education program of the 11th congress of the European Hematology Association

Amsterdam, The Netherlands, June 15-18, 2006
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**Word of welcome**

On behalf of the EHA Education Committee and Scientific Program Committee, we are delighted to welcome you to the beautiful city of Amsterdam. The EHA Congress is the largest and most comprehensive hematology meeting in Europe with a world class line up of invited speakers. The Education Program covers the whole spectrum of clinical hematology and we have assembled a distinguished cast of internationally-recognised speakers. In addition to enjoying the talks, we hope you find the peer-reviewed papers in the Education book a useful source of information and references for the coming year.

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Classification of acute myeloid leukemia

Since the term leukemia was used for the first time by Virchow in 1845 advances in biochemistry, cytogenetics, cytochemistry, immunology, and subsequently molecular biology have led to the identification of different subtypes of acute myeloid leukemia (AML). Especially during the last couple of years, the almost exponentially increase in the understanding of the hematopoietic system has revealed the extraordinary morphological, biological, and clinical heterogeneity of AML. Therefore, clinically relevant classification systems that reflect the underlying tumor biology are needed.

In an attempt to define a biologically and clinically useful working nomenclature, the current World Health Organization (WHO) classification of myeloid neoplasms incorporated those disease characteristics that had been proven to have clinical and biological relevance. This resulted in a more sophisticated classification in which AML is divided into four large sub-classes, that can be further subdivided into several distinct AML subtypes (Table 1). Nevertheless, for many subtypes of myeloid leukemia no specific genetic or pathogenic event has been discovered yet, and within well-defined AML subgroups, such as cases with t(8;21)(q22;q22) or inv(16) (p13q22) considerable clinical heterogeneity is observed. Thus, additional subtypes may exist even within the same cytogenetic category, thereby highlighting the need to further refine the current classification of AML.

Gene expression profiling in leukemias

Genomics currently offers the possibility of identifying the molecular variation underlying the biological and clinical heterogeneity of AML, with DNA microarray-based genome-wide gene expression profiling (GEP) representing one of the most powerful experimental approaches. Notably, the utility and promise of this novel technology was first demonstrated in leukemias. By analyzing AML and acute lymphoblastic leukemia (ALL) samples Golub et al. demonstrated the potential usefulness of a GEP-based classification of leukemias. Using an unsupervised class discovery procedure the authors were able to distinguish AML and ALL without previous knowledge of these classes, and having developed a supervised class predictor, new leukemia cases could be accurately assigned to one or other of these two leukemia classes. Unexpectedly, many of the genes characterizing the different type internal tandem duplications (ITD) of the FLT3 gene, partial tandem duplications (PTD) of the MLL gene, as well as mutations of CEBPA and NPM1 have been shown to be of prognostic relevance, as are the levels of expression of EVI1 and BAALC. Nevertheless, there is still no commonly accepted risk stratification for this group of leukemia patients, nor are the leukemogenic mechanisms fully understood yet.

Studies of molecular genetics have provided several lines of evidence strongly suggesting that AML has a multistep pathogenesis. While the expression of a single fusion gene protein, like e.g. RUNX1-CBFA2T1 resulting from a t(8;21), can block myeloid differentiation without causing leukemia, other events, such as the constitutive activation of FLT3 or RAS family members, can induce a myeloproliferative phenotype. Thus a combination of differentiation-blocking and proliferation-inducing mechanisms might be involved in leukemogenesis.
leukemia subtypes were not markers of hematopoietic lineage, but genes related to cancer pathogenesis. Thus, gene expression patterns that are useful for cancer classification can also provide further insight into cancer biology.

**Gene expression patterns associated with genomic aberrations in acute myeloid leukemia**

Trisomy 8 was one of the first recurrent cytogenetic aberrations in AML to be investigated by GEP. Compared to AML cases with normal cytogenetics, cases with trisomy 8 are characterized by higher average expression of genes located on chromosome 8. Similar gene dosage effects have been reported for other chromosomal gains and losses in AML. Moreover, supervised analytical approaches have also proven to be useful for discriminating characteristic gene expression patterns in AML cases with balanced chromosomal rearrangements, such as cases with **inv(16)**, **t(8;21)**, **t(15;17)** and **t(11q23)/** **MLL**. Similarly, with the aid of supervised statistical algorithms characteristic gene expression patterns have been defined for **FLT3 ITD**, **CEBPA**, and **NPM1** mutations. However, in contrast to cases with translocations involving the **MLL** gene, in larger studies no significant gene expression signature was detected for cases with **MLL PTD**, reflecting the molecular heterogeneity of **MLL PTD** cases and their clear distinction from AML with t(11q23). Likewise, AML cases with **NRAS** mutations do not, apparently, have a characteristic gene expression signature. Thus, have to affect gene expression levels in a characteristic way. A possible explanation might be that additional events in **MLL PTD** or **NRAS-mutated cases result in various different pathomechanisms.**

