia (ET); the genetic mechanisms that contribute to the uniqueness of one versus another form of MPN represent one of the major unsolved issues in the understanding of molecular pathogenesis of MPN. In fact, no unique phenotype-restricted single disease-associated mutation has been discovered. Rather, MPN are characterized by a restricted set of largely overlapping driver mutations, that include the *JAK2*V617F mutation (found in >95% of PV and 60% of ET and PMF), *MPL* mutations, mainly at codon 515 (3-8% in ET and PMF, rare atypical *MPL* mutations also exist, including S505N mutation initially reported as germline mutation), calreticulin mutations (*CALR*; 20-25% in ET and PMF), and also mutations of *JAK2* exon 12 in 3% of *JAK2*V617F-negative PV. [5,6] Non-canonical mutations in *JAK2* and *MPL* have been reported in a minority of 'triple-negative' (TN) patients with ET and PMF, as these patients have operationally been defined by the lack of known driver mutations that currently comprise 10-15% of ET and PMF cases.[9] It is also likely that some cases currently defined as ET are not true clonal disorders, but might include uncharacterized familial variants. When expressed in the mouse, the above driver mutations reproduce an MPN-like disorder, with some differences among models due to the experimental setting and, possibly, the extent of intracellular JAK2 signaling.[10] In fact, abnormal activation of the JAK/STAT signaling pathway is a shared characteristic of the above mentioned driver mutations, and this finding further complicates the understanding of mutation-phenotype correlates. On the other hand, the uniform activation of JAK2 signaling in MPNs helps to explain the reported clinical efficacy of JAK1/2 inhibitors, for which the underlying mutation status is largely irrelevant.

Many patients with MPN (about 20-50% of ET and PV, and even more in PMF) harbor additional mutations (listed in Table 1) that are non-exclusive of MPN, since they are found also in acute leukemia or myelodysplasia, and do not show any selective clustering with driver mutations. The most common mutations target regulators of DNA methylation (*TET2, DNMT3A*), histone modifiers (members of the Polycomb repressor complex 1 & 2, IDH1/2), components of the splicea-

some (*SF3B1, SRSF2*), transcription factors (TP53, CUX1, Ikaros), *bona fide* oncogenes (*NRAS, KRAS*) and proteins involved in signaling (*LNK, CBL*), to name the most frequently involved genes. These mutations usually occur as subclonal events, often with low variant allele frequency and, therefore, their role in disease initiation is difficult to assess. However, they have been associated with important clinical end-points (survival, rate of leukemia transformation).[11,12] Often, these subclonal mutations occur as multiple events, reflecting an intrinsic genetic complexity of the disease and likely expressing a higher propensity to clonal progression. This might contribute to their prognostically negative impact;[11] indeed, patients with PMF have multiple mutations more commonly than patients with PV and ET.[12] The interpretation of the above

Table 1. List of the most frequently detected mutations in patients with myeloproliferative neoplasms.

	Localization	Function	Type of abnormalities
Signaling			
JAK2ex14	9p24	Tyrosine Kinase, signaling	Gain of function
JAK2ex12	9p24	Tyrosine Kinase, signaling	Gain of function
MPL	1p34	Receptor, signaling	Gain of function
CALR	19p13	ER-associated multifunction protein	Gain of function through wild-type MPL
SH2B3(LNK)	12q24	Adaptor, signaling	Loss of function
CBL	11q23	Adaptor, E3 ubiquitin ligase, signaling	Dominant negative
SOCS1	16p13.2	E3 ubiquitin ligase, signaling	Methylation
SOCS2	12q22	E3 ubiquitin ligase, signaling	Methylation
SOCS3	17q25.3	E3 ubiquitin ligase, signaling	Methylation, mutation
Epigenetic			
TET2	4q24	DNA hydroxymethylation	Loss of function
ASXL1	20q11.21	Chromatin modifications	Loss of function
EZH2	7q35	Chromatin methylation	Loss of function
JARID	6p24	Chromatin methylation	Loss of function
SUZ12	17q11.2	Chromatin methylation	Loss of function
IDH1	2q33.3	Metabolism	Neomorphic enzyme
IDH2	15q26.1	Metabolism	Neomorphic enzyme
EED	11q14.2	Chromatin methylation	Loss of function
Splicing			
SRSF2	17q25.1	Spliceasome	Loss of function
U2AF1	21q22.3	Spliceasome	Loss of function
ZRSR2	7q25.1	Spliceasome	Loss of function
SF3B1	2q33.1	Spliceasome	Loss of function
Leukemia progression			
TP53	17p13.1	Cell cycle, apoptosis	Loss of function
SMD4	1q32	TP53 regulator	Amplification
DNMT3A	2p23	Chromatin modifications	Loss of function
RB	13q14	Cell cycle, apoptosis	Deletion
IKZF1	7p12	Transcription factor	Deletion
RUNX1	21q22.3	Transcription factor	Loss of function
NRAS	1p13.2	GTPase, signaling	Gain of function

findings is further complicated by the discovery of the phenomenon of clonal hematopoiesis of indeterminate potential (CHIP), reflecting the acquisition of similar mutation profiles as an age-dependent phenomenon in the otherwise healthy population.[13] The two most frequent mutated genes in the settings of CHIP are *TET2* and *DNMT3A*; it is hypothesized that by increasing the self-renewal capability of HSC, these mutated genes might favor the acquisition of secondary mutations eventually resulting in clonal predominance. Recently, evidence has also been provided that the order in which driver mutations are acquired on the background of other somatic mutations affects disease presentation.[14]

Little is known about the genetic events that promote transformation of a chronic disease to acute leukemia; these aggressive secondary leukemias do not display, if rarely at all, the typical genetic abnormalities found in *de novo* leukemia (such as FLT3, NPM1), but are enriched in mutations of the *TP53* gene, including missense mutations or deletions.[15] Also, amplification of the locus of MDM4, a TP53 transcriptional inhibitor, has been reported as a mechanism of functional insufficiency of TP53. Clones harboring mutated *TP53* may sporadically be found even in the early chronic phase of MPN, thus they are not strictly predictive of the risk to develop leukemia in the individual patient; rather, the transition from mutated *TP53* heterozygosity to homozygosity likely represents one key mechanism responsible for the development of leukemia.

Analysis and characterization of driver and non-driver mutations currently deserves a remarkable role for diagnosis and has increasing relevance for prognostication in patients with MPN. Driver mutations are listed as major diagnostic criteria in the revised 2016 World Health Organization classification,[16] while the detection of non-driver mutations may support evidence of clonal hematopoiesis existing in patients who present a hematologic phenotype suggestive of an underlying MPN but lack driver mutations. Furthermore, there are phenotypic and prognostic correlates with driver mutations (for example the lower risk of thrombosis in *CALR* mutated patients with ET compared to the *JAK2*V617F/*MPL* mutated ones[17] or the negative impact of triple negativity on survival in PMF[18]) as well as with non-driver mutations (the adverse prognostic significance of the so called 'high mutation risk' (HMR) phenotype, represented by mutations in *ASXL1, EZH2, SRSF2, IDH1/2* in PMF[19]). Further efforts are required in order to integrate all of this information with already validated clinical and hematologic variables, and possibly with other genetic phenotype modifiers, in order to obtain a full picture of the epidemiology, clinical significance and pathogenetic role of such a complex genetic background; this knowledge might be key to facilitate identification of novel targets for therapy.[20]

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Targeting specific mutations in myeloproliferative neoplasms

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Take-home messages

- Mutations in three genes (i.e. *JAK2, CALR* or *MPL*) drive the development of an MPN phenotype and all lead to activated JAK-STAT signaling.
- Although the development of JAK inhibitors has significantly advanced MPN treatment, these agents have a number of adverse side effects and are not curative.
- Several avenues of research are being explored with the goal of improving the clonal selectivity of current MPN therapies, including combination treatments with existing JAK inhibitors, developing mutant specific JAK2 inhibitors, and potentially immunological targeting of mutant CALR.

Introduction

The discovery of the JAK2V617F mutation in 2005[1,2] was a major advance in the MPN field and subsequently prompted a closer look into the mutations that drive JAK2V617F–negative MPNs. In the ensuing years, mutations in *JAK2* exon 12 were identified in the majority of V617F-negative polycythemia vera (PV) patients,[3] and mutations in the thrombopoietin receptor, *MPL* were found in a small percentage of essential thrombocythemia (ET) and primary myelofibrosis (PMF) patients.[4] More recently, two groups identified recurrent mutations in the gene calreticulin (*CALR*) in the majority of patients with non-mutated *JAK2* and *MPL*.[5,6] Recent work indicates that *CALR* mutations confer a neomorphic function on mutant CALR that results in activation of MPL signaling.[7] Despite their distinct molecular etiologies, the unifying hallmark of all MPN subtypes is the pathological activation of JAK-STAT signaling. This finding has provided a robust scientific rationale for therapeutic inhibition of the JAK-STAT pathway in MPN patients.

Current state of the art

First-generation JAK inhibitors for treatment of MPN

The discovery of activating *JAK2* mutations changed the landscape of MPN treatment dramatically, quickly prompting the development of small molecule inhibitors of JAK2. First-generation JAK inhibitors are ATP-competitive antagonists of the JAK kinase domain and each inhibits the activity of one or more JAK isoforms to different degrees. Ruxolitinib, a dual JAK1/2 inhibitor, was the first of its class to be approved for treatment of PMF and post-PV/ET MF. Treatment with ruxolitinib has been shown to reduce spleen size and alleviate systemic symptoms of MF,[8] although the myelosuppressive effects of ruxolitinib, particularly anemia, are problematic for MF patients. Subsequent JAK2 inhibitor development efforts sought to overcome the myelosuppressive effects of ruxolitinib, and have had varying levels of success in clinical development. Pacritinib, a JAK2/FLT3 inhibitor, was designed for PMF patients presenting with low platelet counts, and was shown to reduce splenomegaly without worsening thrombocytopenia.[9] Momelotinib, a JAK1/2 inhibitor currently in phase 3 trials for PMF and post PV/ET MF, demonstrated efficacy in reducing splenomegaly while improving anemia in phase II.[10]

Combination therapy

To improve upon JAK inhibitor monotherapy, several preclinical combination treatments are currently being explored. There are a number of potential partners for ruxolitinib, including those that target aberrantly activated pathways that are either downstream of or convergent upon the JAK-STAT pathway (Figure 1A). A number of studies have shown that combined inhibition of JAK2 and the phosphatidylinositol 3-kinase (PI3K) pathway synergistically inhibits MPN cell proliferation.[11] A phase 1b clinical trial is underway to evaluate ruxolitinib in combination with the PI3K inhibitor, buparlisib (NCT01730248). Additionally, given the interaction between the JAK-STAT pathway and the RAS-RAF-MEK pathway, the

effect of combined MEK and JAK2 inhibition has been tested and synergistic effects on MPN cell viability *in vitro* and enhanced survival in a murine model of MPN were demonstrated.[12]

In addition to targeting aberrant signaling pathways that work in concert with the JAK-STAT pathway, combination therapies targeting proteins that stabilize JAK2V617F are also being explored. JAK2 has been shown to be a chaperone client of heat shock protein 90 (HSP90), and treatment with the HSP90 inhibitor, PU-H71 leads to degradation of both JAK2V617F and HSP90.[13] A recent study demonstrated improved efficacy when PU-H71 was combined with JAK2 inhibition in pre-clinical mouse models.[14] This resulted in a phase 2 clinical trial of the HSP90 inhibitor, AUY922 in patients with MF (NCT01668173), which although terminated early due to excess gastrointestinal toxicity, found that all 6 patients treated experienced at least a partial remission (PR).[15]

Limitations of first-generation JAK inhibitors

Despite their clinical benefits, first-generation JAK inhibitors were not the 'home run' they were expected to be. Because none of these agents is selective for the mutant form of JAK2, JAK activity in normal cells is also inhibited, leading to its myelosuppressive effects. Additionally, none of these agents has been shown to eradicate the JAK2 mutant clone or to significantly reduce the mutant JAK2 allele burden, and thus none can cure the disease. Moreover, it has recently been shown that MPN cells can acquire resistance to JAK inhibitors

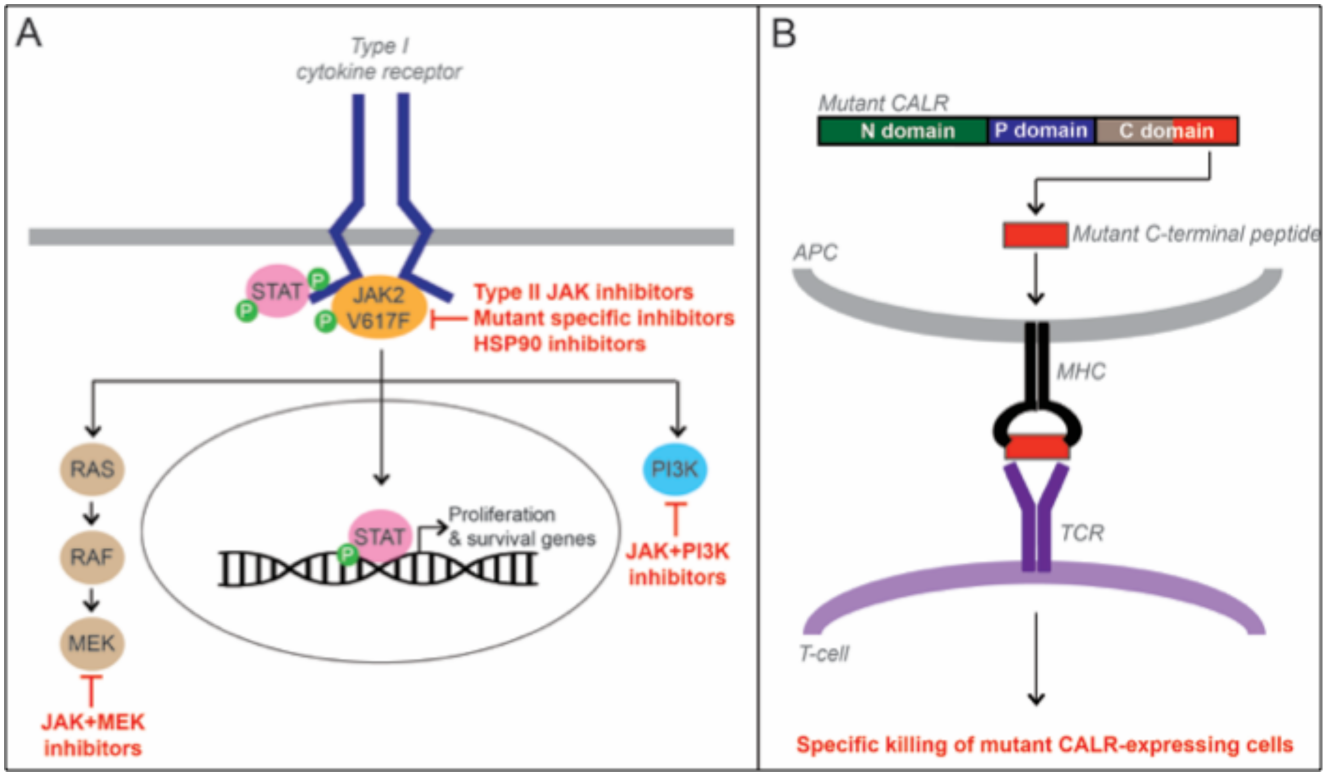

Figure 1. Enhancing the Clonal Selectivity of MPN therapy. (A) Schema showing potential points of intervention for newly developed JAK2 inhibitor monotherapies or combination therapies. Type II JAK inhibitors inhibit JAK2 in its inactive form, and preferentially inhibit JAK2V617F over wild type JAK2. Mutant specific JAK2 inhibitors are being developed to specifically target the JAK2V617F mutant protein. HSP90 inhibitors lead to the degradation of mutant JAK2. Combination therapies currently being explored include JAK inhibitors with PI3K inhibitors, and JAK inhibitors with MEK inhibitors. Both combinations have demonstrated synergistic inhibition of *JAK2*-mutant MPN cells in pre-clinical models. (B) Schema demonstrating one potential mechanism for immune responses to be generated against mutant CALR. Derivatives of the mutant C-terminal peptide of mutant CALR have been shown to elicit T-cell responses in *CALR*-mutant MPN patients following *ex vivo* peptide stimulation, suggesting that the mutant CALR C-terminus contains tumor-specific neo-epitopes that are targeted by T-cells.

through reactivation of JAK-STAT signaling via heterodimeric activation of JAK2 by JAK1/TYK2.[16] Together these limitations highlight the enduring need to identify improved therapeutic avenues for *JAK2*-, *MPL*-, and *CALR*- mutant MPNs.

Future perspectives

Type II JAK inhibitors

Type II JAK inhibitors, which stabilize the inactive unphosphorylated conformation of JAK2 resulting in more potent JAK2 inhibition, were recently developed. In addition to binding the ATP pocket, type II inhibitors bind supplemental adjacent sites, thus enhancing their specificity. A recent study demonstrated that CHZ868, a type II JAK inhibitor, overcame resistance to first-generation JAK inhibitors by binding the inactive conformation of JAK2.[17] CHZ868 was shown to be active in both *in vitro* and *in vivo* models of MPN and although type II inhibitors are not specific for mutant JAK2, CHZ868 was found to preferentially target Jak2V617F cells over Jak2 wild type cells in Jak2V617F mice.[17] If developed for clinical use, type II inhibitors may be more clonally selective for JAK2V617F-mutant cells than current JAK inhibitors.

Mutant-specific JAK2 inhibitors

The identification of the crystal structure of the pseudokinase domain of JAK2, which is the domain in which the V617F mutation occurs, is an important advance towards the development of JAK2V617F mutant-specific inhibitors.[18] Recent biochemical studies have advanced the understanding of the specific requirements for JAK2V617F driven activation and identified specific residues that could potentially be targeted with allosteric small molecule inhibitors.[19] Mutant-specific JAK2 inhibitors should overcome the myelosuppressive effects resulting from inhibition of wild type JAK2 in normal cells.

Mutant CALR immunotherapy

CALR mutations occur as insertions and/or deletions in exon 9, all of which cause a +1 base-pair frameshift. Although more than 50 mutations have been identified to date, all mutant CALR proteins possess the same tumor-specific 36 amino acid C-terminal peptide, making it an attractive target for anti-MPN immunotherapy. A recent study demonstrated that mutant CALR-derived peptides are capable of eliciting T-cell responses in mutant *CALR*-positive MPN patients following *ex vivo* peptide stimulation.[20] This result suggests that the mutant CALR C-terminus is immunogenic, and may represent a promising immunotherapeutic target in *CALR*-mutant MPN patients (Figure 1B).