**GEP-based prediction of acute myeloid leukemia subtypes – a powerful acute myeloid leukemia classification tool**

Importantly, the distinct gene signatures associated with cytogenetic and molecular genetic aberrations can also be used to accurately predict the respective leukemia subgroups. Furthermore, classifiers generated from pediatric AML samples accurately stratified adult leukemia cases exhibiting the same genetic aberrations, thereby indicating age-independent aberration-specific pathomechanisms. Moreover, these diagnostic signatures seem to be quite robust with regard to technical aspects of specimen sampling and target preparation. Therefore, in the future GEP might offer a global, highly accurate approach for the diagnosis of known leukemia subgroups, especially for those associated with recurrent genetic aberrations (see also Table 1), and indeed the first research towards this goal is very promising. In addition, this approach will most likely contribute significantly in to predicting certain leukemia subgroups might be indicative that the current classification system does not fully reflect the underlying biology and that novel tumor classes remain to be discovered.

**Discovery of novel molecularly defined acute myeloid leukemia subclasses**

By applying unsupervised analytical approaches to gene expression data novel clinically significant subtypes of cancer can be identified. One of the first demonstrations of the potential of GEP-based class discovery in AML emerged from studies of therapy-related AML (t-AML) cases. The authors identified novel t-AML subgroups characterized by distinct gene expression signatures, all t-AML cases also displayed a common pattern typical of arrested differentiation in early progenitor cells.

Besides offering novel biological insights, unsupervised cluster analysis also provides a powerful tool for the discovery of new AML subgroups of clinical relevance. In a large study based on unsupervised analysis, 285 AML samples from patients were grouped into sixteen clusters. Some clusters were characterized by high frequencies of certain molecular lesions or mutations, such as two clusters (#1 and #16), that both harbored cases with t(11q23)/**MLL** abnormalities, but also included patients without these molecular lesions. Furthermore, this study identified a distinctive gene expression pattern associated with increased **EVI1** expression and poor treat-
ment outcome. Another cluster associated with shorter survival times included cases with high risk cytogenetic markers, such as monosomies 7 and 5, and the translocation t(9;22). Interestingly, this cluster displayed a signature comparable to CD34+ cells, thereby suggesting a possible common mechanism for resistance to therapy.14

While favorable cytogenetic subgroups were characterized by homogenous clustering, Valk et al. observed molecular variation within these homogeneously grouped cases.14 For example, in cases with inv(16) or t(8;21) clustering was less stringent when more than 2,856 probe sets were included into the unsupervised analysis. In agreement with Valk et al., based on unsupervised clustering using 6,283 genes we also detected some molecular heterogeneity within the cytogenetically well-characterized core binding factor leukemias, with each class, t(8;21) and inv(16), being separated into two main groups.7 Distinct patterns of gene expression within each of these t(8;21) and inv(16) subgroups might reflect alternative cooperating mutations/deregulated pathways leading to transformation, since the primary translocation/inversion events themselves are not sufficient for leukemogenesis.24

In our study, cases with normal karyotype also segregated mainly into two distinct groups, each of which included a small number of cases from other classes.7 FLT3 aberrations were more prevalent in one subgroup, while M4/M5 morphologic subtypes according to the French-American-British (FAB) classification were significantly more represented in the other subgroup. In agreement with these results, Valk et al. also identified normal karyotype-predominated clusters associated with FLT3 ITD, as well as a cluster including mainly specimens from patients with AML of FAB M4 or M5 subtype.14 Notably, in our study Kaplan-Meier analysis identified a statistically significant difference in overall survival between the two subclasses.7