Additional mutations and clinical implications

In addition to the aforementioned MPN phenotypic driver mutations (i.e. *JAK2, CALR, MPL*), co-occurring mutations in epigenetic and splicing genes are found in MPN patients (in particular in MF) and are associated with poor clinical outcome. These include mutations in genes that impact DNA methylation (e.g. *TET2, IDH1/2*), polycomb complex proteins (e.g. *ASXL1, EZH2*) and mutations in splicing factors (e.g. *SRSF2*).[21,22] Studies focused on developing treatment strategies that target these mutations or their molecular consequences are therefore warranted.

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Emerging treatments for classical myeloproliferative neoplasms

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Take-home messages

- An accurate diagnosis is an essential first step in the management of MPN.
- Novel prognostic scores integrating molecular data are being developed.
- Initial data from comparator studies with IFN and HU demonstrate equivalence in their ability to control blood counts and that both agents may deliver molecular and histological responses.
- JAK inhibition is an important modality and data from phase III studies with second line JAK Pacritinib and Momelotinib is important. The management of cytopenia in MF remains challenging and novel agents such as PRM-151, Sotatercept and others are of interest. Telomerase inhibition is also being assessed.

Introduction

The myeloproliferative neoplasms essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF, collectively termed MF) have overlapping clinical and biological features. However, an accurate diagnosis is important as increasingly management is nuanced to very specific features of the disease. The recent revision of the WHO diagnostic criteria further emphasizes the distinction between ET and PV and recognizes pre-fibrotic MF as a separate entity.[1] Furthermore, a decision regarding treatment intensity: watch and wait, vs aspirin; vs aspirin with cytoreductive therapy vs experimental therapy follows a risk adapted strategy for PV and ET. For PMF prognostic scoring is used for transplantation only and then a problem-based approach is employed. Currently available prognostic scores are summarized in Table 1. Gaps currently exist with regard to patients who have myelofibrosis following a prior diagnosis of ET or PV and in the integration of data regarding non-driver mutations.

Current state of the art

Survival for high-risk PV patients receiving contemporary care is 10.9 years;[2] in contrast for low-risk ET a standardized mortality ratio of 1 has been reported.[3] Despite current therapy there is an on-going risk of thrombosis, hemorrhage, impaired quality of life and risk of transformation. For example, in treated high-risk PV, residual thrombosis risk is 2.93 per 100 patient-years, with overall risk of PPV-MF (26%), and AML (10%) at 20 years, respectively.[4]

Aspects meriting specific consideration are changes in prognostic scores with CALR mutated ET potentially needing less intensive treatment, refinement of treatment targets and the emergent importance of the leucocyte count as a marker of disease risk. An on-going question has been the relative benefit of interferon alpha (IFN) *vs* hydroxycarbamide (HU). Results from PROUD PV[5] a study with peg-proline interferon alpha2b (PEGINVERA) *vs* HU and an interim analysis from the MPDRC-112 study comparing pegylated-interferon alpha-2a (PEGASYS) vs HU (in both ET and PV)[6] were recently presented. PROUD PV demonstrated better tolerability of PEGINVERA, however rates of hematological adverse events with HU were unexpectedly high. Hematological control was equivalent but 2 cases of acute leukemia, and 3 non-squamous cell skin malignancies occurred on the HU arm.[5] Interestingly the interim analysis from MPDRC-112 study also demonstrated equivalent outcomes, the striking finding from MPDRC-112 was that both arms were equivalent in achieving molecular and histological remission.[6] Further data is required from longer-term follow-up of these studies as at present both agents look equivalent. Stopping IFN has also been reviewed in some detail with data to suggest around 40% of patients who stop may remain off interferon for over 6 months, without further therapy.[7]

Experimental therapies for ET and PV include the JAK inhibitors, histone deacetylase inhibitors and imetelstat. Ruxolitinib has been evaluated in 3 phase III studies, RESPONSE, RESPONSE2 and RELIEF showing that in second line after HC failure/intolerance ruxolitinib effectively controls blood count, spleen size, symptoms and interestingly

Myeloproliferative neoplasms - Section 3

there may be molecular responses in some patients (reviewed in [4]). All of these agents are of interest for both ET and PV but further data is needed regarding long-term safety and efficacy regarding thrombosis and transformation to MF.

Future refinement of prognostic scores is also likely in MF with for several reasons: first *CALR* mutations, especially type1/type1-like, are associated with longer survival and so-called triple-negative (TN) disease much shorter,[8] lastly the presence of mutations in *ASXL1, EZH2, SRSF2, IDH1* or *IDH2* carries a poor prognosis.[9] How this might impact on therapeutic decisions relates mainly to stem cell transplant (SCT). For example, *ASXL1* or TN mutation status in a DIPSS intermediate-1 patient might make them a putative candidate for HSCT, and their absence in an intermediate-2 risk patient perhaps the opposite.

The JAK1/JAK2 inhibitor (JAKi) ruxolitinib as evaluated in

Table 1. Current prognostic scores for patients with PV, ET and PMF.

Polycythemia vera	Essential thrombocythemia	Primary myelofibrosis
Conventional Thrombosis score	**Conventional Thrombosis score**	**IPSS**
Age >60y Previous thrombosis Presence of either variables define a high-risk patient	Age >60y Previous thrombosis Presence of either variables define a high-risk patient	Age >65 Anemia (Hb <100g/L) Leukocytes >25x10^9/L Blood blasts >1% Constitutional symptoms Each variable= 1 point *Categories:* Low-risk= 0 point Intermediate-1 risk= 1 point Intermediate-2 risk= 2 points High risk= 3-5 points
Survival score	**IPSET score**	**DIPSS**
Age >67 (5 points) Age 55-66 (2 points) Leukocytes >15x10^9/L (1 point) Venous thrombosis (1 point) *Categories:* Low-risk= 0 Intermediate-risk= 1-2 High risk= ≥3	Age >60y (1 point) CV risk factors (1 point) Previous thrombosis (2 points) JAK2V617F mutation (2 points) *Categories:* Low-risk= 0 Intermediate-risk= 1-2 High risk= ≥3	Age >65 Anemia (Hb <100g/L) Leukocytes >25x10^9/L Blood blasts >1% Constitutional symptoms Each variable= 1 point, except anemia=2 points *Categories:* Low-risk= 0 point Intermediate-1 risk= 1-2 point Intermediate-2 risk= 3-4 points High risk= 5-6 points
		DIPSS-plus
		DIPSS score RBC transfusion dependency Platelets <100x10^9/L Unfavorable karyotype[§] DIPSS low= 0 point DIPPS int-1= 1 point DIPSS int-2= 2 points DIPSS high = 3 points Each additional variable = 1 point *Categories:* Low-risk= 0 point Intermediate-1 risk= 1 point Intermediate-2 risk= 2-3 points High risk= 4-6 points

CV= cardiovascular; [§]Unfavorable karyotype: complex karyotype or sole or two abnormalities that include +8, -7/7q-, i(17q), -5 /5q-, 12p-, inv(3), or 11q23 rearrangement.

the COMFORT studies delivers spleen volume reduction and improvement in quality of life; prolongation of survival has also been suggested. However, a Cochrane Review concluded that the evidence was insufficient to allow any conclusion regarding survival, mainly due to lack of statistical potency of the phase 3 trials to measure a possible survival gain. This review was conducted before mature data was available and indeed both studies recently reported updates.[10,11] Dose-limiting myelosuppression and an increased risk of infection ranging from common to rare severe infections such as progressive multifocal leucoencephalopathy (reviewed in [12]) can be problematic. A current question pertains to the earlier use of Ruxolitinib which has successfully been used in selected patients with intermediate-1 risk disease and the ReTHINK trial is currently underway for patients with lower risk disease and adverse mutational profile.[13] For higher risk patients a number of studies are assessing the benefit of combining Ruxolitinib with other drugs to either allow adequate dosing or to improve response (reviewed in [4]).

Concerning other JAKi; Pacritinib and Momelotinib are of interest, as is NS018[14] and INCB039110.[15] Pacritinib was evaluated in the PERSIST trials,[16,17] myelosuppression was not as marked as anticipated and 23% of transfusion dependent patients became transfusion independent. In January 2017 an FDA clinical hold due to safety concerns with Pacritinib was lifted. Momelotinib a JAK1/2i delivered anemia-related, spleen and symptom responses.[18] Peripheral neuropathy was reported and might impact its place in the therapeutic algorithm. Currently results of 2 phase III studies (SIMPLIFY-1 & -2), are expected.

Regarding non JAKi therapies Imetelstat, a telomerase inhibitor induced both molecular and fibrosis responses and is being assessed in the IMBARK study. PRM-151, a recombinant human pentraxin-2 (PTX-2) is also being assessed in a phase 2 study (PROMOTE). Sotatercept has shown some activity for anemia in a proportion of patients[19] and the SMAC mimetic (LCL161) is also being assessed in early phase studies.[20]

Future perspectives

Improvements in our understanding of basic biology in the MPNs has driven changes in diagnostic criteria, prognostic stratification and has now delivered important new therapeutic options for patients. Yet gaps persist; longer term data is lacking for most therapies used for PV and ET further data is to be expected regarding IFN and HU and is needed for Ruxolitinib. Concerning MF there is opportunity to deliver even more improvement for example myelosuppression is limiting for some patients with Ruxolitinib and studies with novel agents will deliver important information.

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New approaches to indolent lymphoma

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Introduction

Indolent B-cell lymphomas have a good prognosis with a low rate of mortality, but relapses are frequent even late and for the advanced stages they are usually not curable diseases. Molecular techniques have shed new light on the pathobiology of indolent lymphoma and helped to identify lesions with prognostic implications and therapeutic targets. Some specific biological abnormalities may help for targeted therapies; like in hairy cell leukemia (HCL) the high frequency of BRAF mutations may suggest a key role in the pathogenesis of the disease or MYD88 in lymphoplasmacytic lymphomas (LPL). The survival of follicular lymphoma (FL) has dramatically increased since 1997 with the use of rituximab initially in relapse setting than in first line. Patients with a high-tumor burden need chemotherapy (CT) combined with rituximab and this CT may expose them to long term complication (secondary malignancies, cardiac toxicity, etc.) mostly for patients experiencing multiple relapses. New agents are studied in this setting, e.g. antibodies drug conjugates, intracellular targets or drugs targeting the microenvironment. The high efficacy of these drugs used alone or with antiCD20 antibodies let us hope that they will soon be used in first line delaying the time to CT. Management of mucosa-associated lymphoid tissue (MALT) lymphomas has evolved among time but for gastric lymphoma it must begin with antibiotics for *Helicobacter pylori* eradication, stage I disease can receive radiation therapy at 30 gys and for advanced stages if a treatment is required the best published combination is represented by rituximab/chlorambucil, but new agents have shown efficacy like lenalidomide and ibrutinib.

Learning goals

1. ***There is still a place for watchful waiting in asymptomatic patients.***
2. ***Major changes in identification of new therapeutic targets in indolent lymphomas.***
3. ***New agents studied in this setting (antibodies, intracellular targets or drugs targeting the microenvironment).***
4. ***Development of targeted treatments delaying the time to first chemotherapy in advanced patients requiring therapy.***

EUROPEAN HEMATOLOGY ASSOCIATION

New approaches to indolent lymphoma - Section 1

Molecular profiling of indolent lymphoma

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Take-home messages

- Indolent lymphomas are characterized by a clinical course measurable in years or even decades but remain incurable for the vast majority of patients with a high tumor burden.
- Molecular and cytogenetic techniques have significantly expanded the bio-pathological knowledge of these neoplasms by identifying lesions that are provided with diagnostic, prognostic and/or therapeutic relevance.
- The perspective is the development of targeted therapies that can eradicate indolent lymphomas leading to patients' cure and delay the time to first chemotherapy.

Introduction

Molecular techniques have shed new light on the pathobiology of indolent lymphomas and evidenced lesions provided with diagnostic, prognostic and/or therapeutic relevance, especially in the setting of chronic lymphocytic leukemia (CLL), lymphoplasmacytic lymphoma (LPL), hairy cell leukemia (HCL), marginal zone lymphomas [(MZL), splenic (S), nodal (N) and extranodal (EN)], and follicular lymphoma (FL) (Table 1).[1]

CLL:[1-4] Patients with mutated IGHV have a better prognosis than those with unmutated genes. The commonest alterations in CLL are deletions in 13q14.3 (miR-16-1 and miR-15a) (~50%), trisomy 12 (~20%) and, less commonly, deletion in 11q22-q23 (*ATM* and *BIRC3*), deletion in 17p13 (*TP53*), and deletion in 6q21. The most commonly mutated genes, detected in 3-15% of cases, are *NOTCH1*, *SF3B1*, *TP53*, *ATM*, *BIRC3*, *POT1*, and *MYD88*. Deletion in 11q and, particularly, deletion in 17p confer a worse clinical outcome whereas isolated deletion in 13q14 is associated with a more favorable clinical course. *TP53* abnormalities (i.e., deletion in 17p13 and *TP53* mutations) are predictive of lack of response to fludarabine-containing regimes. Mutations in *TP53*, *ATM*, *NOTCH1*, *SF3B1*, *BIRC3,* among others are associated with a poor outcome.

LPL:[1,5-8] IGHV turn hypermutated. No specific chromosomal abnormalities are recognized in LPL; however, >90% and ~30% of cases have *MYD88* L265P and *CXCR4* mutations, respectively. *ARID1A* mutations are recorded in 17% of patients, and less commonly, mutations of *TP53, CD79B, KMT2D/MLL2* and *MYBBP1A*. Documenting a *MYD88* L265P mutation may be helpful in diagnosing LPL, although it can be recorded also in other B-cell lymphomas. Similarly, *CXCR4* mutations are not exclusive of LPL. These mutations are important in the pathogenesis of LPL by causing NF-kB activation. Notably, cases lacking a *MYD88* L265P mutation

Table 1. Summary of the main cytogenetic and molecular aberrations in the setting of indolent lymphomas.

Lymphoma category	Main cytogenetic findings	Main somatic gene mutations
Chronic lymphocytic leukemia	del13q14.3, +12, del11q22-q23, del17p13, del6q21.	*NOTCH1, SF3B1, TP53, ATM, BIRC3, POT1, MYD88*
Lymphoplasmacytic lymphoma	del6p, +4	*MYD88, CXCR4, ARID1A, TP53, CD79B, MLL2, MYBBP1A*
Hairy cell leukemia	-	*BRAF V600E*
Splenic marginal-zone lymphoma/leukemia	t(2;7)(p12;q21), del7q, +3q	*MLL2, NOTCH2, KLF2, MYD88*
Nodal marginal-zone lymphoma	+3, +18, del6q23	*MLL2, NOTCH2, KLF2, MYD88, PTPRD*
Extranodal marginal-zone lymphoma	t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21), t(3;14)(p14.1;q32), +3, +18, del6q23	*TNFAIP3, MYD88, PIM1, TP53, MYC*
Follicular lymphoma	t(14;18)(q32;q21), del1p36	*EZH2, KMT2D/MLL2, CREBBP, RRAGC, TP53, CDKN2A, MYC*

are reported to have an adverse prognosis and a lower response to ibrutinib. Gene expression profiling (GEP) studies suggest upregulation of IL6 and its downstream MAPK signaling pathway.

HCL:[9] Most HCL cases show hypermutated IGHV. A unique feature of HCL is the common co-expression of multiple clonally related Ig-isotypes, suggesting arrest at some point during isotype switching. No cytogenetic abnormality is specific for HCL. The high frequency of *BRAF* V600E mutation suggests a key role in the pathogenesis of the disease and has prompted to the usage of BRAF-inhibitors in patients with HCL resistant to previous lines of therapy. Whether cases that lack *BRAF* V600E mutation, use the IGHV4-34 family and have *MAP2K1* mutations are most closely related to classic HCL or HCL-variant remains to be established.

SMZL:[1,10] SMZL lacks recurrent chromosome translocations observed in other lymphomas. A small number of cases carries a t(2;7)(p12;q21) activating the *CDK6* gene. Approximately 30% of SMZL show a heterozygous deletion in 7q. In addition, gain of 3q is present in a considerable subset of cases. *NOTCH2* and *KLF2* are mutated in 10-25% and 10-40% SMZLs, respectively. Both mutations, however, are also found in other small B-cell neoplasms and have been associated with SMZLs carrying deletion in 7q. *MYD88* mutations are rare in SMZL, and therefore may contribute to the differentiation from LPL. The observation that the most frequently mutated genes in SMZL (*NOTCH2*, *KLF2, KMT2D/MLL2*) are physiologically involved in proliferation and commitment of mature B-cells to the MZ, points to homing to the spleen compartment and MZ differentiation as the major programs deregulated in this lymphoma. Consistently, SMZL has an expression signature characterized by the upregulation of genes belonging to the MZ differentiation program.

NMZL:[1,11,12] IGH are clonally rearranged with a predominance of mutated VH3 and VH4 families, particularly VH4-34. Cases associated with HCV preferentially use VH1-69. NMZL shares gains of chromosome 3 and 18 and loss of 6q23-q24 with ENMZL and SMZL. GEP analysis has demonstrated an increased expression of NFkB-related genes. *MYD88* L256P mutation has been detected in occasional cases. A recent publication has shown that, although NMZL shares with SMZL a common mutation profile, NMZL harbors *PTPRD* lesions that are otherwise absent in SMZL.

ENMZL:[1,13,15] IGHV are rearranged and hypermutated. There is biased usage of certain IGVH families at different anatomic sites, suggesting antigen-induced clonal expansion. Chromosomal translocations associated with ENMZLs include t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21), and t(3;14)(p14.1;q32), resulting in the production of a chimeric protein (*BIRC3/MALT1*) or in transcriptional deregulation (*BCL10, MALT1, FOXP1*) respectively. Trisomies 3, 18 or less commonly of other chromosomes are not infrequent in ENMZL, although unspecific. The prevalence at which the translocations or trisomies occur, varies with the primary anatomic site and geographic area. Abnormalities of *TNFAIP3* on chromosome 6q23 occur in 15-30% of cases but are not specific for ENMZL. *MYD88, PIM1, TP53,* and *MYC* mutations have been reported in 6-9% ENMZL.