**Monitoring drug effects – drug discovery in acute myeloid leukemia**

Analyzing the effects of all-trans retinoic acid (ATRA) in acute promyelocytic leukemia (APL)-derived cell lines, such as NB4 cells, showed that ATRA-regulated genes include members of the tumor necrosis factor (TNF) pathway suggesting that this pathway might intersect with ATRA signaling.25,26 Indeed, the interaction between ATRA and TNF involved increased NF-κB activity followed by a synergistic induction of NF-κB target genes.26 This supports the idea that ATRA primes cells to become more susceptible to the differentiation effects of other pathways. In addition, many promoters of ATRA target genes contain NF-κB binding sites, thereby providing further evidence that this pathway might play a role in regulating cell survival in response to ATRA.27

Besides monitoring drug effects, GEP has also proven to be a powerful means for discovering both novel drug targets as well as novel drugs. For example gene expression-based high-throughput screening approaches can be used to screen for chemical compounds with differentiation-inducing activity in AML.28 A microarray-based five-gene differentiation signature formed the cornerstone for a high throughput screening method using multiplexed reverse transcriptase polymerase chain reaction (RT-PCR), single base extension reaction and matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) mass spectrometry. In HL-60 cells treatment with 1,739 different compounds revealed eight chemicals that reliably induced this differentiation signature. These drugs included 4,5-dianilinophthalimide (DAPH1), a compound with epidermal growth factor receptor (EGFR) kinase inhibiting activity. Therefore, the authors hypothesized in a subsequent study that the Food and Drug Administration (FDA)-approved EGFR inhibitor gefitinib might also promote differentiation in AML.29 In accordance with this hypothesis, in vitro gefitinib treatment of AML cell lines and primary patient-derived AML blasts promoted cellular differentiation even in the absence of EGFR expression suggesting an EGFR-independent mechanism of gefitinib-induced differentiation.29

**Prognostic signatures in acute myeloid leukemia**

As already mentioned, GEP allows the identification and prediction of specific signatures correlated with low-risk and high-risk cytogenetics, as well as with prognostically relevant molecular genetic aberrations.5,14,20 However, supervised approaches have also been used to identify novel gene signatures predictive for response to chemotherapy. Although not statistically significant, an early attempt to explore candidates with potential biological significance overexpressed in AML patients with treatment failure included HOXA9.5 HOXA9, a gene known to be frequently activated in AML,50 has recently been associated with NPM1 mutations,18 which have been shown to be of prognostic relevance.19,51

Recently, Heuser et al. also attempted to identify a characteristic gene expression profile, that distinguishes AML samples from patients with good or poor response to induction chemotherapy.25 Based on supervised data analysis, the authors successfully characterized a gene expression pattern associated with induction chemotherapy resistance. Importantly, this signature provided significant prognostic information in a previously published independent set of AML patients,7 and in multivariate
analysis this treatment-response signature proved to be an independent prognostic factor.32

However, other supervised approaches looking for signatures correlated with AML outcome have been less successful as survival and survival time in acute leukemia represent imprecise surrogates for the underlying prognostically relevant tumor subclasses. For example, a *prognostic signature* generated in childhood AML by comparing patients with *good* and *poor* outcome,33 did not allow significant risk stratification when the respective gene expression pattern was applied to an independent data set.33

**Semi-supervised outcome prediction approaches**

To discover new prognostically relevant, and biologically meaningful subclasses of AML, a strategy combining the strengths of both supervised and unsupervised approaches has been shown to provide better outcome prediction.34 Using such a *semi-supervised method*, called semi-supervised clustering, we devised an outcome class predictor in AML that was an independent prognostic factor in multivariate proportional hazards analysis.35 Importantly, this predictive gene expression signature also defined good and poor outcome classes when applied to AML samples with normal karyotype only, and this result has recently been validated by an independent study group analyzing 68 AML cases with normal karyotype.36

On the other hand, as in the study by Heuser et al.,37 our AML data also served as an independent test set for a prognostic signature defined in prostate cancer that displays a stem cell-like expression profile.38 This signature had prognostic power in independent samples obtained from 1,155 cancer patients diagnosed with 11 different types of cancer including AML. Thus, several prognostic signatures might be found in gene expression data sets, clearly demonstrating the importance of making data sets publicly available, as ongoing data mining of existing data sets will contribute significantly to our better understanding of leukemogenesis.

**Future challenges in acute myeloid leukemia: integration of GEP and whole genome approaches**

Hematological malignancies have been an attractive field for genomic approaches, such as DNA microarray technology,39,40 and GEP has contributed an important new facet to the exploration of AML.39 Nevertheless, while the above-mentioned findings are definitely encouraging, further validation of these observations in larger cohorts and in independent studies is clearly required before clinical implementation becomes feasible in AML.