FL:[1,16-20] IGH and IGL are rearranged; variable region genes show ongoing somatic hypermutation. The BIOMED-2 approach is recommended to detect clonality. FL is associated with the development of multiple sub-clones: transformation develops in an earlier common progenitor, rather than one of the later sub-clones. FL is genetically characterized by the translocation t(14;18)(q32;q21) between the IGH and *BCL2* genes: it is present in up to 90% of grade 1-2 FL. Given the variation in breakpoint regions, FISH is more sensitive than PCR to detect the translocation. Using GEP, FL lacking *BCL2* rearrangement usually has a late germinal center profile and is more frequent graded 3B. In addition to the t(14;18), other genetic alterations are found in 90% of FL. One of the most commonly affected regions is 1p36, which contains *TNFRSF14*. The number of additional alterations increases with histological grade and transformation. Mutations of *EZH2* are relatively common in FL, and appear to be an early event. Additionally, driver mutations in the chromatin regulator genes *CREBBP,* and *KMT2D/ MLL2* play a key role, and *EZH2*, *KMT2D/MLL2*, and *CREBBP* have all been proposed as possible therapeutic targets. More recently, activating somatic mutations in *RRAGC* were found in approximately 17% FL. GEP studies have shown the importance of the microenvironment in the pathogenesis, evolution and prognosis of FL. Transformation to DLBCL may follow different pathways including inactivation of *TP53*, *CDKN2A*, and activation of *MYC*.

In the present era of druggable genome, molecular studies give for the first time the chance to effectively cure indolent lymphomas.

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New approaches to indolent lymphoma - Section 2

Update on follicular lymphoma: Time beyond chemotherapy?

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Take-home messages

- Immunochemotherapy is still the standard of care in frontline and first relapse.
- Efficacy, toxicity, quality-of-life and costs of new approaches have to be compared in long-term follow-up with existing regimens.
- There is a clear need for identification of predictive markers enabling individually tailoring of treatment regimens for subgroups of patients.

With the introduction of CHOP chemotherapy in 1976 this regimen has become a worldwide accepted standard in the treatment of non-Hodgkin's lymphoma. A big jump in the outcome was achieved when the CD20 antibody rituximab entered to the marked in the nineties. Nowadays, using immunochemotherapy the overall survival in patients with advanced follicular lymphoma (FL) is getting close to 20 years. However, most patients relapse within 5 to 7 years, and about 20% of patients' experience progression of disease within 2 years. Especially this group of patients has a poor outcome.[1] In 2013, Rummel *et al*. published their landmark paper showing that reducing the intensity of chemotherapy does not consequently results in a loss of efficacy.[2] In a randomized non-inferiority trial the authors compared bendamustine/rituximab (BR) with R-CHOP in patients with newly diagnosed stage III and IV indolent lymphoma. It was clearly shown the BR was well tolerated and prolongs progression-free survival (PFS) compared to R-CHOP. Based on these results, BR is used by most investigators as first-line chemotherapy in FL.

With the expanding knowledge of the biology and pathogenesis of B cell malignancies, several new compounds acting through a variety of mechanisms have been investigated in clinical trials. In contrast to cytostatic agents, these agents are characterized by a specific target on the surface of the lymphoma cell, in the intracellular pathway or in the microenvironment of the lymphoma cell (an overview is given in Table 1). But why to change an effective, well-known chemotherapeutic regimen? In general, a chemotherapy-free approach has to demonstrate to be more active and less toxic compared to standard therapies. Ideally, new approaches should offer innovative options for high-risk patients with early relapse, they should have the potential to overcome disease resistance that develops over time, they should avoid cumulative toxicities from successive therapies, they should reduce the risk of transformation and they should raise a chance of cure.

Targeting the cell surface

In recent years, the search for CD20 antibodies with improved activity compared to rituximab has been of particular interest. Ofatumumab is an anti CD20 agent which may have an increased complement dependent cytotoxicity. However, its role in FL is still unclear since there is no significant improvement in rituximab-refractory patients.[3] Obinutuzumab is another CD20 antibody which is claimed to have a higher antibody-dependent cellular cytotoxicity and more effective apoptosis induction compared to rituximab. In combination with chemotherapy, obinutuzumab achieve a significant better PFS in first-line compared to a rituximab-containing regimen.[4] In rituximab-refractory patients, the combination of obinutuzumab and bendamustine documented an effective treatment with a deep level of remission and was recently approved by the authorities.[5] However, in a head to head comparison with rituximab in rituximab pretreated patients, obinutuzumab failed to show a survival benefit.[6]

A variety of further monoclonal antibodies, antibody drug conjugates and bispecific antibodies (which also interact with the microenvironment) are under evaluation in FL (Table 1). Most of them show activity but it is still unclear what will be their definitive role.

Intracellular targets

At this time, idelalisib is the only compound which already received approval as monotherapy in Europe for relapsed/refractory FL. It is an orally available inhibitor of the

New approaches to indolent lymphoma - Section 2

delta isoform of phosphatidylinositol 3-kinase (PI3K) targeting key signaling molecules downstream of the B cell receptor. Approval based on results from a pivotal phase II study by Gopal *et al*.[7] In this study, 125 heavily pretreated patients (FL: 72 patients) received idelalisib until progression or intolerance. Patients achieved a median PFS of 11.0 months and a median OS of 20.3 months. It was also shown that idelalisib may have significant clinical activity in high-risk and doubly-refractive FL following early relapse.[8] However, because of an excess of atypical infections, the safety of idelalisib is still under discussion. Duvelisib is an inhibitor of the gamma and delta isoform of PI3K, showing a median PFS of 8.4 months and a median OS of 18.4 months in a phase II study in patients refractory to rituximab.[9] However, 63% of patients had dose modifications due to adverse events and 17% of patients discontinued treatment.Bruton Tyrosine Kinase (BTK) is a molecule which is crucial for function and survival of the B cell receptor. Ibrutinib is a first-in-class, selective, and irreversible inhibitor of the BTK and already plays an important role in the treatment of CLL, mantle cell lymphoma, and M. Waldenström. In FL, several trials evaluate its activity as a single agent and as part of a combination regimen. In a phase II trial of ibrutinib and rituximab, the overall response rate (ORR) in 60 patients with untreated FL was 85% with a rate of complete remission (CR) of 35%.[10] Ibrutinib was discontinued in 15% of patients due to adverse events. In immunochemotherapy-refractory FL, ibrutinib achieved an ORR of 20.9% (10.9% CR), and a median duration of response of 19.4 months.[11] Serious adverse events were reported in 48.2% of patients.

Venetoclax is a highly selective orally available inhibitor of BCL2, which is typically overexpressed in FL. In 29 pretreated patients with FL, the ORR was 38% (14% CR) with a median PFS of 11 months.[12]

Table 1. Selected targeted drugs in follicular lymphoma.

Targeting the cell surface	
Target	**Agent**
CD20 (type I antibody)	Rituximab*, Ofatumumab, Ocrelizumab, Ublituximab, Veltuzumab
CD20 (type II antibody)	Obinutuzumab*
CD22	Epratuzumab, Inotuzumab Ozogamicin°
CD79b	Polatuzumab Vedotin°
CD19	Coltuximab Ravtansine°
CD37	Otlertuzumab
CD80	Galiximab
HLA-DR	IMMU-114
CD3/CD19	Blinatumomab
Intracellular targets	
Target	**Agent**
PI3K	Idelalisib* (PI3Kδ), Duvelisib (PI3Kδγ), Copanlisib (PI3Kαδ)
BTK	Ibrutinib, Acalabrutinib
BCL-2	Venetoclax
Syk	Entospletinib, Fostamatinib
HDAC	Vorinostat
Proteasome	Bortezomib, Carfilzomib
MTOR	Temsirolimus, Everolimus
MDM2	Idasanutlin
EZH2	Tazemetostat
Targeting the microenvironment	
Target	**Agent**
'Immunomodulation'	Lenalidomide
PD-1	Nivolumab, Pembrolizumab, Pidilizumab
PD-L1	Atezolizumab, Durvalumab
KIR	Lirilumab
CD137	Urelumab

**EMA-approved in follicular lymphoma; °antibody drug conjugates.*

New approaches to indolent lymphoma - Section 2

Targeting the microenvironment

The interaction between lymphoma cells and the microenvironment plays a critical role in the pathogenesis of FL. Immunotherapeutic agents and checkpoint inhibitors have the potential to enhance the immunocompetence directed against the lymphoma cell.

Lenalidomide has only limited activity as single agent in relapsed FL, but efficacy is greatly enhanced in the combination with rituximab. In frontline, this combination achieved a CR rate of 87% with a PFS (3 years) of 78.5%.[13] The most common grade III/IV toxicity was neutropenia in 35% of patients. The results of the phase III Relevance trial comparing rituximab/lenalidomide with rituximab/chemotherapy are expected soon.

To date, several trials with so called checkpoint inhibitors are ongoing. The combination of pidilizumab, a presumed PD1 inhibitor, with rituximab in relapsed FL shows a ORR of 66% (CR 52%).[14] The regimen was well tolerated.

Are we now ready to abandon chemotherapy in FL? There is no doubt that non-cytotoxic agents are active in FL. There is also no doubt that these agents have side effects and the combination of new drugs may result in unacceptable toxicity.[15,16] When considering a chemotherapy-free approach in FL, it is important to keep in mind that:

- immunochemotherapy is still the standard of care in front-line and first relapse;
- efficacy, toxicity, quality-of-life and costs of new approaches have to be compared in long-term follow-up with existing regimens;
- there is a clear need for identification of predictive markers enabling individually tailoring of treatment regimens for subgroups of patients.

It is really exiting to move away from standard immunochemotherapy and to move in the era of targeted therapies, but the target patient has not been defined yet.

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New approaches to indolent lymphoma - Section 3

Treatment of extranodal marginal zone B-cell lymphomas (MALT-lymphomas)

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Take-home messages

- Eradication of *Helicobacter pylori* remains the preferred first-line therapy in patients with gastric MALT lymphoma irrespective of stage. Antibiotic therapy can also be given in patients with ocular adnexal MALT-lymphomas as sole initial management.
- The rate of HP-negative gastric MALT-lymphomas, however, is dramatically increasing in large series. Nevertheless, such patients may also be managed with (clarithromycin-based) antibiotic therapy.
- Systemic treatment has increasingly been investigated also in localized disease, and current guidelines have stated curative potential both for systemic therapy as well as local irradiation.
- While promising results have been published for various chemotherapies including cladribine, rituximab plus bendamustine and also chemo-free approaches, no standard therapy has been defined so far. The only randomized data have been generated in a trial comparing rituximab plus chlorambucil versus chlorambucil alone, and a third arm on rituximab alone has been added.

Introduction

According to the recent WHO classification,[1] extranodal marginal zone B-cell lymphomas of the mucosa associated lymphoid tissue (MALT lymphomas) account for 7- 8% of all newly diagnosed lymphomas. While most prominently encountered in the gastrointestinal tract, MALT lymphoma may arise in virtually any organ of the human body with a different diagnostic, but also therapeutic approach for different localizations. The stomach still remains the most commonly involved organ in patients with MALT lymphoma (amounting for 35-60% of newly diagnosed MALT lymphomas in larger cohorts), followed by the ocular adnexa, lung and salivary glands.

While initially thought to be a localized disease in the majority of patients,[2] more recent data have suggested MALT-lymphoma as a potentially systemic disease from the onset,[3] owing to the homing properties of MALT-lymphoma cells within mucosal structures as well as the potential for late/systemic relapses following local therapy. In view of this, various systemic approaches have been tested in recent years, and guidelines have actually advocated systemic therapies as having equally curative potential in patients with localized disease.[4,5] In addition, the usually highly indolent course of the disease has resulted in increasing attempts to minimize toxicities with application of chemo-free approaches.

Current therapeutic concepts

One of the most striking properties of MALT lymphoma is the high association of MALT lymphomas with antigenic drives such as bacterial infections and autoimmune diseases. Especially, a high rate of infection with the gram-negative rod *Helicobacter pylori* (HP) reported in up to 90% of gastric MALT lymphomas[1] had been documented, leading to early attempts for HP-eradication as sole management of gastric MALT lymphoma. To date, HP-eradication is still the standard first line treatment for HP-associated gastric MALT-lymphomas irrespective of stage,[4,5] and remissions can be seen in up to 75% of patients. The time to optimal remission, however, may be more than one year in selected cases, so an interval of at least 12 months after documented eradication of HP is necessary to judge the success of antibiotic therapy. Patients who respond to antibiotics alone should not be given further therapy, even in case of histological lymphoma remnants on follow-up biopsies due to the favorable course of such patients.[4-7] A randomized trial had shown no benefit of chemotherapy using chlorambucil over a wait-and-see strategy in patients after HP-eradication,[6] and a retrospective analysis of 107 patients with minimal disease after an interval of one year following antibiotics showed a favorable course in 96% of patients (complete regression with prolonged follow-up in 30% and at least stable disease in 60%), respectively.[7]

New approaches to indolent lymphoma - Section 3

Of late, some concern has been raised by the increasing rate of patients with HP-negative gastric MALT lymphoma, which has drastically increased to up to 35-50% in larger series. Interestingly, responses to antibiotics have been reported in a relevant percentage of patients with de facto absence of any parameter whatsoever to suggest prior HP infection, including histology, breath-test, stool antigen and serologic testing.[3,8] Thus, antibiotic therapy alone is thought to be a reasonable first line option also in patients with gastric MALT lymphoma without evidence of HP-infection in the relevant guidelines.[4,5] A possible association between *Chlamydophila psittaci* (CP) and ocular adnexal MALT lymphoma (OAML) was detected in an Italian series.[9] While some authors have shown almost 100% CP-positivity in patients with OAML, these findings could not be reproduced in other studies and countries, where CP was totally absent from this cohort of patients. Nevertheless, the use of doxycycline for upfront treatment of OAML has been associated with a rate of CP-eradication of 48%, an overall response rate of 65%, and a 5-year PFS of 55% in patients with stage-I ocular adnexal MALT lymphoma. A retrospective analysis of data from 131 patients receiving doxycycline in OAML has disclosed a CR in 23 (18%), a PR in 36 (27%) and stable disease in 55 (42%) patients, with only 6% progressing.[10] While there was a trend towards better responses in CT-positive patients, doxycycline was rated as a reasonable empirical first-line approach in patients with OAML irrespective of CP-status. For the time being, however, antibiotic therapy remains experimental in other non-gastric MALT lymphomas.

Apart from HP-eradication in gastric MALT lymphoma, no clear recommendations are put forward in the various guidelines following resistance to antibiotics or relapse. In the latter, however, another attempt at eradication may be feasible. Various options exist for treatment of such patients depending on the localization and the clinical presentation.[11,12] In asymptomatic patients, a wait and see strategy might be feasible, as spontaneous 'wax-and-wane' phenomena have been reported especially in OAML and pulmonary MALT lymphomas. In localized disease, excellent results have been reported with radiotherapy with good local control rates, and the 5-year failure-free survival ranges from 60-65% for ocular adnexal MALT lymphoma to 100% for thyroid MALT lymphoma.

More recently, systemic approaches are increasingly being used not only in advanced, but also localized MALT lymphomas due to their potentially curative nature,[4,] and a recent study on 185 patients with extragastric MALT lymphoma followed for a median time of 49 months has also shown no difference in outcome between various therapeutic approaches in localized non-gastric lymphomas in terms of response rates and PFS. Various agents have been used with long-term response rates of up to 100% in some series,[13] but no standard has been defined so far. Interesting options appear application of cladribine, rituximab plus bendamustine or chemo-free approaches including lenalidomide plus rituximab.[14] Only one recent randomized trial (IELSG 19) comparing rituximab plus chlorambucil versus chlorambucil alone[15] showed a response rate of 87% for chlorambucil and 94% for the combination (p=0.069). Complete response rate and 5-year event free survival were significantly higher with the combination (78% versus 65%, p=0.025 and 68% versus 50%, respectively). The IELSG19 study was subsequently amended to add a third arm (rituximab alone). The long term results confirmed superiority of the combination versus either chlorambucil or rituximab monotherapy in terms of event-free and progression-free survival, however, no overall survival benefit was seen.[16]

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Stem cell transplantation - GvHD

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Introduction

Graft-versus Host Disease (GvHD) is a major cause of morbidity and mortality after allogeneic stem cell transplantation and it mainly caused by alloreactive donor T-cells. However, alloreactive donor T cells are also targeting recipient hematopoietic cells including the malignant cells. Several studies have shown a close correlation between the occurrence of GvHD and a reduced risk of relapse suggesting absence of GvHD implies also a less stronger graft-versus leukemia (GvL) effect. In this Educational Update, new insights into the pathogenesis of GvHD will be presented with special focus of the increasing role of the intestinal microbiota and its modulation by antibiotics, which may have a greater impact in preventing GvHD (*CK Stein-Thoeringer and MRM van den Brink*). Furthermore, in the recent years new possibilities in preventing and treatment of GvHD by targeting JAK pathway or chemokine receptors are becoming available or are currently tested in clinical trial (*R. Zeiser*) and finally elegant ways of immunmodulation by selecting specific T cells as donor lymphocyte infusion may allow a better balance between GvHD and GvL effect (*F. Falkenburg*) will be presented.

Learning goals

1. ***Understand pathogenesis of graft-versus Host Disease (GvHD) including new therapeutic options in prevention and treatment.***
2. ***Understand the emerging role of intestinal microbiota in GvHD and the impact of microbiota modulation by antibiotics.***
3. ***Understand how Graft-versus Leukemia (GvL) effect can be balanced to the risk of Graft-versus Host Disease.***

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Stem cell transplantation - GvHD - Section 1

GvHD prophylaxis and treatment, new modalities

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Take-home messages

- Recent advances in understanding the pathophysiology of GvHD are being discussed.
- Certain findings in the mouse model could not be translated into the clinical application. Therefore, the advantages and shortcomings of different animal models for GvHD are being elucidated.

Introduction

Despite the advances in our understanding of the pathogenesis of acute graft-versus-host disease (aGvHD) and the prophylactic treatment with a wider array of immunosuppressive medication, about 30-50% of our patients that undergo allogeneic hematopoietic cell transplantation (alloHCT) develop grade 2-4 aGvHD.[1] aGvHD patients who are refractory to standard steroid treatment have a dismal long-term prognosis with only 5-30% overall survival.[2-4] Here we discuss different prophylactic and therapeutic modalities against aGvHD that are based on pharmacological or cellular strategies.

Current state of the art

Based on the observation that the release of pro-inflammatory cytokines is a hallmark of aGvHD many investigators have focused their work on the role of multiple cytokines in the pathophysiology of aGvHD. Highly pro-inflammatory cytokines such as IL-1β,[5] IL-6 (6,7) and TNF-α[8,9] and protective cytokines IL-10[10] and IL-11[11,12] were identified to be functionally involved in murine aGvHD. However, the findings in the mouse models are often not directly translatable into the human situation. For example in the mouse model of GvHD, IL-11 promoted T cell polarization towards a Th2 phenotype which was protective against GvHD.[11,12] However in a phase I/II double-blind, placebo-controlled study for mucositis and aGvHD prevention, recombinant human interleukin-11 was connected to a high mortality based on severe fluid retention that caused pulmonary edema.[13] This example indicates that a cytokine that was well tolerated by the mice induced severe side effects in humans. Conversely, IL-1β was shown to be a proinflammatory cytokine in some murine GvHD models,[5,14,15] while other studies in mouse models showed only a minor role for IL-1 in GvHD pathophysiology.[16] Early clinical studies using IL-1 antagonism in the therapeutic setting suggested a benefit for patients suffering from GvHD,[5,17] while a later prospective, randomized controlled trial failed to show a protective effect of IL-1 blockade in the prophylactic setting.[18] In different mouse models of GvHD, TNF-α was shown to be operational in GvHD[8,9,19] and to downmodulate the function of regulatory T cells (Treg).[20] Clinical studies using TNF-α antagonism with etanercept[21] or infliximab[22] in the therapeutic setting showed some activity against GvHD. Infliximab combined with steroids reduced GvHD severity, however the reported non-relapse mortality (NRM) was high.[22] Etanercept given as a combination therapy with inolimomab (anti-IL-2Rα) for the treatment of steroid-refractory aGvHD was connected estimated rates of 2-year overall survival of 10%.[21] Other reports on TNF-α blockade after allo-HCT showed a high incidence of fungal infections[23] and reduced GVL effects.[24] These findings are in keeping with mouse studies indicating TNF antagonism reduced GvL effects against P815 cells.[8] Another pro-inflammatory cytokine, IL-6 was shown to be responsible for aGvHD in mice.[6,7] The later prospective single-institution phase 1/2 clinical study testing the IL-6R antagonist tocilizumab for aGvHD prophylaxis showed an incidence of grade 2-4 acute GvHD in patients treated with tocilizumab at day 100 of 12% which is lower than expected.[25]

Besides blocking individual cytokines, the costimulation of T cells was recognized as a potential powerful target against aGvHD. Blockade of a major costimulatory molecule, CTLA4 was shown to reduce lethal murine GvHD.[26] CD28:CD80/86 costimulation blockade with abatacept caused low GvHD rates.[27] Another negative regulator of T cell activation namely programmed death-1 (PD-1) using checkpoint inhibition showed promising results in the mouse model[28,29] that have so far not been investigated in the clinic.

Stem cell transplantation - GvHD - Section 1

Another potential target of GvHD are chemokines that guide the migration of T cells towards GvHD target organs.[30,31] However this strategy is seen controversial as high radiation can affect the principles of chemokine mediated tissue migration of T cells. For example CCR5 inhibition was protective against GvHD in a non-irradiated GvHD mouse model[30] while in the presence of total body irradiation (TBI) an earlier time to onset and a worsening of GvHD was observed when CCR5$^{-/-}$ T cells were applied.[32] In the GvHD prophylaxis setting a single institution phase-I trial reported that CCR5 inhibition prevents aGvHD of liver and gut before day 100.[33] T cell egress from the lymph node[34,35] and DC migration[36] were both potently inhibited by the sphingosine 1-phosphate receptor agonist FTY720 in the mouse model of GvHD. This important therapeutic concept is currently investigated by using a sphingosine 1-phosphate receptor type 1 agonist[37] in a clinical study on patients undergoing alloHCT (ClinicalTrials.gov Identifier: NCT01830010).

As aGvHD is a multifactorial disease, it is likely that inhibition of multiple layers of the disease, *e.g.* by blocking downstream signals of multiple cytokine and chemokine receptors could be more effective than classical approaches targeting an individual cytokine, chemokine or co-stimulatory molecule. Signalling of multiple cytokine receptors relies on intact Janus kinase (JAK) 1 and 2 activity (Figure 1). Based on this observation different groups could show that pharmacological inhibition of JAK1/2 reduced aGvHD in the mouse.[38,39] A later retrospective survey that included 19 stem cell transplant centers in Europe and the United States showed that the use of the JAK1/2 inhibitor ruxolitinib for steroid refractory GvHD[40] was connected to overall response rates of 81.5% (44/54) in steroid refractory aGvHD including 25 complete responses (46.3%). JAK1/2 inhibition for steroid refractory cGvHD was connected to an overall response rate of 85.4% (35/41), consistent with data in a cGvHD mouse model.[40]. Ruxolitinib is currently being investigated in a prospective trial in Germany

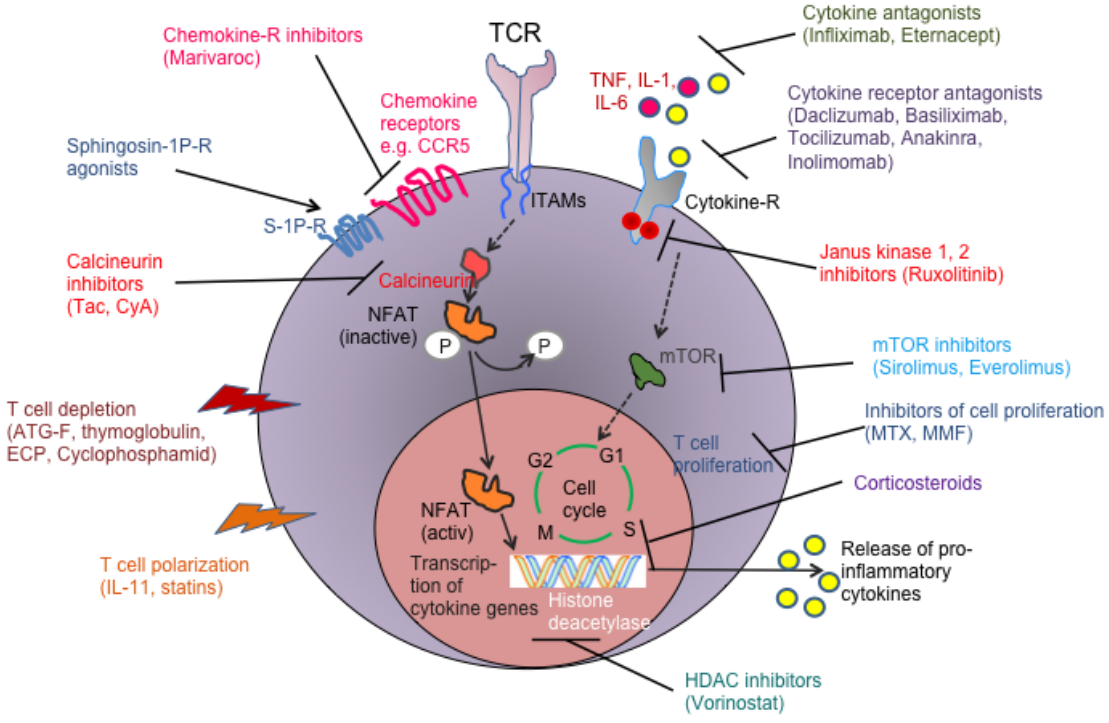

Figure 1. The different pathways for T cell activation, cytokine production and proliferation are shown in the context of their inhibitors used in GvHD prophylaxis and therapy.

(NCT02396628) and a clinical trial using the JAK1 selective inhibitor INCB39110 has begun for the treatment of GvHD (NCT02614612). Also a recent pre-clinical study indicates that topical ruxolitinib suppresses GvHD and protects skin follicular stem cells.[41] Another promising approach to reduce aGvHD in the mouse model is based on the NFkB inhibition, thereby reducing inflammatory protein production via the proteasome inhibitor bortezomib.[42] Clinical trials using a short-course, bortezomib-based GvHD prophylaxis yielded low aGvHD rates.[43,44]

Besides approaches that target the effector cells, strategies that aim at protecting target tissues were investigated. One examples is enhanced regeneration of the epithelial barrier by using a growth factor called keratinocyte growth factor (KGF).[45,46] KGF reduced aGvHD in mouse models as shown by different groups,[45,46] but the survival benefit did vary between the different reports raging from a modest improvement of the survival[45] to very potent protective effects.[46] Based on these pre-clinical data, the drug Palifermin was analyzed in a clinical study where it did not reduce aGvHD but the need for parenteral nutrition after TBI.[47,48] Another approach that aimed at enhancing epithelial regeneration via stimulation of intestinal stem cells was via R-spondin-1 which yielded promising results in the mouse model of aGvHD.[49]

The multiple approaches developed from the mouse model into a clinical application for aGvHD are summarized in Figure 1 and listed in Table 1.

We apologize to those investigators whose work could not be cited due to space restrictions.

Table 1. Translation of immunosuppressive strategies from animal models of acute GvHD into clinical trials.

Main conclusion from the preclinical model of GvHD (year)	Ref.	Main conclusion from the clinical trials (year)	Ref.
IL-11 down-regulated IL-12, and reduced aGvHD-related mortality (1998, 1999).	(11, 12)	IL-11 leads to increased mortality in patients (2002). Phase I/II double-blind, placebo-controlled study.	(13)
IL-1 blockade reduces GvHD in mice in some but not all models (1991).	(5)	IL-1 antagonist is not effective in the GvHD prophylaxis setting (2002). Phase III prospective placebo-controlled study.	(18)
TNF-α antagonism reduces GvHD (1999, 2003).	(8, 9)	Infliximab and corticosteroids are effective as initial treatment of GvHD 2009: Prospective phase III study, 2011: Retrospective analysis.	(22, 50)
IL-6 blockade reduces acute GvHD in mice (2009).	(6, 7)	Early IL-6 inhibition with tocilizumab leads to a low risk of aGvHD (2014) Phase 1/2 single institution trial.	(25).
Anti-CCR5 antibody treatment protects against aGvHD-related mortality (1999, 2003).	(30, 31)	CCR5 inhibition prevents aGvHD of liver and gut before day 100 (2012). Phase 1/2 single institution trial.	(51)
The sphingosine 1-phosphate receptor agonist FTY720 reduces GvHD (2003, 2009).	(34, 35)	Active clinical study on KRP203 in patients undergoing alloHCT (2016). Randomized, Open-label Phase 1/2 study.	(52)
CTLA4-Ig reduces lethal murine GvHD (1994).	(26)	CD28:CD80/86 costimulation blockade with abatacept leads to low GvHD rates (2013). Single-arm feasibility study.	(27)
KGF reduces but does not uniformly eliminate GvHD lethality in mice (1998, 1999).	(45, 46)	Palifermin does not reduce aGvHD severity (2012) but the need for parenteral nutrition after TBI (2013). 2012: Randomized, double-blind, placebo-controlled trial. 2013: Retrospective analysis.	(47, 48)
HDAC inhibition reduced GvHD severity in mice (2008).	(53)	Vorinostat in combination with standard GvHD prophylaxis is associated with a low incidence of severe aGvHD (2014). Phase 1/2 trial.	(54)
JAK1/2 inhibition reduces aGvHD (2014, 2015).	(38, 39)	JAK1/2 inhibition reduces aGvHD in patients refractory to multiple therapies (2015). Retrospective analysis.	(40)
Proteasome inhibition with bortezomib reduces GvHD (2004).	(42)	Short-course, bortezomib-based GvHD prophylaxis yields low aGvHD rates (2009, 2012). 2009: phase 1 trial. 2012: prospective phase I/II trial.	(43, 44)
α-GalCer reduces GvHD (2005).	(55)	RGI-2001 is tested for GvHD prevention (2016). Phase 1/2 trial.	(33)
Cyclophosphamide can induce tolerance towards skin allografts (1989)(56) and post-transplant CP reduced GvHD severity in mice (2014).	(56, 57)	Post-transplantation cyclophosphamide is effective as single-agent aGvHD Prophylaxis (2014). Open Label multi-institutional trial.	(58, 59)

Stem cell transplantation - GvHD - Section 1

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Stem cell transplantation - GvHD - Section 2

The role of the intestinal microbiota in GvHD

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Take-home messages

- Profound perturbations of the intestinal microbiota have been discovered in patients undergoing allo-HSCT.
- Antibiotic induced loss of bacterial diversity and shifts in the microbiota are significantly associated with allo-HSCT related morbidity and mortality.
- Microbiota modulation by narrow-spectrum antibiotics, selected microbial ecosystems and metabolites may induce superior benefits regarding survival and gastrointestinal health.

Introduction

The impact of the intestinal microbiota on health and disease has become increasingly clear in the last decade, and imbalances in the gut microflora are highly relevant for a variety of diseases.[1] The human body is colonized by 10^{13} - 10^{14} microbes, and the vast majority resides in the gut with a biomass of mainly anaerobic bacteria.[2] The gut contains also archaea, eukarya and viruses, but their clinical relevance has been less studied. Over 1,200 different bacterial species have been identified in the human intestines, and each individual is host to a set of at least 160 species with a predominance of the bacterial phyla Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia[3]. Environmental factors like xenobiotics, especially antibiotics, or diet, host genetics and the immune system shape an individual's microbiota.[4,5] In a bidirectional manner, the intestinal microflora, innate and adaptive immunity co-develop after birth and cross-talk during life to achieve homeostatic balance between tolerance to commensal microorganisms and immunity to pathogens.[5] Considering this complex bacteria–immunity interaction, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been shown to induce profound gut microbiota perturbations, which, in turn, are highly relevant for transplant-related clinical outcomes.

Current state of the art

Allo-HSCT has been established as a curative therapy for patients with hematopoietic malignancies, hematological deficiencies and immune disorders. Although this therapy has significantly increased survival prospects for many patients, it poses substantial risk to the patient due to infections through immunocompromising of the host, pre-transplant conditioning-induced organ failure, and graft-versus host disease (GvHD) affecting skin, liver, lung and the intestines. In particular, moderate to severe intestinal GvHD occurring in up to 10% of allo-HSCT patients,[6] conveys a substantial risk for transplant-related mortality (TRM), and several lines of evidence point to a major role of the intestinal microbiota in this process. An impact of the gut microflora on GvHD development has been first described in the 1970's by van Bekkum et al. demonstrating that mice kept under germ-free conditions and undergoing allogeneic bone marrow transplantation showed significantly reduced mortality.[7] In parallel, first clinical studies on antibiotic gut decontamination or laminar-airflow isolation also pointed to superior survival after allo-HSCT;[8] however, subsequent clinical trials produced rather mixed results again highlighting the complexity of microbiota – host interactions in allo-HSCT, that we have recently started to understand in more detail since the development of deep-sequencing techniques to uncover the microbiome.

A major advance in the understanding of the impact of the microbiome on GvHD came from observations that a loss of gut microbial diversity after transplant was significantly associated with worse overall survival after allo-HSCT[9] and increased mortality due to GvHD.[10,11] Prophylactic administration of antibiotics or diet changes, e.g., total parental nutrition[10], in the peri- and early post-transplant setting may account for this diversity loss. In addition, we recently reported that use of broad-spectrum antibiotics to treat febrile neutropenia in allo-HSCT patients significantly increases TRM in

contrast to the use of antibiotics with more narrow antibacterial activity[12]. A similar effect of increased TRM was reported by Weber *et al*.[13] regarding the use of broad-spectrum bacterial decontamination by ciprofloxacin/metronidazol *vs*. restricted, gram-positive bacteria targeting using rifaximin.

The post-transplant decrease in gut microflora diversity was not only observed on the level of a reduction of species abundance in the patients' stool specimens, but concomitantly in a reduction of the bacterial metabolite 3-indoxyl sulfate measured in the patients' urine.[11]

The loss of intestinal diversity observed in allo-HSCT patients and in mouse models of GvHD is generally associated with a loss of *Clostridia* species known to have beneficial effects on the host through fermentation of dietary fibers and the release of short chain fatty acids (SCFA).[14] These monocarboxyl acids, especially butyrate, are an important energy source for the epithelium, but also regulate innate and adaptive immune responses, especially regulatory T cells.[15]In line with a *Clostridia* loss, allo-HSCT in mice is associated with a significant reduction of intraepithelial butyrate in the intestinal mucosa.[15] Oral butyrate supplementation or administration of 17 rationally selected strains of high butyrate – producing *Clostridia* to mice subjected to allo-HSCT significantly reduced GvHD-related mortality and enhanced epithelial integrity.[15]

On the level of gut microbiome changes in humans after allo-HSCT, we observed a loss of *Clostridia* and *Bacteroides* species[12] and, notably, the genus *Blautia*[10] that was associated with increased GvHD and TRM. In addition, Proteobacteria, *Lactobacillales*, *Streptococcus* and *Enterococcus* species dominate the post-transplant intestinal flora of allo-HSCT patients, and can lead to severe blood stream infections.[11,14,16]

In addition to the outgrowth of facultative pathogenic bacteria, microbiota perturbances in allo-HSCT also significantly elevate the risk for *Clostridium difficile* infections (CDI) as another clinically relevant post-transplant condition and co-

Table 1. Overview of clinical trials on microbiota manipulation in allo-HSCT setting.

Study title	Study type	Treatment arms	Primary outcomes	Secondary outcomes	Sponsor
Gut decontamination in pediatric allogenic hematopoietic stem cell transplant patients (NCT02641236)	interventional, phase 2; randomized	No gut decontamination vs. vancomycin-polymyxin B	Gut microbiome changes (2 weeks post HSCT)	Incidence of acute GvHD; survival	Dana-Farber te Cancer Institu
Fecal microbiota transplantation after HSCT (NCT02733744)	Interventional, Phase 0; single group assignment	FMT of fecal microbiota in capsules for 15 days	Feasibility of delivery	Incidence of acute GvHD; survival	Massachusetts General Hospital
Autologous fecal microbiota transplantation (Auto-FMT) for prophylaxis of Clostridium difficile infection in recipients of allogeneic hematopoietic stem cell transplantation (NCT02269150)	Interventional, phase 2; randomized	FMT with pre-transplant feces in patients with low post-transplant microbiota diversity vs. standard care	Incidence of CDI	Incidence of acute GvHD; microbiome changes	Memorial Sloan Kettering Cancer Center
Lactobacillus rhamnosus GG in reducing incidence of graft-versus-host disease in patients who have undergone donor stem cell transplant (PERFECT trial) (NCT02144701)	Interventional, phase 2; randomized	No treatment vs. Lactobacillus rhamnosus GG daily for 1 year	Incidence of acute GvHD	Gut microbiome changes; inflammation markers	Rutgers Cancer Institute, NCI
Modification of the intestinal microbiome by diet intervention to mitigate acute graft-versus-host disease (NCT02763033)	Interventional, phase 2; randomized	Standard BMT diet vs. potato-based resistant starch diet (Bob's Red Mill®)	Incidence of acute GvHD	Gut microbiome changes; fecal butyrate; inflammation markers	University of Michigan Cancer Center

Summary compiled according to clinicaltrials.gov.

factor for subsequent development of intestinal GvHD.[17] Fecal microbiota transfer (FMT) can be used to restore a disrupted intestinal flora as proven in recurrent CDI or CDI in allo-HSCT patients.[18] In addition, Kakihana et al. performed a small FMT series patients with steroid-refractory intestinal GvHD and found complete remission of gastrointestinal GvHD symptoms in 3 out of 4 subjects.[19]

Future perspectives

There is a growing understanding of the profound impact of the intestinal microbiota on allo-HSCT patients. As loss of bacterial diversity and shifts in the microbiome profoundly influence morbidity and mortality in allo-HSCT patients the development of novel strategies to monitor and modulate the microbiome are required (see Table 1, summarizing clinical trials on microbiome interventions in allo-HSCT). These include antibiotic regimens with narrow-spectrum antibiotics (e.g., rifaximin[13]), dietary interventions including prebiotics and probiotics,[18] and postbiotics providing bacterial metabolic products as fecal filtrate transfers (FFTs), a novel, experimental approach recently introduced in the treatment of CDI.[20] However, all these microbiological interventions, especially choosing the right antibiotic regimen, require careful clinical decisions given that we are treating individual patients with different risks for infectious complications.