In the future, GEP may contribute further to a comprehensive molecular leukemia classification, as characteristic expression patterns may support individualization of cancer treatment, and enable an improved risk-adapted AML management. Ultimately, one microarray experiment could be sufficient to diagnose leukemias, predict their course, and indicate individualized treatment strategies.

An outstanding challenge for the future is the integration of DNA microarray technology and other whole genome approaches to validate the numerous biological hypotheses generated by GEP in AML. Integrative analyses evaluating the AML transcriptome in the context of other data sources derived, for example, from single nucleotide polymorphism (SNP) arrays, comparative genome hybridization (CGH) arrays, tiling arrays, promoter arrays, and proteomics, will provide new insights into leukemogenesis. However, in order for the integration to be successful, a common language for communicating genomic profiles across diverse experimental systems will have to be defined, and integrative bioinformatics solutions for sharing and analyzing the data will have to be developed.

**References**

Hematology, in general, and the study of hematological malignancies in particular, is one of the areas of medicine in which the application of flow cytometry has undergone a major development in the last decade. This is related to the fact that flow cytometry requires single cell suspensions, which are easily obtained from peripheral blood samples and bone marrow aspirates. Moreover, single cell suspensions can also be prepared from lymph node biopsies and fine needle aspirates, bone marrow biopsies and biopsies from other lymphoid tissues. In addition, since the late 1970s, haematopoietic cells have been used as a source of antigens to develop monoclonal antibodies, leading to the availability of an increasingly high number of reagents directed against hematopoietic cell markers. These unique features, together with the continuous advances in laser technology, optics, fluorochrome chemistry, bead technology, informatics and the production of monoclonal antibodies, have reshaped the way flow cytometry is used in hematology and have expanded its applications. Accordingly, flow cytometry is currently the method of choice for immunophenotypic characterization of hematological malignancies at diagnosis and for the immunophenotypic monitoring of minimal residual disease, during and after therapy. In addition, it is also a primary laboratory diagnostic tool in patients suspected of having paroxysmal nocturnal hemoglobinuria, systemic mastocytosis and primary thrombocytopenias such as Glanzmann disease and Bernard-Soulier syndrome, for the detection of anti-platelet antibodies and the quality control of both leukocyte contamination in transfusion products and of CD34+ hematopoietic stem and precursor cells and CD3+ T-lymphocytes in transplant cell products. The increased diagnostic use of flow cytometry is certainly related to its relative simplicity, high sensitivity and specificity and the possibility of providing clinically useful results in a short period of time.

In this paper we review the currently most promising applications of flow cytometry in the diagnosis of hematological malignancies (Table 1), particularly those related to the multiparameter immunophenotypic identification, enumeration and characterization of leukemic cells.

Immunophenotyping of hematological malignancies

Immunophenotyping of leukemias and other hematological malignancies has become one of the most relevant clinical applications of flow cytometry. Initially, its utility was mainly focused on further characterization of leukemic cells and classification of the disease once the diagnosis of leukemia/lymphoma had already been established. Antigen expression was commonly evaluated with relatively restricted panels of single, unconjugated monoclonal antibody reagents, using mononuclear cell-enriched samples which contained high percentages of neoplastic cells. A clear example of such use is the establishment of the lymphoid versus myeloid origin of blast cells in acute leukemia and the subclassification of both T-cell and B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) according to the expression of maturation-associated antigens, as proposed in 1995 in the EGIL classification:

- pro-T/TI (CD7+ and CyCD3+, negative for other T-cell markers), pre-T/TII (CD7+, CyCD3+, sCD3+, CD1a+ and CD2+ and/or CD8+ and/or CD5+), common-T/TIII (CD7+, CyCD3+, CD1a+), mature-T/TIV (CD7+, sCD3+, CD1a+) and pro-B/B1 (CD19+, CyCD79a+, Ig+, CD10+), common-B/BII (CD19+, CyCD79a+, CD10+, Ig-) and mature-B/BIV (CD19+, CyCD79a+, slg+). Progressively some individual immunophenotypic markers were also associated with disease prognosis. Among others, CD38 and, more recently intracellular ZAP70 expression, were shown to be related to a worse clinical outcome in B-
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<table>
<thead>
<tr>
<th>Type of medical indication</th>
<th>Disease category</th>
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<td>Diagnostic screening</td>
<td>B- and T-cell chronic lymphoproliferative disorders (CLPD)</td>
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<tr>
<td></td>
<td>Myelodysplastic syndromes (MDS)</td>
</tr>
<tr>
<td></td>
<td>Plasma cell dyscrasias (PCD)</td>
</tr>
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<td></td>
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<td></td>
<td>Immunologic classification of acute lymphoblastic leukaemias</td>
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<td></td>
<td>New subtypes of acute non lymphoblastic leukaemias</td>
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<td>B, T and NK-cell chronic</td>
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<td></td>
<td>lymphoproliferative disorders</td>
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<td></td>
<td>Plasma cell dyscrasias</td>
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<td>Screening for genetic abnormalities</td>
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<td></td>
<td>Acute lymphoblastic leukaemias (childhood and adult)</td>
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<tr>
<td></td>
<td>B-cell chronic lymphoproliferative disorders</td>
</tr>
<tr>
<td>Others</td>
<td>Prognostic stratification (e.g. CLPD, MDS)</td>
</tr>
<tr>
<td></td>
<td>Staging and evaluation of disease extension (e.g. CLPD)</td>
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<tr>
<td></td>
<td>Minimal residual disease detection (e.g.: AL, CLPD, PCD)</td>
</tr>
<tr>
<td></td>
<td>Prediction/monitoring of response to therapy (e.g.: CLPD, AML)</td>
</tr>
</tbody>
</table>

| * For further information please see references 2, 3 and 4. |

Emerging applications of multiparameter flow cytometric immunophenotyping in the diagnosis of hematological malignancies

For many years now, efforts have been made to identify tumor-specific antigens in leukemia and lymphoma cells. This has led to the identification of tumor-specific antigens such as the idiotypic protein in mature neoplastic B cells and plasma cells or fusion proteins resulting from specific chromosomal translocations (e.g.: BCR/ABL, TEL/AML1, PML/RARα, among others) particularly in acute leukemias. However, from a practical point of view, most of these tumor-specific antigens have not been introduced in routine diagnostics for the specific identification of neoplastic cells.1,14 This is mainly due to: i) the lack of high quality reagents for the identification of these tumor-specific markers, ii) their relatively low levels of expression in neoplastic cells and iii) their complexity, due to the occurrence of different genomic breakpoints or individual variability in the case of idiotypic proteins. In contrast, the identification of altered expression patterns of one or more normal proteins in leukemic cells – leukemia/lymphoma-associated aberrant phenotypes – has proven to have a much greater and easier applicability in discriminating leukemic cells from normal cells.1,14 Accordingly, previous studies indicate that in virtually all patients with T-ALL (>99%), BCP-ALL (>95%), B-CLPD (>95%), plasma cell dyscrasias (>90%) and most AML (>80%), neoplastic cells display aberrant phenotypes which allow their discrimi-
The exact aberrant phenotypes displayed by leukemic cells usually differ between distinct disease groups. In addition, they may also vary between different patients within a disease category and even between different neoplastic cell populations within a single patient. In general, within a diagnostic category, lymphoid-lineage malignancies show common and more stable aberrant phenotypes than do AML or other myeloid neoplasias. As an example, patients suffering from a typical B-CLL consistently (>95% of the cases) show dim co-expression of CD22, CD20 and/or CD81 among CD5+/CD23+ B cells, while overexpression of BCL2 in neoplastic B cells with a follicular immunophenotype (CD10+, CD38+) represents a hallmark of follicular B-cell non-Hodgkin’s lymphoma (NHL) with t(14;18). Based on these observations, unique multicolor combinations of monoclonal antibodies (e.g. Bcl2/CD10/CD38/CD20 for t(14;18)+ follicular B-cell NHL) can be defined for the identification of malignant cells in patients suffering from a specific hematological malignancy. In contrast, in AML, the most common aberrant phenotype (e.g. CD33high, HLADR–, CD34–, CD15–, CD14, CD11b+ neutrophil lineage cells) may be present in less than one third of all cases (Table 2). However, a more detailed analysis of the aberrant phenotypes identified in AML, as well as in BCP-ALL, shows that a high concordance exists between specific aberrant phenotypes and genetic lesions that define diagnostic subcategories of both ALL and AML according to the WHO classification.