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FMT 2.0: Transfer of bacterial and host metabolites from fecal waters from healthy donors to patients with CDI significantly reduces infectious burden.

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Balancing graft versus leukemia and graft versus host responses

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Take-home messages

- Specific targeting of the alloimmune response towards hematopoiesis of recipient origin results in GVL with no or limited to GvHD.
- Due to the high susceptibility of hematopoietic cells for recognition by T cells, limiting the magnitude and diversity of the alloimmune response can result in GVL with limited GvHD.
- Limiting inflammation *in vivo* may result in skewing the alloimmune response towards specific GVL reactivity.

Introduction

The main therapeutic effect of allogeneic hematopoietic stem cell transplantation (alloSCT) is control of the disease by the graft versus leukemia/lymphoma (GVL) reactivity mediated by an alloimmune response of donor T cells recognizing hematopoietic cells from the recipient including the malignant population.[1] However, alloreactive T cells recognizing polymorphic antigens on non-hematopoietic tissues may also result in the development of graft-versus-host disease (GvHD). Therefore, as expected, complete removal of T cells from the graft or in vivo purging of T cells will abrogate GvHD but also GVL. Fortunately, hematopoietic cells usually reside in tissues relatively readily accessible to T cells, and hematopoietic cells are relatively susceptible to recognition by T cells due to their high expression of HLA class I, sometimes HLA class II, and expression of adhesion and costimulatory molecules. A variety of clinical observations has illustrated that GVL reactivity can occur in the presence of limited GvHD.

Current state of the art

The balance between GvHD and GVL depends on many factors. Both reactivities are dependent on genetic polymorphic differences between donor and recipient, and therefore disparity for HLA alleles as well as disparity for minor histocompatibility antigens (MiHA) are dominant factors in this balance. If donor and recipient are fully HLA identical, donor anti-recipient alloimmune responses are the result of differences in presentation of polymorphic peptides presented in the groove of HLA molecules (defined as MiHA), since thymic selection has excluded recognition of non-polymorphic peptides in the context of self HLA molecules.[2] T cells are not exposed to non-self HLA alleles during thymic selection, and therefore any peptide presented in the context of non-self HLA molecules may theoretically be immunogenic for T cells. [3] Since the peptidome presented in HLA molecules contains at least 10 times more monomorphic peptides than polymorphic peptides, frequencies of T cells capable of recognizing non-polymorphic peptides in alloHLA alleles are a magnitude higher than of T cells recognizing MiHA.[4]

Following HLA identical sibling transplantation, the development of donor anti-recipient immune responses mimics the development of T cell responses against other nonself antigens like viral antigens. If the donor has not been exposed to alloantigens by transfusions or pregnancy, T cell responses recognizing MiHA are likely to be present in the naïve and not in the memory T cell compartment.[5] As a consequence, the T cell response has to be provoked by activated dendritic cells (DC) (Table 1).[6] These DC will present endogenous peptides and will cross-present antigens picked up during inflammation and from damage tissue. Since DC are cells derived from the hematopoietic system, donor T cell responses provoked by patient DC are likely to react with the hematopoietic compartment of the recipient.[1] This reaction is identical to the GVL effect. Donor T cells may also react with MiHA derived from broadly expressed genes presented in DC, and with MiHA from damaged tissue in which the DC reside. The more tissue damage, inflammation and danger signals, the greater and more diverse the alloimmune responses.[7] We recently demonstrated in a clinically defined model of development of alloimmune responses, i.e. preemptive donor lymphocyte infusion (DLI) following T cell depleted alloSCT, that the balance

between GvHD+ GVL versus GVL only is highly influenced not only by the antigen specificity of the immune response, but more significantly by the magnitude and diversity of the T cell response against MiHA.[8] This may explain why dosing and timing of T cell infusion may dictate relative GvHD or relative GVL. An alloimmune response directed against DC under limited inflammatory circumstances can result in a restricted GVL reactivity. In contrast, under highly inflammatory circumstances with significant tissue damage in organs where the DC reside, highly diverse immune responses with major magnitude will take place resulting in GvHD, with organ specificity depending on the site of inflammation and tissue damage.[9] These results may also explain why delayed DLI may lead to more specific GVL reactivity, and why late after transplantation higher doses of T cells can be infused with a lower likelihood of GvHD.[10]

Following alloSCT with 'matched' unrelated donors (MUD), donor and recipient are usually matched for HLA class-I alleles. Therefore, alloimmune responses against HLA class I restricted antigens behave in principle similar to following HLA identical sibling alloSCT. However, since only HLA-ABC, -DR and -DQ but not HLA-DP are usually taken into account in HLA matching, 80% of donor-recipient pairs will have disparity for HLA-DP alleles. This results in a significant increase of the possibilities of alloimmune responses.[11,12] Since in the absence of inflammation, HLA class-II expression is mainly restricted to cells of the hematopoietic system, allo-HLA-DP responses under non-inflammatory circumstances may lead to a specific GVL reactivity.[13] However, under inflammatory circumstances HLA class-II is upregulated on non-hematopoietic tissues, which may result in an amplification of GvHD.[14] Since allo-HLA responses are T cell responses which can also reside in the memory compartment, the threshold of activation may be significantly lower, and young donors with a lower diversity of the memory T cell repertoire may provoke less GvHD (Table 1). Thus, in unrelated transplants the balance between the development of GvHD and GVL is less predictable.

Following multiple major HLA allele mismatching between donor and recipient, the amplitude and diversity of the immune response will greatly increase, and the alloreactive T cells are likely to be present both in the naïve and the memory T cell repertoire, unless umbilical cord blood (UCB) is used as source.[15,16] Following UCB transplantation, frequencies of alloreactive T cells recognizing the mismatched HLA alleles will be high, but since the UCB T cells will all be naïve, the threshold of activation will be relatively high, and activated DC's are probably necessary to provoke the immune response. This may explain in part the low intensity of GvHD following UCB transplantation. When adult major HLA mismatched donors are used in haplo-identical transplantation, significant T-cell depletion is necessary to reduce the incidence of severe GvHD.[17] This can be performed by complete or partial T-cell depletion of the graft, by in vivo T-cell depletion of the graft using antibodies like anti-T cell globulin (ATG) or alemtuzumab, or by *in vivo* removal of the majority of rapidly activated allo-HLA reactive T cells by the administration of cyclophosphamide post-transplant.[18] Rapidly proliferating T cells will then be depleted, resulting in lower magnitude and diversity of the alloimmune response, resulting in less GvHD. Hopefully, not too many alloreactive T cells will be depleted thereby also removing the GVL reactivity. Obviously, it will be difficult to precisely orchestrate these immune responses. If all T cells are removed from the graft or *in vivo*, the GVL response may completely depend on the presence of alloreactive NK cells.[17] Especially myeloid malignancies have been reported to be susceptible to recognition by alloreactive NK cells capable of killing these cells in the absence of inhibitory self HLA molecules.

Table 1. Dependency of alloreactivity on recipient professional antigen presenting cells.

Donor T cell source	Alloreactivity present in		Alloreactivity dependency on recipient dendritic cells
	Memory compartment	Naive Compartment	
HLA identical sibling	--	++	++
Matched unrelated donor (10/10 HLA match)	+	++	+/-
HLA-mismatched donor/Haplo identical donor	++	++	---
Umbilical cord blood	---	++	+

Future perspectives

Future improvements of alloSCT will depend on successful manipulation of the immune response post-transplant. Approaches after HLA identical transplantation may include the use of relatively purified stem cell populations, in combination with the memory T cell compartment from the donor which contains mainly pathogen specific T cells[19] or the use of purified pathogen specific T cells followed by postponed administration of naïve tumor reactive T cells. Following HLA mismatched transplantation, major depletion of the broad anti-HLA repertoire from both the memory and naïve T cell compartment may be necessary. This may be performed by *in vitro* or *in vivo* T-cell depletion using antibodies, or by the use of cyclophosphamide post-transplant. However, also after HLA mismatched transplantation reintroducing antitumor reactivity may be necessary. This may be performed by specifically targeting HLA molecules with limited tissue expression like HLA-DP or HLA-DQ under non-inflammatory circumstances, or by the use of other alloreactive immune cells like alloreactive NK cells. *In vivo* manipulation of the magnitude of the immune response by tempering inflammation or tissue damage may help to direct the immune response towards more specific GVL reactivity.

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Thrombosis

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Introduction

Inflammation is a protective tissue response to injuries, which involves blood vessels and leukocytes, together with several mediators. Inflammatory processes have a role in the initiation and evolution of atherosclerosis and in the progression to acute thrombotic complications. There is also growing evidence on the association between inflammation and venous thromboembolism (VTE). Inflammatory processes influence initiation and propagation of coagulation activation, downregulation of anticoagulant pathways and inhibition of fibrin removal.

The diagnostic approach to patients with clinically suspected VTE has evolved over time. Initially, diagnosis was only made on clinical grounds, but because signs and symptoms of VTE are nonspecific, objective diagnostic methods were required for a correct diagnosis. However, only a minority of patients referred for imaging tests is actually confirmed to have VTE, thus questioning the cost-effectiveness of their routine use in clinical practice. To improve the diagnostic approach to patients with suspected VTE, algorithms integrating clinical data, laboratory, and imaging tests have been developed and implemented.

The improved diagnostic yield of imaging tests has contributed to an increased detection of small clots with uncertain clinical impact. This is in particular the case of isolated distal deep vein thrombosis and subsegmental pulmonary embolism. Whether these patients require routine anticoagulant treatment remains a matter of debate.

Learning goals

After attending this lecture, participants should be able:

1. ***To understand the role of coagulation in inflammation and the impact of inflammation on thromboembolic disorders.***
2. ***To select the most appropriate diagnostic approach for patients with clinically suspected deep vein thrombosis or pulmonary embolism, with the aim to safely reduce the need for imaging tests.***
3. ***To decide the correct therapeutic strategies for patients with small clots such as isolated distal deep vein thrombosis and patients with subsegmental pulmonary embolism.***

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Thrombosis - Section 1

Cross-talk between inflammation and coagulation

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Take-home messages

- Coagulation and inflammation are closely linked and the FXII-driven contact activation system is an example for this intimate crosstalk.
- Platelet-released polyP forms nanoparticles that are retained on platelet surfaces where they directly initiate FXII activation..
- A routine assay for platelet polyP exists and may be used to establish the polymer as a biomarker for thrombosis.
- Targeting polyP protects from thrombosis in an FXII-dependent manner in vivo without elevating the bleeding risk.

Introduction

Coagulation and inflammation are considered as two distinct pathologies but they closely interact at multiple levels. The end result of proinflammatory and procoagulant reactions constitutes the unifying principle for a variety of disorders affecting the cardiovascular system including atherothrombosis, acute coronary artery disease, ischemia/reperfusion injury and infectious diseases such as bacterial sepsis. Understanding the role of coagulation in inflammation and the impact of inflammation on coagulation will introduce new perspectives to improve diagnostics and therapies for both disease states.

The plasma contact system is a pro-inflammatory and procoagulant protease cascade that is initiated by factor XII (FXII), in a reaction involving high molecular weight kininogen (HK) and plasma kallikrein (PK).[1] Upon contact with anionic surfaces, a conformational change occurs in zymogen FXII resulting in active FXII (FXIIa). FXIIa initiates the intrinsic pathway of coagulation via activation of the FXII substrate factor XI (FXI), and the bradykinin (BK) producing kallikrein-kinin system via plasma kallikrein.[2] Binding of BK to B2 receptor (B2R) activates intracellular signaling pathways that increase vascular permeability. C1 esterase inhibitor (C1INH) is the major plasma inhibitor of activated FXII and PK. Factor XII contact activation triggers coagulation in the diagnostic clotting assay 'activated partial thromboplastin time' (aPTT), however, deficiency in the protease is not associated with hemostatic abnormalities in humans and mice. Challenging the concept of the coagulation balance, thrombus formation is defective in FXII deficient (*F12*$^{-/-}$) mice.[3] Consistently, pharmacological inhibition of FXIIa-driven coagulation provides thromboprotection in large animals and humans[4] without an increase in therapy-associated bleeding. The biochemistry of the contact system is well-described but its biological functions, however, have just emerged within the last years. This short review summarized major findings in the contact system area of the last year. A more detailed overview is given in the latest reviews.[5,6]

Current state of the art

For decades, blood platelets have been known to activate FXII-driven coagulation the underlying mechanisms, however, have remained unknown. Until recently polyphosphate (polyP) was identified as a novel platelet derived FXII activator with critical implications for platelet-driven thrombosis and inflammatory reactions *in vivo*.[7,8] PolyP is an inorganic polymer of orthophosphate units linked by phosphoanhydride bonds. The polymer is ubiquitously found in all living cells and varies in chain length from just a few to several thousand phosphate units. In mamls, polyP stimulates an array of procoagulant mechanisms and drives fibrin formation by activation of the FXII-driven contact system.[9] Experimental animal models suggest a role of polyP in platelet-driven arterial and venous thrombosis. However, the potential therapeutic implications of interference with this substance have remained to be elucidated.

We have recently developed specific inhibitors of polyP and shown that this strategy confers thromboprotection in a FXII-dependent manner.[10] Exopolyphosphatase (PPX) is an intracellular enzyme that specifically hydrolyses polyP. To target the FXII activator polyP, we developed recombinant E. coli PPX

Thrombosis - Section 1

mutants that specifically neutralized circulating polyP. Interference with polyP abolished platelet-driven fibrin formation and thrombus formation in human blood. Targeting polyP interferes with FXII activation and selectively reduces the activated FXII-driven 'intrinsic' pathway of coagulation. Neutralizing blood-borne polyP in mice potently interferes with experimental arterial thrombosis and provides protection from venous thromboembolism in a pulmonary embolism model. Despite its potent antithrombotic activity, specific ablation of circulating polyP, similar to targeting FXII, does not prolong the bleeding time in mice and does not cause excess blood loss from injury sites. The study provides mechanistic insight as to the procoagulant properties of polyP and introduces ways to specifically interfere with its function. Disruption of blood-borne polyP proved to not interfere with hemostasis and may thus pave the way for the development of novel anticoagulation approaches with an improved benefit-to-risk profile in comparison with currently available agents.

Recently elegant imaging studies have visualized platelet polyP on living cells.[11] Platelets contain two pools of polyP: short polymers of a chain length ranging between 60-100 that are soluble and released into the supernatant. The majority of platelet polyP, however, is long chain molecules of chain length >500 that are bound to calcium ions and are not soluble in plasma. Calcium-polyP forms procoagulant nanoparticles of 100-200nm diameter that are retained on the platelet surface and readily activate FXII. Additionally, a promising assay to analyze polyP in human samples has been recently established.[12] A flow cytometry (FACS)-based assay using a recombinant polyP specific probe (PPX_Δ12) quantifies polyP exposed on cells such as activated platelets. Using this FACS-based system, the authors showed that platelets expose polyP aggregates e.g. nanoparticles on their surface that function as powerful activator of coagulation.

In addition to its procoagulant activities the contact system is a powerful pro-inflammatory pathway. The life-threatening swelling disorder hereditary angioedema (HAE) develops in individuals who are deficient in functional C1 esterase-

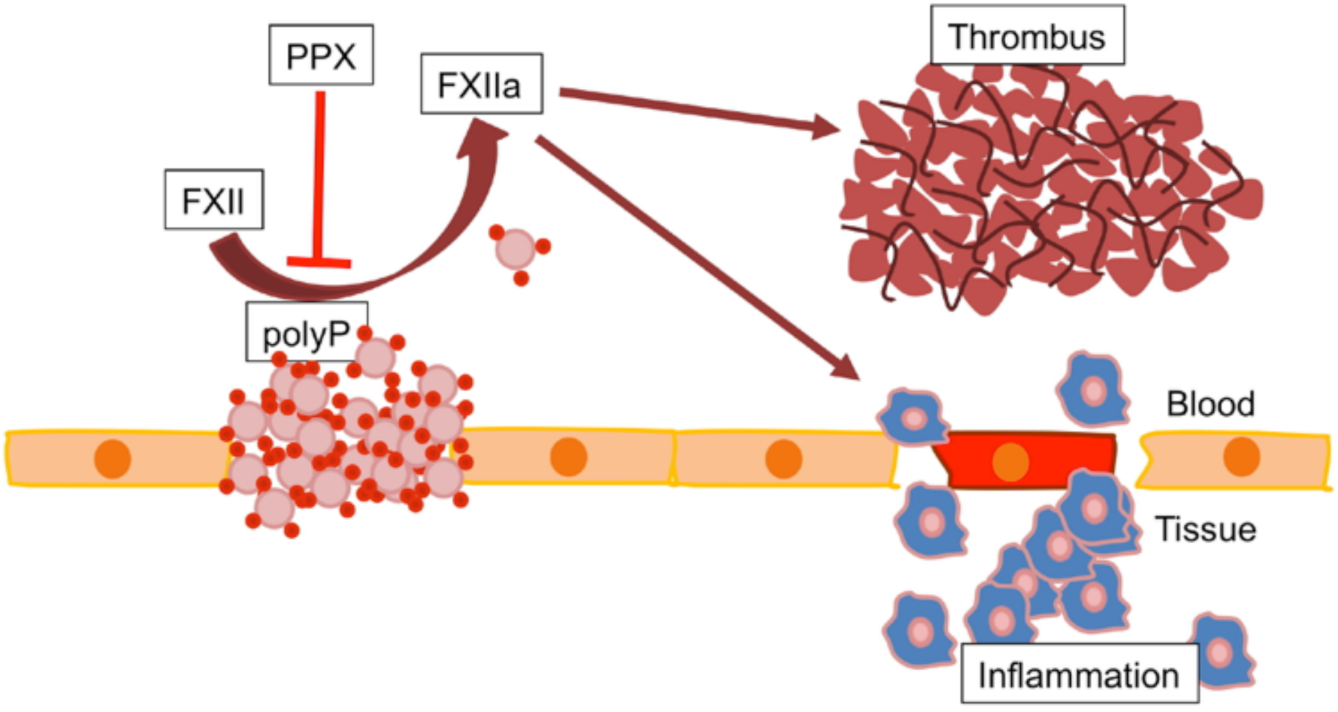

Figure 1. The polyphosphate-factor XII pathway in thrombosis and inflammation. Activated procoagulant platelets release the inorganic polymer polyphosphate (polyP) that is retained as calcium ion-rich nanoparticles at the cell surface. Binding of factor XII (FXII) zymogen to platelet polyphosphate induces contact activation and produces the active protease (FXIIa). Activated FXII drives thrombosis and vascular inflammation via the intrinsic coagulation pathway or kallikrein kinin system, respectively. The exopolyphosphatase PPX specifically degrades polyP and thus interferes with thrombosis and inflammation in an FXII-dependent manner.

inhibitor (C1INH; HAE types I and II). In addition to these two classic HAE types a third variant, HAE type III, exists in patients that have a completely normal C1INH but similarly suffer from edema attacks. The pathophysiology of HAE type III has remained unresolved, which limited therapy for this condition. GWAS studies have associated specific FXII point-mutations (Thr309Lys, Thr309Arg) with HAE type III. Using mouse edema models and patient materials, we have recently identified the mechanisms of HAE type III.[13] FXII-derived from HAE type III patients is defective in a mucin-type Thr309-linked glycosylation. Mutant FXII displays aberrant activation, which leads to excessive production of bradykinin via the kallikrein-kinin pathway. Intra-vital laser-scanning microscopy shows increased contact-driven microvascular leakage in both *F12*$^{-/-}$ mice reconstituted with recombinant FXII mutants and in humanized HAE type III mouse models with inducible liver-specific expression of Thr309Lys-mutated FXII. A FXII-neutralizing antibody, but not C1-esterase inhibitor that interferes with edema in HAE types I and II, abolished bradykinin generation in HAE type III patient plasma and blunted edema in HAE type III mice. The study characterized the mechanism of HAE type III and established FXII inhibition as a novel therapeutic strategy to interfere with excessive vascular leakage in HAE and potentially, other causes of edema and anaphylaxis.[14]

Recently a natural trigger for FXII activation, which causes uncontrolled bradykinin production in patients with HAE type III has been identified.[15] Recombinant variants of FXII revealed, that the HAE type III-associated mutations collectively introduce new sites that are sensitive to enzymatic cleavage by the protease plasmin. FXII mutants found in HAE type III rapidly activate following cleavage by plasmin, escape inhibition through C1 esterase inhibitor, and drive excessive bradykinin formation indicating that plasmin modulates disease activity in patients with HAE type III. These findings indicate a new pathway for bradykinin formation in patients with HAE in which FXII is cleaved and activated by plasmin. Taken together, the contact system has emerged as a promising drug target for interference with inflammation and coagulation *in vivo* (Figure 1). Understanding the interplay of coagulation and inflammation triggered by the FXII-driven contact system will open perspectives for safe anticoagulant drugs with additional anti-inflammatory activities.

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Thrombosis - Section 2

Novel aspects in the diagnostic management of deep vein thrombosis and pulmonary embolism

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Take-home messages

- Validated diagnostic algorithms should be used in every patient with suspected deep-vein thrombosis or pulmonary embolism.
- The YEARS diagnostic algorithm is an easy algorithm for suspected PE, with parallel assessment of D-dimer and clinical decision items, leading to improved logistics and less need for CTPA.
- The ADJUST rule of using adjusted D-dimer above the age of 50 years is safe and leads to less need for CTPA.
- In patients with suspected recurrent ipsilateral DVT, MRDTI may prevent over diagnosis and treatment.

Introduction

Because the diagnosis of clinically suspected deep vein thrombosis (DVT) and pulmonary embolism (PE) is nonspecific, standard diagnostic algorithms for patients with suspected venous thromboembolism (VTE) have been developed over the years, which include sequential use of both non-invasive bedside tools (clinical decision rules and D-dimer blood tests) for patients with low pre-test probability and imaging techniques - which comprises of compression ultrasound for DVT and computed tomography pulmonary angiography for PE - for patients with a high pre-test probability based on the bedside tools (Huisman MV. J Thromb Haemost 2013;11:412-22; Huisman MV. Blood 2013;121:4443-8.) Recently, the YEARS algorithm has been developed and validated in a prospective management outcome study (van der Hulle T. Lancet 2017, in press). The YEARS algorithm involves the simultaneous assessment of three most contributing items of the original Wells rule – symptoms of DVT, hemoptysis and diagnosis of PE most likely – and a D-dimer level. Dependent on the result of this assessment a CT pulmonary angiography (CTPA) is indicated. In the validation study, this novel algorithm proved efficient – leading to an absolute 14% reduction in CTPA - and safe-incidence of recurrent VTE was 0.61% and fatal PE was

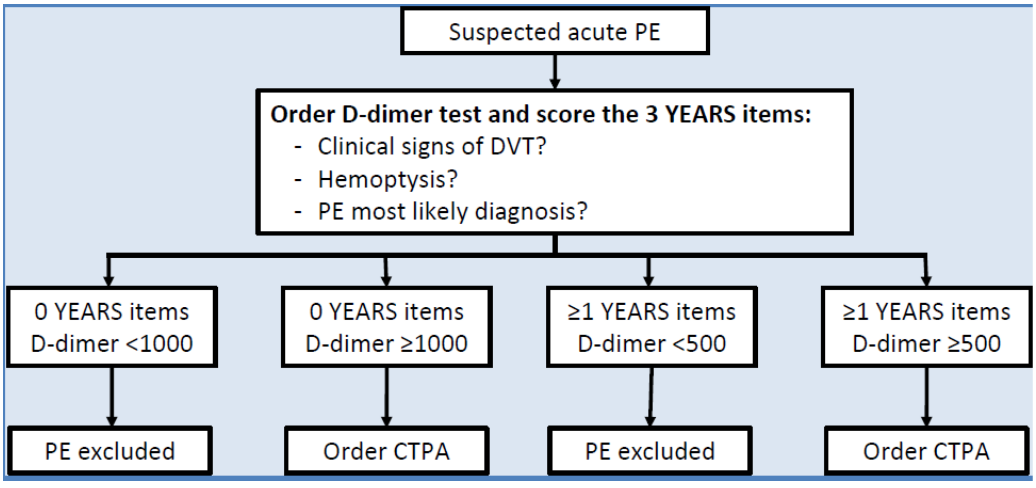

Figure 1. YEARS algorithm.

Thrombosis - Section 2

0.20%. This combination has led to standardized diagnostic approaches with proven safety and efficiency for excluding VTE. At the same time, it has become apparent that there are several areas of diagnosis where there is controversy as to the best approach. This includes patients with clinically suspected arm vein thrombosis, patients with clinically suspected recurrent VTE, elderly and pregnant patients with suspected VTE. These patient groups all present with special diagnostic challenges, since either the current standard diagnostic algorithms are not sufficient or they involve unwanted radiation. In a recent management study a comprehensive algorithm for suspected arm vein thrombosis proved to be efficient and safe (Kleinjan A. Ann Int Med 2014;160:451-7). In 87 of 406 patients (21%), an unlikely score combined with normal d-dimer levels excluded upper extremity DVT. Superficial vein thrombosis and arm vein thrombosis were diagnosed in 54 (13%) and 103 (25%) patients, respectively. Only one of 249 patients with a normal diagnostic work-up developed a thrombosis at follow-up, for a failure rate of 0.4% (95% CI, 0.0% to 2.2%). Of note, the efficiency of the algorithm - defined as not needing a CUS - was lower in patients with cancer (11%) or older age (>75 years -13%). In patients with suspected recurrent DVT of the leg, CUS is often not decisive, because old thrombi render the CUS to stay abnormal. A novel technique called MR direct thrombus imaging was proved to be highly sensitive and specific for first and recurrent DVT. In a proof of principle study, the MRDTI showed sensitivity of 95% and specificity of 100% with a kappa of 99% (Tan M. Blood 2014;124:623-7). A clinical outcome study using MRDTI as the sole imaging test to rule out ipsilateral DVT is currently including patients. In elderly patients, there is a need for improved efficiency of the algorithm since the clinical utility of the D-dimer test is limited in them. A new cut-off of D-dimer measurement - 10 times the age above 50 years has been successfully evaluated as part of an algorithm in more than 3000 patients (Righini M. JAMA 2014;311:1117-24). Using this rule, an absolute extra 10% of patients could be managed without imaging tests and recurrent VTE occurred in 0.3% of patients. Finally, in pregnancy there are few validated algorithms. Currently the ARTEMIS study - based on the YEARS algorithm - is currently including pregnant patients.

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Thrombosis - Section 3

Controversies in treating small clots in the leg and in the lung

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Take-home messages

- Isolated distal DVT, and possibly subsegmental PE, are conditions that differ with regard to recurrence rate, mortality, and chronic sequelae from more extensive disease. A considerable proportion of both diagnoses is likely to represent false positive imaging results.
- Uniform anticoagulation for all patients has a substantial risk for an unfavorable harm-benefit-ratio.
- Risk profiling as basis for well-balanced treatment decisions is warranted but lacks firm data to be based on. All experts accept active cancer as high-risk condition necessitating anticoagulation. Others will have to be defined by future studies.

Introduction

'Small clots in the legs and in the lung' can be translated into two distinct disease entities, i.e. isolated distal calf vein thrombosis (ICVT) and subsegmental pulmonary embolism (SSPE). ICVT is being diagnosed mostly in symptomatic patients with suspected DVT. Thrombosis is confined to the calf muscle veins and/or the paired deep calf veins without involving the popliteal vein.

SSPE is being diagnosed in two different patient populations: first, in symptomatic patients, with the thrombembolus only in one or a few minor branch(es) of the pulmonary artery tree, supplying less than one segment; and second, in asymptomatic patients undergoing CT scans for follow up examinations in currently or previously treated cancer.

The clinical impact of small clots has been questioned in both cases, and thus, the need for anticoagulation is under debate.

Current state of the art

ICVT

Known from pathophysiology, most episodes of symptomatic deep vein thrombosis (DVT) start in the calf and propagate to the thigh veins.[1] Once having reached the proximal veins DVT has a considerable risk for pulmonary embolism. Conversely, as long as ICVT does not propagate the risk of pulmonary embolism (PE) is negligible.[2] Apart from propagation to proximal, ICVT is a relatively benign disease: recurrence rates are reportedly lower in ICVT than in proximal DVT or PE, except if associated with malignancy.[3] In addition, the frequency and severity of the post thrombotic syndrome (PTS) as a late sequela is less than half as compared with proximal DVT.[1]

The key question, therefore, is the estimated risk of propagation from distal to proximal. Different rates of extension of symptomatic ICVT to the proximal veins have been reported. A recent meta-analysis resulted in an estimate of around 9%.[4] This means that around 90% of all cases would not need anticoagulation because of a self-limiting natural course.[5]

Two different attitudes towards the diagnosis of DVT - and thereby ICVT - have emerged: serial imaging of the proximal leg veins with anticoagulation only in case of proximal DVT[6] *versus* complete compression ultrasound of the leg (CCUS) as a single examination,[7] followed by anticoagulation of proven ICVT in most cases. Neither the first nor the second strategy has proven superiority over each other regarding safety or efficacy.[8,9] However, serial testing of proximal veins is not resource saving, whereas routine examination of distal veins carries a substantial risk of overtreatment due to both false positive ultrasound results and anticoagulation of a self-limiting condition.

Up to now, randomized trials on treatment of ICVT failed to demonstrate any benefit of anticoagulation[10] (Table 1). The most recent example of such a RCT was the CACTUS trial that showed no difference in efficacy but significantly more

bleeding in patients with anticoagulation.[11] Like others, it suffered from the fact that only patients with an obviously low risk of propagation had been included. Consequently, international guidelines give only low-grade recommendations for a highly individualized treatment algorithm based on supposed risk factors for propagation.[12]

SSPE

With the event of multiple detector computed tomographic pulmonary angiography (CTPA) the rate of detection of subsegmental pulmonary embolism, due to higher resolution, has increased. In parallel, doubts have arisen as to whether these SSPEs deserve the same treatment as segmental or even more proximal PEs.[13] The source of uncertainty is threefold. First, false positive SSPE detection in CTPA remains a matter of concern.[14] Second, the safety of single detector vs multiple detector CTPA for the exclusion of PE seemed to be equal despite a rate of SSPE double as high in the latter, thereby providing indirect evidence that the 'missed' SSPEs in single detector CTPA had no prognostic relevance in the following three months.[15] Third, epidemiologic studies demonstrated an increasing rate of incident PEs over the years without an increase in mortality due to PE. This provides indirect evidence that the case fatality of PE dropped down, indicating that the surplus of PEs can attributed to benign and clinically less relevant cases.[16] In consequence, therapeutic anticoagulation for all patients with a SSPE diagnosis might have a significant potential for harm.

There are no randomized controlled trials addressing the issue. In 2012, a systematic review identified 60 patients with SSPE in whom anticoagulation was withheld. None of these patients suffered recurrent symptomatic VTE (PE or DVT) during a 3-month follow-up.[17] By contrast, indirect evidence for a greater clinical relevance of SSPE was provided by the finding that, in a large cohort of patients with suspected PE, the prevalence of risk factors, the 3 months' recurrence risk and mortality of 116 SSPE patients was similar to 632 with more proximal PE but dissimilar to 2980 patients in whom PE had been excluded.[18] All patients with SSPE in this series had received anticoagulation. This is in concordance with the result of a survey in which most experts were in favor of prescribing anticoagulants to patients with SSPE.[19] Finally, in a pooled cohort of 926 cancer patients with incidental PE from 11 different studies, 197 had had SSPE. Again, the 6 months' recurrence rate was similar to patients with incidental, more proximally located PE. In the subgroup of 42 patients left untreated, the recurrence rate of SSPE was numerically comparable between SSPE and other localisations.[20]

Like for ICVT, international guidelines support a management algorithm that takes risk factors for propagating or relapsing VTE into account when assessing the need for anticoagulation. Unequivocally, patients with active cancer are considered to be at high risk.

Future perspectives

Despite the lack of direct evidence, the expert view is consolidating that for both entities, ICVT as well as SSPE, anticoagulation is indicated in patients with active cancer. A potential for withholding anticoagulation, however, does exist for non-cancer patients without other high-risk constellations for VTE propagation or recurrence. However, the definition of 'high risk' is far from being established and is likely to be different in ICVT and SSPE. Since any RCT will require firm exclusion criteria a priori, no additional insights about 'high risk' can be gained from such type of future study. Instead, better knowledge may be derived from well-characterized cohorts of patients with either ICVT or SSPE who are left untreated but receive close surveillance in order to attribute adverse outcomes to given risk factor profiles.

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Thrombosis - Section 3

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This very large pooled patient series strongly supports the view that at least in cancer patients SSPE does require treatment comparable to symptomatic more proximal PE.

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Update on hemoglobinopathies

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Introduction

During the last 2 decades, the treatment of thalassemia and sickle cell disease (SCD) improved significantly, extending the survival and providing better quality of life for the affected patients, however some unmet needs still remain and further progresses are warranted. Bone marrow transplantation is curative for up to 90% of selected patients, but serious complications can occur, especially without an HLA-matched sibling donor. Gene therapy is promising either for thalassemia and SCD but has not yet proven to be fully effective. Dr M. Weiss will discuss a future perspective for curing SCD and beta-thalassemia by genome editing of hematopoietic stem cells (HSCs), either to repair the causal mutations or to create new mutations that suppress disease phenotypes by inducing fetal hemoglobin (HbF) production in adult red blood cells (RBCs). Dr. V Viprakasit will update on iron chelation (ITC) in hemoglobinophaties that, at present, remains a pillar of the conventional treatment for these diseases in order to control morbidity and mortality. By optimizing doses, formulations and drug combinations of available iron chelators,the results in clinical practice are satisfactory although the patient's compliance remains the main driver. Dr F. Kirkham will focus on neurological complications in SCD in children and in adults. There appears to be a familial predisposition to stroke and to high blood flow velocities in SCD, indicating that genetic factors may play a role. Monitoring and preventative measures for neurological complications in hemoglobinophaties will be discussed.

Learning goals

1. ***To update on future perspectives for treating hemoglobinopathies by genome editing.***
2. ***To update on iron chelation modalities having 3 iron chelators available; suggestions on how to tailor the ITC to the patient need.***
3. ***To update on neurological complications in SCD: how to monitor and prevent stroke in children and in adults.***

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Update on hemoglobinophaties - Section 1

Genome editing in hemoglobinopathies

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Take-home messages

- Genome editing is initiated by programmed nucleases that introduce a double-stranded DNA break at a specified sequence.
- Cells repair double-stranded DNA breaks through error-prone non-homologous end joining (NHEJ) or through homology directed repair (HDR), which utilizes an exogenous DNA template to create precise nucleotide alterations. Strategies to treat β-hemoglobinopathies include NHEJ to induce fetal hemoglobin or HDR to repair the pathogenic mutation.
- In hematopoietic stem cells, NHEJ occurs at approximately 10-fold greater frequency than HDR.
- Challenges to clinical genome editing include optimizing programmed nuclease delivery into cells, defining and minimizing deleterious off target effects, and maximizing the efficiency of on target editing.

β-hemoglobinopathies, including sickle cell disease (SCD) and ß-thalassemia are common, devastating autosomal recessive disorders caused by mutations in the *HBB* gene, which encodes the β-globin subunit of adult hemoglobin (HbA, α_2 β_2).[1,2] Medical therapies extend the lives of patients and reduce suffering, but do not eliminate major clinical problems. Allogeneic bone marrow transplantation (BMT) is curative for up to 90% of selected patients, but serious complications can occur, particularly for BMT recipients without an HLA-matched sibling donor. Gene therapy via lentiviral vector replacement of *HBB* is promising, but has not yet proven to be fully effective.