<table>
<thead>
<tr>
<th>Group</th>
<th>Disease</th>
<th>% Expression of aberrant phenotype</th>
<th>Most frequently expressed aberrant phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute leukaemias</td>
<td>B-cell precursor acute lymphoblastic leukaemia (ALL)</td>
<td>&gt;95%</td>
<td>Asynchronous antigen expression: TdT+/CD10+; CD38+/CD34+</td>
</tr>
<tr>
<td></td>
<td>T-ALL</td>
<td>&gt;99%</td>
<td>Ectopic phenotypes: TdT+ and CyCD3+ and CD99+ cells outside the thymus</td>
</tr>
<tr>
<td></td>
<td>Acute myeloblastic leukaemia</td>
<td>&gt;80%</td>
<td>Asynchronous antigen expression: CD33+, HLADR, CD34, CD15, CD14, CD11b, CD13, CD33, CD117, HLADR</td>
</tr>
<tr>
<td>Mature T-cell neoplasias</td>
<td>T-cell prolymphocytic leukaemia</td>
<td>93%</td>
<td>Antigen overexpression: CD7+ in mature T cells</td>
</tr>
<tr>
<td></td>
<td>Sézary syndrome</td>
<td>100%</td>
<td>Antigen underexpression: CD3+; CD20+, CD7+; CD4–, CD26</td>
</tr>
<tr>
<td></td>
<td>Angioimmunoblastic T-cell lymphoma (AITL)</td>
<td>90%</td>
<td>Asynchronous antigen expression: CD10+, CD3– in CD4+ mature T-cells</td>
</tr>
<tr>
<td></td>
<td>T-cell large granular lymphocyte leukaemia</td>
<td>To be determined</td>
<td>Abnormally high number of CD4+, CD8–, CD56+, CD57+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD56−, CD94+, HLADR+</td>
</tr>
<tr>
<td>Mature NK-cell neoplasias</td>
<td>NK-large granular lymphocyte leukaemia</td>
<td>60%</td>
<td>Antigen overexpression: CD7+ in mature T cells</td>
</tr>
<tr>
<td>Mature B-cell neoplasias</td>
<td>Chronic lymphocytic leukaemia</td>
<td>&gt;90%</td>
<td>Antigen over or underexpression: mature CD23+ B-cells with CD5–, CD22–, CD20+, CD38+, CD70+</td>
</tr>
<tr>
<td></td>
<td>Prolymphocytic leukaemia</td>
<td>80%</td>
<td>Antigen overexpression: sIg+</td>
</tr>
<tr>
<td></td>
<td>Hairy cell leukaemia</td>
<td>100%</td>
<td>Asynchronous antigen expression: CD10+, CD11c–, CD22+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aberrantly high FSC/SSC</td>
</tr>
<tr>
<td></td>
<td>Lymphoplasmacytic lymphoma</td>
<td>100%</td>
<td>Asynchronous antigen expression: CD22–, CD10–</td>
</tr>
<tr>
<td></td>
<td>Mantle cell lymphoma</td>
<td>100%</td>
<td>Asynchronous antigen expression: CD22–, CD10–, CD5+</td>
</tr>
<tr>
<td></td>
<td>Follicular lymphoma</td>
<td>97%</td>
<td>Overexpression of Bcl2 in CD10+, CD38+ B lymphocytes with follicular phenotype.</td>
</tr>
<tr>
<td></td>
<td>Splenic marginal zone lymphoma</td>
<td>100%</td>
<td>Antigen overexpression: FMC7+ (variant epitope on CD20 molecule)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aberrantly high FSC/SSC</td>
</tr>
<tr>
<td></td>
<td>Burkitt lymphoma</td>
<td>&gt;75%</td>
<td>Antigen overexpression: CD56− in CD38+, CD45−, CD19+ plasma cells</td>
</tr>
<tr>
<td>Plasma cell neoplasias</td>
<td>Multiple myeloma</td>
<td>98%</td>
<td>Antigen overexpression: CD56− in CD38+, CD45−, CD19+ plasma cells</td>
</tr>
</tbody>
</table>
Diagnostic screening of hematological malignancies using multiparameter flow cytometric immunophenotyping