We are poised to cure SCD and β–thalassemia by genome editing of hematopoietic stem cells (HSCs), either to repair the causal mutations or to create new mutations that suppress disease phenotypes by inducing fetal hemoglobin (HbF) production in adult red blood cells (RBCs). Genome editing is based on programmable sequence-specific DNA nucleases that create targeted double-stranded breaks (DSBs).[3,4] Classes of gene editing nucleases include clustered regularly interspaced short palindromic repeats (CRISPR)/Cas, transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs). Cells typically repair nuclease-induced DSBs according to two distinct and competing endogenous enzymatic pathways: 1) error-prone non-homologous end joining (NHEJ) can rejoin the broken DNA ends, but often introduces insertions or deletion mutations (indels) that can disrupt coding genes or noncoding regulatory sequences; 2) in the presence of a user-supplied homologous "donor" DNA template, DSBs can be repaired precisely by homology directed repair (HDR). By varying the HDR donor template, it is possible to correct deleterious mutations. Both HDR and NHEJ-mediated genome editing strategies are under investigation for treating β-hemoglobinopathies. The general strategy is to remove HSCs from the patient, edit these cells *ex vivo* to induce therapeutic genetic alterations, then return them to the patient after administration of a myelotoxic "conditioning" agent to enhance engraftment of the altered HSCs. This therapy modifies somatic cells so that the induced genetic changes are not passed through the germline.

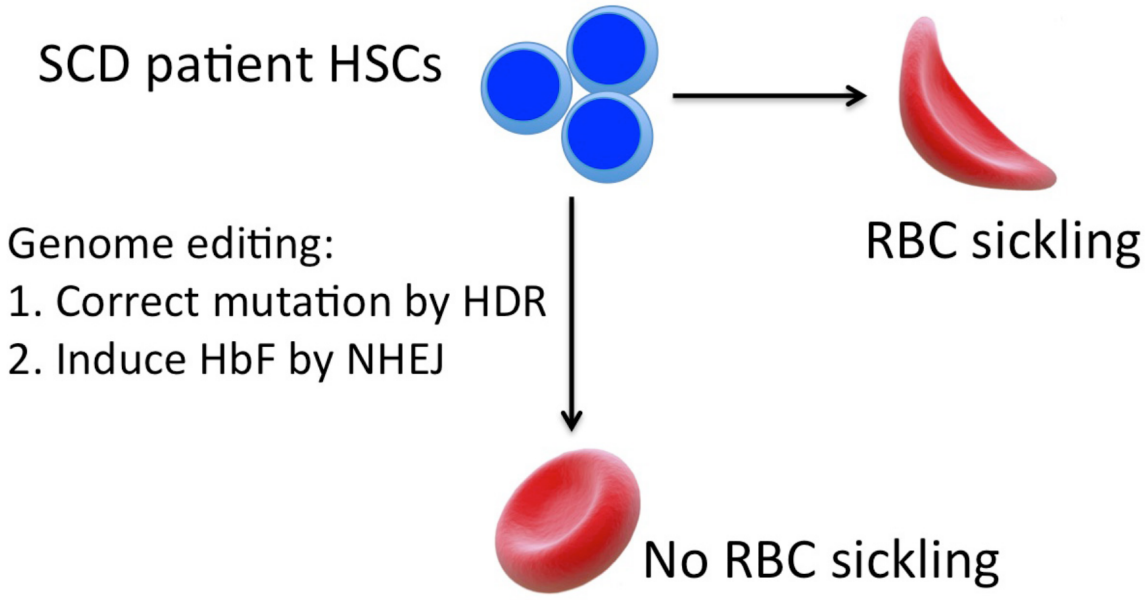
Correction of β-hemoglobinopathymutations by HDR

The major form of SCD is caused by a homozygous A-to-T mutation resulting in a glutamic acid-to-valine substitution at β-globin amino acid 6. It is possible to correct this mutation through genome editing by creating a DSB in mutant *HBB* exon 1 and supplying a donor DNA template containing the normal sequence.[5-7] Preliminary results are promising, but several challenges exist for clinical application. First, NHEJ occurs more efficiently than HDR at DSBs,[8,9] particularly in quiescent HSCs, resulting in low rates of gene correction combined with NHEJ-mediated null (β^0) alleles. Second, HDR requires co-delivery of a donor template and may require selection of edited cells, both of which may complicate production of therapeutic cell products. In contrast to SCD, β-thalassemia is caused by many different mutations, making it more challenging to create a general correction strategy.

Induction of fetal hemoglobin via NHEJ

Fetal Hb (HbF, $\alpha_2\gamma_2$) is the main RBC oxygen carrier during late gestation. After birth, γ-γlobin synthesis declines and β-globin increases, thereby switching HbF to HbA. The γ-globin genes *HBG1* (Aγ) and *HBG2* (Gγ) are located upstream of *HBB* (β) and their relative expression is controlled by regional cis elements and key trans-acting transcription factors, both of which have been identified by human genetic studies.[10,11] Residual HbF in adult RBCs is expressed as a quantitative genetic trait and β-hemoglobinopathy patients with higher HbF levels experience fewer symptoms and reduced mortality.[10,12,13] Hydroxyurea, the only approved drug for SCD, acts partly by raising HbF levels though unknown mechanisms. Genome editing provides new opportunities to raise HbF levels therapeutically by manipulating known regulators. In a rare genetic condition termed hereditary persistence of fetal hemoglobin (HPFH), relative HbF levels exceed 20% in adult RBCs.[14] HPFH is a benign condition typically caused by deletions or point mutations in the extended β-globin locus. Individuals with SCD or β-thalassemia genotypes who co-inherit HPFH mutations experience few or no disease pathologies. Several groups, including ours, are using genome editing-mediated NHEJ to recapitulate HPFH mutations, with promising preliminary results.[15,16]

It is also possible to raise HbF by targeting transcription factors that repress γ-globin production. The leading candidate is BCL11A, which was identified through genome wide association studies to regulate HbF levels in adult RBCs.[17-19] Because HSCs and B cells require BCL11A,[20-23] therapeutic strategies to inhibit its expression must be specific to erythoid lineages. This may be achieved via NHEJ-mediated disruption of an erythroid-specific *BCL11A* enhancer, which raises HbF to potentially therapeutic levels in adult $CD34^+$ cell-derived erythroblasts.[19] Excision of the orthologous enhancer in mice increases embryonic/fetal globin expression in adult RBCs, with no deleterious effects on other cell lineages.[24] Thus, genome editing-mediated disruption of the*BCL11A* erythroid enhancer via NHEJ represents a potential therapeutic strategy.



Genome editing for sickle cell disease (SCD). Patient hematopoietic stem cells (HSCs) produce red blood cells (RBCs) that undergo pathological sickling due to adult hemoglobin (HbA, α2β2) polymerization at low oxygen tension. HSCs may be modified by genome editing to prevent sickling of their RBC progeny. Two approaches are under investigation: correction of the *HBB* gene SCD mutation by homology directed repair (HDR) and induction of fetal hemoglobin (HbF, α2γ2) by non-homologous end joining to de-repress γ-γlobin transcription from the *HBG1* and/or *HBG2* genes.

Toxicities

While gene editing is quite specific, nuclease-induced off-target DSBs can cause unintended mutations.[25] Additionally, genomic deletions or rearrangements can occur between two DSBs that occur simultaneously in the same cell. While some off-target DSBs are predicted by sequence homology to the on-target site, current *in silico* prediction algorithms fail to capture the majority of *bona fide* off-target effects.[25] Numerous cell-based unbiased genomic approaches can detect off target DSBs and genomic rearrangements in cells to a sensitivity of about 0.1%. Most low frequency off-target DSBs are likely to be inconsequential. However, a major concern is that rare off-target mutations in HSCs could confer selective growth advantages and thereby induce hematopoietic malignancies. In these cases, very low-frequency mutations occurring below current limits of detection could be deleterious. Ideal preclinical assays to assess such mutations are lacking. Currently, patients receiving any form of HSC gene therapy, including genome editing, must undergo myelotoxic conditioning prior to infusion of modified autologous HSCs in order to facilitate their engraftment. Conditioning regimens can cause acute organ damage, sterility, hair loss and leukemia.

How much correction is enough?

The fraction of correctly edited HSCs required to cure β-hemoglobinopathies is unknown. Genetic correction will enhance the survival of erythroblasts and RBCs over those with β-thalassemia or SCD mutations. Therefore, disease-free RBC populations can arise from relatively low levels of corrected HSCs. Assessment of mixed donor chimerism after allogeneic BMT for β-hemoglobinopathies[26-32] and mathematical modeling[33] estimate that as low as 20% corrected HSCs could be curative. However, these models assume that every gene-edited cell will undergo complete correction, which may not be the case. For example, HDR to correct the *HBB* SCD mutation will likely be accompanied by NHEJ-induced null alleles and gene editing to inhibit γ-to-β globin switching may not induce HbF to fully therapeutic levels in all RBCs. Thus, while preclinical benchmarks for % HSC editing are important to consider, clinical trials are necessary to define more precisely the therapeutic requirements for HSC modification.

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Update on hemoglobinophaties - Section 2

Iron chelation in hemoglobinopathies

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Take-home messages

- Iron overload (IOL) is highly prevalent among patients with hemoglobinopathies; both transfusion dependent and non-transfusion dependent thalassemia (TDT & NTDT).
- Serum ferritin (SF) could be used to screen and identified patients with IOL, however a direct tissue iron measurement using magnetic resonance imaging (MRI) has become more effective for a better long term iron monitoring and guiding management.
- With appropriate tailoring based on tissue iron monitoring, three current iron chelators, deferoxamine (DFO), deferasirox (DFX) and deferiprone (DFP), could be used as a monotherapy and in a combination to provide effective iron chelation.

Introduction

Thalassemia is characterized by a reduction of either or both α and β globin chain synthesis, represents the most common and most significant form of hemoglobinopathies. Based on a recent classification, thalassemias are divided based on their clinical presentation, phenotypic severity and transfusion requirement into transfusion dependent thalassemia (TDT)[1] and non-transfusion dependent thalassemia (NTDT).[2] In TDT, iron overload (IOL) was directly resulted from transfused blood (transfusional iron overload) leading to iron related mortality and morbidities. IOL also develops in NTDT patients, even without frequent blood transfusion. Iron dysregulation and clinical management of IOL are somewhat different between TDT and NTDT in several aspects (summarized in Table 1) and would be the main subjects of this review. Iron overload and management in Sickle cell disease (SCD) has recently been reviewed and not covered.[3]

Current state of the art

Role of iron overload and its relevance of clinical complications

At present, management guidelines for transfusional iron overload are generally derived from clinical experience and trials in TDT.[1,4] Due to a lack of physiologic mechanism to remove iron acquired from transfused blood (each unit of blood contains 200-250 mg iron), regularly transfused patients accumulate iron of 0.4-0.5 mg/kg/day and IOL can occur after 10-20 transfusions (Table 1).[4] Despite, infrequent or no transfusion, IOL occur in NTDT due to increased gastrointestinal iron absorption, driven by hepcidin suppression and erythron expansion leading to hepatic iron loading.[5] In TDT, heme catabolized iron will readily saturate transferrin generating non transferrin bound iron (NTBI) in plasma. This NTBI can be rapidly taken through calcium channels into primarily hepatocytes and extra-hepatic iron accumulation i.e. heart and endocrine glands leading to several iron related complications (Table 1).[5] Although cardiac siderosis is a major cause of morbidity and mortality and a key factor in management decisions in patients with TDT, it does not seem to be a major concern in NTDT.[2] However, IOL in NTDT was associated with several morbidities (Table 1) leading to a recommendation of treatment.[2] Interestingly, a subgroup of NTDT patients who were previously transfused, splenectomized and high transferrin saturated (>70%) have increased NTBI and can be significantly susceptible to extrahepatic IOL.[5]

Role of iron monitoring: serum ferritin (SF) vs magnetic wresonance imaging (MRI)

Non-invasive iron monitoring using MRI for liver iron concentration (LIC) and cardiac T2* have become the gold standard to diagnose IOL and guide iron chelation therapy (ICT).[6] Several clinical surveys have demonstrated a geographical difference on prevalence of IOL among different regions of the world.[7-9] β-thalassemia major (TM) patients in Southeast Asia had the highest prevalence of cardiac IOL (T2*<20 msec), followed by Europe and the Middle East.[7] This result was consis-

tent with another survey using baseline SF to determine IOL in TDT.[8] Further evidence of considerable cardiac and liver iron burden across regions was reported.[9] The underlying mechanism of this difference remains unknown but it may suggest different local and regional ICT practice. Although, MRI can provide a direct organ specific iron determination, however its clinical use should be optimized for a cost-benefit in real-life practice.[10-12] To this regard, SF cut-offs of 1900, 1100 and 650 ng/mL for detection of liver IOL were proposed in β–TM, transfused and non-transfused β–TI respectively.[11] However, SF is not appropriate for diagnosing cardiac IOL, but can be used for exclusion when SF <2500 ng/mL.[12]

Role of iron chelation and its relevance to morbidity and mortality

Currently, the primary goal of ICT has shifted from treating or rescuing IOL to maintaining at all time the safe levels of body iron.[1] To achieve this, iron intake must be balanced with iron excretion by chelators to prevents iron accumulation and end-organ complications leading to normal survival and quality of life.[1] Therefore, appropriate, tailoring ICT with chelator choices and dose adjustment must be implemented at a timely manner, especially in pediatric patients.[4] It leads to a dramatic improvement of life expectancy in TDT patients in the last 50 years.[13]Three iron chelators, deferoxamine (DFO), deferasirox (DFX) and deferiprone (DFP), are currently available as

Table 1. Iron overload (IOL) and iron chelation therapy (ICT) in patients with transfusion dependent thalassemia (TDT) *vs.* non-transfusion dependent thalassemia (NTDT).

	Factors	TDT[1]	NTDT[2]
Underlying disease	Common types of thalassemia	β-thalassemia major (β-TM), severe Hb E/β-thalassemia, transfusion dependent Hb H disease, Hb Bart's hydrops	β-thalassemia intermedia (β-TI), Hb E/β-thalassemia, Hb H disease, Hb C/ β-thalassemia
IOL	Mechanism	Major: Blood transfusion Minor: Increased intestinal absorption	Major: Increased intestinal absorption Minor: Occasional blood transfusion
	Rate of iron accumulation	Rapid with marked generation of NTBI and LPI	Slow and lower NTBI
	Onset	Usually after 10-20 units of blood transfusions	Later on, usually after 10 yrs (or 15 yrs in patients with Hb H disease)
	Risk of extrahepatic IOL*	High	Low
	Common IOL related complications	Cardiac siderosis, heart failure and cardiac arrhythmia Liver fibrosis, cirrhosis and carcinoma Endocrinopathies i.e. diabetes, hypothyroidism, hypoparathyroid, adrenal insufficiency, hypogonadism, low bone mass, osteoporosis and growth failure	Liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) Associated with increased risk of thrombosis, pulmonary hypertension (PHT), osteoporosis, hypothyroidism, hypogonadism, right heart failure, gallstones and infections
ICT	Indication to initiate ICT	SF ≥ 1000 ng/mL	SF ≥ 800 ng/mL and/or LIC ≥ 5 mg/g dry weight liver
	Optimal levels of iron status after ICT	SF <1000 ng/mL and LIC <7 mg/g dry wt. liver and Cardiac T2* ≥ 20 msec.	NA
	Indication to intensify ICT	SF ≥ 2500 ng/mL and/or LIC >7 mg/g dry wt. liver and/or Cardiac T2* < 20 msec.	LIC after 6 months of treatment > 7 mg/g dry wt. liver or SF >1500-2000 ng/mL and < 15% decrease from baseline
	Indication to stop ICT	SF < 300 ng/mL and/or LIC <3 mg/g dry wt. liver	SF < 300 ng/mL and/or LIC < 3 mg/g dry wt. liver
	Choices of ICT** Monotherapy	First line of treatment: DFO 40-60 mg/kg/day DFX 20-40 mg/kg/day Second line of treatment: DFP 75-100 mg/kg/day	DFX 10-20 mg/kg/day NA
	Combination***	DFO+DFP, DFO+DFX and DFP+DFX	NA

*NTBI: non-transferrin bound iron; LPI: labile plasma iron; SF: serum ferritin; LIC: liver iron concentration; yrs; years; DFO: deferoxamine; DFX: deferasirox; DFP: deferiprone; wt.: weight; NA: not available; *including pancreas, endocrine glands and kidney; **based on current drug registration in Europe, USA and Asia; ***only compassionate use based on published data.*

monotherapy. Clinical decision to initiate, adjust and stop (or maintaining) of ICT are based on SF, MRI-LIC and cardiac T2* (Table 1). Development of new tridentate molecules of desferrithiocin class such as FBS0701[14] and SP-420[15] were now on hold due to reported toxicities. Therefore, recent clinical studies of ICT focused on current chelators by improving formulation, optimizing dose administration and adapting different chelator combination. A new film-coated tablet (FCT) of DFX was studied in a randomized trial comparing with original dispersible tablets (DT).[16] Based on Patient-Reported Outcomes (PROs), FCT showed greater adherence and satisfaction, better palatability and higher compliance than DT. However, it remains to be seen whether this improvement could be translated into a meaningful clinical efficacy. By adjusted administrative dose of DFX; from once into twice daily, improvement on efficacy was observed.[17] Combination of iron chelators have been reported with improving effectiveness.[18,19] A rapid decrease in LIC from heavily iron-overloaded TDT patients was observed in DFX-DFO.[18] A study of DFX in NTDT provided evidence of better ICT by early dose escalation.[20] However, these studies did not change on official label of the drugs and clinicians must warn their patients for an off-label use if they apply these reported experiences into their clinical practice.

Future perspectives

Further longitudinal studies are needed to assess causal relationship between iron overload and certain morbidities in NTDT patients in particular, those with higher risk of generating NTBI and extrahepatic IOL. Although safe and adequate blood transfusion with optimal chelation can normalize survival and improve quality of life in TDT patients. However, applying this approach to 'all thalassemia patients' might be limited in several parts of the world where resources are restricted. A long term randomized study comparing between current standard practice and a more proactive transfusion and chelation is highly required. Moreover, several practical questions remain unanswered; i.e., roles of early chelation in younger children (<2 yrs.) before IOL developed, the best optimal levels of iron status balancing risk of chelators *vs.* iron toxicities and which strategy between 'stop and re-start' or continuous ICT when iron level reach a certain threshold. To interrogate these unsolved puzzles, an international effort on multicenter international collaborating studies would be valuable and lead to further evidence based recommendations for ICT in hemoglobinopathies.

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Update on hemoglobinophaties - Section 2

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This article further demonstrated dose escalation of DFX in NTDT patients based on baseline LIC and LIC response at 6 months with the maximum DFX dose of 30 mg/kg/day could provide a better chelation efficacy. DFX was registered for NTDT at dose of 10-20 mg/kg/day.

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Update on hemoglobinophaties - Section 3

Neurological complications of sickle cell disease

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Take-home messages

- Pathologies underlying acute neurological events in sickle cell disease include (1) arteriopathy: cervical carotid and vertebral and intracranial arterial stenosis, dissection, occlusion and aneurysm, (2) venous sinus thrombosis (3) posterior reversible encephalopathy syndrome (4) shunting at pulmonary and cardiac level
- While emergency transfusion is the mainstay of management of acute neurological problems, consultation with intensive care and the stroke unit enables appropriate supportive management, while thrombolysis is not contraindicated in adults with SCD and stroke within the 4.5 hour window from onset and if hemorrhage has been excluded by CT scan
- Velocities >200 cm/sec on transcranial Doppler ultrasound (TCD) predict stroke in children; indefinite transfusion or 1 year of transfusion followed by Hydroxyurea in those with normal magnetic resonance angiography prevents stroke.

Abstract

Children with sickle cell disease may have a wide variety of neurological syndromes, including ischemic and hemorrhagic stroke, anterior and posterior territory transient ischemic attacks (TIAs), seizures, headache, coma, visual loss, altered mental status, 'silent' cerebral infarction (SCI) on neuroimaging and cognitive difficulties. Those with clinical ischemic stroke usually have stenosis or occlusion of the cervical and intracranial arteries. Venous sinus thrombosis may cause coma with or without ischemic or hemorrhagic stroke. Seizures are associated with cerebrovascular disease and SCI but may also occur secondary to posterior reversible encephalopathy syndrome (PRES). For hemorrhagic stroke, aneurysms are common in adults but children may present with hypertension secondary to transfusion or corticosteroids, possibly PRES. Long term transfusion prevents recurrent infarction in those with SCI but does not appear to improve intelligence quotient. Velocities >200 cm/sec ontranscranial Doppler ultrasound (TCD) predict stroke in children; indefinite transfusion or 1 year of transfusion followed by Hydroxyurea in those with normal magnetic resonance angiography prevents stroke. The interaction between genetic and modifiable environmental effects should be investigated. As hemoglobin oxygen desaturation and airway obstruction appear to be risk factors, randomised trials of overnight respiratory support in older children and adults, and of Montelukast in preschool children, are underway.

Introduction

There is a broad spectrum of acute presentation with CVA and other neurological complications in patients with SCD.[1] Without preventative strategies, clinical stroke, with focal signs lasting >24 hours (Table 1), is 250 times more common in children with SCD than in the general pediatric population, and commonly presents 'out-of-the-blue' in an apparently well child. Patients with SCD also have transient ischemic attacks (TIAs) with symptoms and signs resolving within 24 hours, although many of these individuals are found to have recent cerebral infarction or atrophy on imaging (Table 1). In addition, seizures, headache[2] and coma are common in patients with SCD. Altered mental status can occur in numerous contexts, including acute chest syndrome (ACS),[3] acute anemia e.g. aplastic secondary to parvovirus,[4] after surgery, transfusion[5] or immunosuppression, and apparently spontaneously. These patients may have had an ischemic or hemorrhagic cerebrovascular accident (Table 1), although there is a wide differential of alternative focal and generalized vascular and non-vascular pathologies, including posterior reversible encephalopathy syndrome (Table 1),[6] and shunting at pulmonary or cardiac level.[7]

As well as those with obvious acute neurological events, patients with SCD accumulate 'silent' cerebral infarction (SCI) on MRI from infancy through to adulthood,[8-10] characteristically in the anterior and/or posterior borderzones (Table 1), without having had a clinical stroke, although they may have had subtle TIAs, headaches or seizures. Cognitive diffi-

culties affect processing speed, attention and executive function,[11] as well as intelligence.[12]

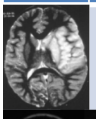
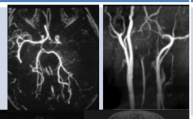
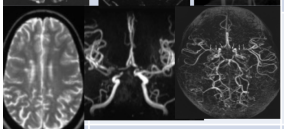
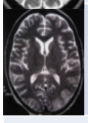
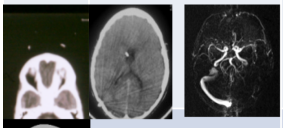
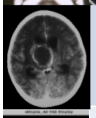
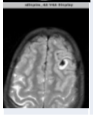
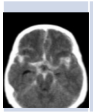
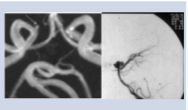
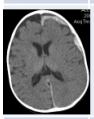
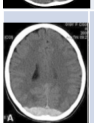
Current state of the art

Acute neurological problems

When a patient with SCD presents with acute neurological problems, the priority is to transfuse to improve brain tissue oxygenation. Attention to fluid balance, blood pressure and oxygenation is also likely to improve neurological outcome and there should be consultation with Intensive Care and with the local Stroke Unit, as in adults, in the absence of hemorrhage on emergency CT scan, thrombolysis is not contraindicated within a 4.5-hour window.[13]

As these patients are often admitted to a peripheral hospital without facilities for emergency imaging under general anesthesia, acute imaging is often not performed. Except in hemorrhage (Table 1), CT may not show abnormalities within the

Table 1. Neurological complications in sickle cell disease.

MRI	Vascular: MRA/MRV	Clinical and pathological findings	Treatment
		Sudden onset **stroke** with arterial territory infarct: stenosis, occlusion, dissection ICA, MCA. Exclude shunting	Transfuse, O2, Intensive care Stroke Unit -TL
		**Silent cerebral infarction**: no stroke but may have had seizures. Stenosis, occlusion, moyamoya ICA,MCA. Shunt	?Transfuse; ?Hydroxyurea
		**PRES: Posterior reversible encephalopathy syndrome** after rapid transfusion, acute chest, hypertension	Treat seizures, hypertension, hypoxia
		**Venous sinus thrombosis**: presents c hemiplegia, seizures, coma. CT : empty delta, thrombus, CTV /MRV	?Transfuse; rehydrate, anticoagulate
		**Abscess:** seizures, headaches, coma, raised intracranial pressure, fever	Antibiotics Neurosurgeon Intensive care
		**Intracerebral haemorrhage:** sudden onset very severe headache, coma. Venous , hypertension, aneurysm	Neurosurgeon Intensive care
		**Subarachnoid haemorrhage:** sudden onset very severe headache, coma . Aneurysm, venous , hypertension	Neurosurgeon Intensive care
		**Subdural haemorrhage:** headache, coma, raised intracranial pressure, skull infarction. Exclude trauma /NAI	Neurosurgeon Intensive care
		**Extradural haemorrhage:** headache, coma, raised intracranial pressure, skull infarction. Exclude trauma /NAI	Neurosurgeon Intensive care

first 24 hours after the onset of neurological symptoms. Diffusion-weighted MRI (DWI) can show ischemic regions within minutes, i.e. before irreversible infarction has occurred, and can also help to distinguish between alternative pathologies, while T2-weighted MRI is usually abnormal within a few hours. There is therefore a case for emergency MRI (Table 1), which might reveal:

- acute infarct (detected on DWI) in the distribution of an artery (cervical or intracranial stenosis, occlusion or dissection; shunting)
- abnormality in the basal ganglia, or deep white or grey matter of the border zones (old 'SCI' secondary to cervical or intracranial stenosis, occlusion or dissection; shunting)
- occipito-parietal or thalamic involvement (sinovenous thrombosis)
- posterior reversible encephalopathy syndrome
- subarachnoid or intracerebral hemorrhage[14] (acute hypertension/sinovenous thrombosis/aneurysm or moyamoya collateral rupture)

Between 60% and 90% of patients with SCD and acute stroke in an arterial distribution have abnormal findings on magnetic resonance (MRA) angiography (Table1). Typical abnormalities include

- stenosis or occlusion of the cervical or intracranial carotid or middle cerebral arteries
- vertebral or carotid dissection[15]
- moyamoya (bilateral severe stenosis or occlusion of the internal carotid arteries with collateral formation)
- small vessel vasculitis
- aneurysm[10,14]

Venous sinus thrombosis (Table 1)[16] is probably underdiagnosed; if emergency MRA is normal, MR or CT venography should be considered.

Secondary prevention

Clinical stroke

Despite a lack of high quality evidence,[17] blood transfusion, ideally erythrocytapheresis because of the lower rate of iron accumulation, has been the mainstay for secondary stroke prevention. Moyamoya syndrome is associated with an increased risk of stroke recurrence which appears to be reduced by revascularisation.[18] The majority of patients have stable findings on neuroimaging after hematopoietic stem cell transplant but although some improve, around 1 in 6 deteriorate.[19]

Silent cerebral infarction

The Silent Cerebral Infarct Transfusion was conducted to determine whether blood transfusion therapy for 36 months prevents progression of infarct recurrence (stroke or SCI) in children with SCA (5 to 15 years of age) and pre-existing SCI. In participants receiving regular blood transfusion, there was 58%relative risk reduction in cerebral infarct recurrence (stroke or new or progressive silent cerebral infarcts) when compared to the children in the observation arm.[20] The evidence is of moderately good quality,[17] but the number of children with SCA and SCI who need to be transfused to prevent one recurrent infarct is 13.8 and there is no evidence of benefit on intelligence so the high burden of regular blood transfusion may decrease enthusiasm for this management strategy.

Primary prevention of stroke

Time-averaged maximum velocity on transcranial Doppler ultrasound (TCD) >200 cm/sec predicts clinical stroke in children with SCD not receiving regular blood transfusion therapy (3-6weekly to hemoglobin S level<30%). The number needing indefinite transfusion to prevent one stroke is 7 but the strategy has dramatically reduced the stroke rate.

The Transfusions Switching to Hydroxyurea (TWiTCH) trial,[21] was a primary stroke prevention trial for SCD children who had received at least 12 months of blood transfusion for TCD velocities >200 cm/sec. Standard therapy was continuation with blood transfusion therapy and chelation and experimental therapy was hydroxyurea therapy and phlebotomy, although there was a median overlap of 6 months. The primary outcome was TCD velocity; after the first interim analysis, the trial was ended early because non-inferiority was demonstrated. Trial design limitations included the short period of time on hydroxyurea therapy at maximum tolerated dose, approximately 18 months, and the exclusion of c.10% of children with abnormal TCD *and* MRA, meaning that management for this group cannot be determined from the TWiTCH trial, but it does appear that hydroxyurea is effective in maintaining a lower TCD in those with normal MRA.

Future perspectives

Genetic and environmental risk factors for stroke in sickle cell disease

There appears to be a familial predisposition to stroke and to high blood flow velocities in SCD, indicating that genetic fac-

tors probably play a role. Siblings might, however, also share adverse environmental conditions, including poverty, air pollution and poor nutrition. Difficulties in sleeping are well-recognized in SCD and the prevalence of sleep-disordered breathing, including snoring, arousals, obstructive sleep apnea (OSA) and nocturnal desaturation, is higher in SCD than in the general population and is associated with cerebral vasculopathy[22] and cognitive dysfunction. These may be useful biomarkers alongside quantitative MRI for randomised controlled trials of treatment, e.g. of overnight respiratory support in older children and adults, and of Montelukast in preschool children.

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Celgene (Advisory Board); Roche (Advisory Board, Research Support, Honoraria); Novartis (Advisory Board); Sandoz (Advisory Board); Spectrum (Advisory Board, Research Support); Takeda (Honoraria)

Pieters R
Affiliations to disclose: No Affiliations

Pileri S
Affiliations to disclose: Yes
Takeda (Advisory Board)

Pilo F
Affiliations to disclose: Unknown

Platzbecker U
Affiliations to disclose: No Affiliations

Pospisilova S
Affiliations to disclose: No Affiliations

Rachmilewitz E
Affiliations to disclose: No Affiliations

Raderer M
Affiliations to disclose: Yes
Celgene (Speaker); Janssen (Advisory Board); Pfizer (Advisory Board); Sunesis (Consultancy); CTI (Consultancy)

Rambaldi A
Affiliations to disclose: No Affiliations

Renné T
Affiliations to disclose: No Affiliations

Ribera JM
Affiliations to disclose: No Affiliations

Rodríguez-Otero P
Affiliations to disclose: Unknown

Rosenwald A
Affiliations to disclose: No Affiliations

Rossi M
Affiliations to disclose: No Affiliations

Rule S
Affiliations to disclose: Yes
Janssen (Advisory Board, Research Funding); Roche (Advisory Board); Astra Zeneca (Advisory Board); Celgene (Advisory Board)

Saglio G
Affiliations to disclose: No Affiliations

Salles G
Affiliations to disclose: Yes
Amgen (Advisory Board); BMS (Honoraria); Celgene (Advisory Board, Honoraria); Gilead (Advisory Board, Honoraria); Janssen (Advisory Board); Novartis (Advisory Board); Roche (Advisory Board, Research Support); Servier (Honoraria); Merck (Advisory Board, Research Support)

Salles G
Affiliations to disclose: Yes
Amgen (Advisory Board); BMS (Honoraria); Celgene (Advisory Board, Honoraria); Gilead (Advisory Board, Honoraria); Janssen (Advisory Board); Novartis (Advisory Board); Roche (Advisory Board, Research Support); Servier (Honoraria); Merck (Advisory Board, Research Support)

Samuelsson J
Affiliations to disclose: Yes
Novartis (Advisory Board)

San-Miguel JF
Affiliations to disclose: Yes
Millennium (Advisory Board); Celgene (Advisory Board); Novartis (Advisory Board); Onyx (Advisory Board); Janssen (Advisory Board); BMS (Advisory Board); MSD (Advisory Board); Amgen (Advisory Board)

Schellong SM
Affiliations to disclose: Yes
Bayer (Advisory Board, Speaker Fees); BMS (Speaker Fees); Boehringer (Advisory Board, Speaker Fees); Daiichi (Advisory Board, Speaker Fees); Pfizer (Speaker Fees)

Schlatt S
Affiliations to disclose: No Affiliations

Schlenk R
Affiliations to disclose: Yes
Novartis (Advisory Board, Speaker's Bureau, Research Funding); Arog (Speaker's Bureau, Research Funding); Daiichi Sankyo (Advisory Board)

Sevilla J
Affiliations to disclose: Yes
Novartis (Advisory Board, Speaker's Bureau); Terumo BCT (Speaker's Bureau)

Shimamura A
Affiliations to disclose: No Affiliations

Soverini S
Affiliations to disclose: Yes
Incyte (Consultancy); Bristol-Myers Squibb (Consultancy); Novartis (Consultancy)

Squizzato A
Affiliations to disclose: No Affiliations

Stanworth S
Affiliations to disclose: No Affiliations

Stein-Thoeringer CK
Affiliations to disclose: Unknown

Stevenson F
Affiliations to disclose: No Affiliations

Sureda A
Affiliations to disclose: Yes
Takeda (Advisory Boards, Honoraria Consultancy); BMS (Advisory Boards, Honoraria Consultancy)

Szalat R
Affiliations to disclose: Unknown

Tabanelli V
Affiliations to disclose: Unknown

Tamary H
Affiliations to disclose: No Affiliations

Tausch E
Affiliations to disclose: Unknown

Valk P
Affiliations to disclose: No Affiliations

van den Brink MRM
Affiliations to disclose: Yes
Seres (Research Support); Evelo (Consultant); Novartis (Consultant); Regeneron (Consultant); Flagship Ventures (Consultant); Boehringer Ingelheim (Consultant); Merck (Consultant)

Vannucchi AM
Affiliations to disclose: Yes
Novartis (Lectures, Advisory Board, Institutional Research Funding); Gilead (Lectures); Shire (Lectures)

Vey N
Affiliations to disclose: Yes
Novartis (Advisory Board, Honoraria); Amgen (Advisory Board); Seattle Genetics (Advisory Board); Sunesis (Advisory Board); Roche (Advisory Board); Bioknesis (Consultant); Boerhinger (Advisory Board)

Viprakasit V
Affiliations to disclose: Yes
Novartis Pharmaceuticals (Research Support, Honoraria); Genzyme-Sanofi (Research Support, Honoraria); Sebia (Research Support, Honoraria); Roche Diagnostics (Research Support, Honoraria); Shire (Research Support); Sideris (Research Support); Siriraj Hospital (Research Support)

Vora A
Affiliations to disclose: Yes
Jazz (Advisory Board, Meeting Support); Pfizer (Advisory Board); Amgen (Advisory Board); Medac (Advisory Board, Meeting Support)

Wallace WH
Affiliations to disclose: No Affiliations

Weiss MJ
Affiliations to disclose: Yes
Glaxo SmithKline (Consultant); Biogen (Research Support); Rubius (Advisory Board); CRISPR therapeutics (Consultant); Editas (Consultant)

Wendtner CM
Affiliations to disclose: Yes
Hoffmann-La Roche (Research Support, Advisory Boards, Consultant); Celgene (Research Support, Advisory Boards, Consultant); Mundipharma (Research Support, Advisory Boards, Consultant); Janssen (Research Support, Advisory Boards, Consultant); Gilead (Research Support, Advisory Boards, Consultant); Morphosys (Research Support, Advisory Boards, Consultant); Abbvie (Research Support, Advisory Boards, Consultant)

Whyte CS
Affiliations to disclose: Unknown

Younes A
Affiliations to disclose: Yes
Bayer (Honoraria); Celgene (Honoraria); Incyte (Honoraria); Janssen (Honoraria); Sanofi (Honoraria); Seattle Genetics (Honoraria); Takeda Millenium (Honoraria); Genentech (Honoraria); Merck (Honoraria); Novartis (Research Support); J&J (Research Support); Curis (Research Support); Roche (Research Support); BMS (Honoraria, Research Support)

Zeiser R
Affiliations to disclose: No Affiliations

Zucca E
Affiliations to disclose: No Affiliations

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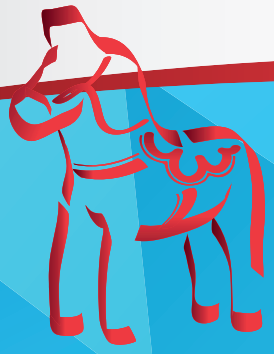

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