Recent reports indicate that multiparameter flow cytometric immunophenotyping is a fast, sensitive and specific approach in routine diagnostic screening for the presence of neoplastic lymphoid cells in peripheral blood bone marrow and other hematological and non-hematological samples. Accordingly, we have recently shown that the use of a single four-color, seven-monoclonal antibody tube (CD19/CD56-sIg/CD3/CD13/CD34/CD4) for the screening of absolute lymphocytosis, allows the identification of B-cell neoplasias with an extremely high sensitivity and specificity when compared to conventional cytomorphology, immunophenotyping and molecular diagnostic approaches. A high diagnostic efficiency was also observed when this approach was applied to the evaluation of fine-needle aspirates from lymph nodes, (Orfao et al., unpublished observations). Recently, it has been shown that multiparameter flow cytometric immunophenotyping has high diagnostic value for the identification of leptomeningeal involvement in B-NHL patients with cytomorphologically occult disease, allowing the identification of small numbers of neoplastic cells in spinal fluid (down to 1 cell/3 µL). In parallel, the use of monoclonal antibody panels directed against different members of the TCRβ and TCRγ/δ families is of great utility for the diagnosis of T-cell clonality in both peripheral blood and lymph node samples, particularly when combined with antibodies for the identification of cells expressing leukemia/lymphoma-associated phenotypes.

Immunophenotypic classification of hematological malignancies

Once the presence of leukemic cells in a sample has been demonstrated, immunophenotyping allows their precise characterization and enumeration. For many years, specific attention was paid to the evaluation of the similarities between leukemic and normal haematopoietic cells. Based on this information, the leukemic cells of most hematological malignancies, could be assigned to the B- and T-lymphoid vs. myeloid lineages and could be classified according to their stage of maturation (for example CD13+ and/or TdT- neoplastic cells are classified as immature, while cells expressing sIg, sCD3/TCR in the absence of CD34, are more likely to be classified as mature). This information is of great help for the diagnostic classification of hematological malignancies. Clear examples of such utility are the phenotypic diagnosis of lineage involvement in acute leukemias, the identification of biphenotypic acute leukemias and the phenotypic classification of ALL.

In recent years, the availability of more detailed and complete knowledge about the normal phenotypic patterns of different hematopoietic lineages and maturation-associated cell compartments, has contributed to the identification of new subtypes of acute non-lymphoblastic leukemias (ANLL). As an example, at present it is well-established that in a small subset of ANLL, blast cells show immunophenotypic features that are characteristic of normal plasmacytoid dendritic cells, including strong reactivity for both HLA-DR and CD123, and positivity for CD4 and CD36, in the absence of other highly-specific neutrophil and monocytic lineage-associated intracellular markers (e.g.: CyMPO, CyLysozyme). However, in contrast with their normal counterpart, neoplastic plasmacytoid dendritic cells typically show coexpression of CD56 and NG2 (7.1) together with abnormally low CD45 levels in the absence of CD34 expression. Consequently, new combinations of markers, introduced to detect early commitment of neoplastic cells, for specific hematopoietic cell lineages has proven to be of value for a more sensitive subclassification of AML, according to the myeloid lineages involved. As an example, the combination of CD64 and CD36 expression appears to be more sensitive than CD14 alone for identifying commitment of myeloid blast cells towards the monocytic lineage. Similarly, cytoplasmic expression of tryptase has been associated with specific subtypes of AML in which a variable degree of involvement of the basophil and/or mast cell lineages exists.

In addition to the similarities observed between neoplastic and normal hematopoietic cells, aberrant patterns of protein expression have been identified in most leukemic cells, and have been shown to reflect underlying cytogenetic abnormalities. Consequently, the detailed characterization of such aberrant phenotypes, not only allows discrimination between normal and neoplastic cells, but is also being increasingly used for a more accurate classification of the disease and for identification of cases carrying specific genetic abnormalities in which rapid, cost-effective confirmatory molecular studies must be performed to enable prompt initiation of therapy; this applies both to acute leukemias and B-CLPD. For example, blast cells in acute promyelocytic leukemia patients carrying the t(15;17) show commitment into the neutrophil lineage (cMPO+, CD33+, CD13+) with a maturation arrest at the promyelocytic stage (HLADR–/dim+, CD13–dim, CD33–/+, CD117–/+, CD11b+). Similarly, cytoplasmic expression of tryptase has been associated with specific subtypes of AML in which a variable degree of involvement of the basophil and/or mast cell lineages exists.
TELA/ML1 typically show a common/BII BCP-ALL phenotype associated with heterogeneous CD34 expression, in the absence of reactivity for CD20.28,34 In turn, cases with MLL gene rearrangements frequently have a pro-B/BI phenotype with reactivity for NG2 (7.1) and the CD15 and CD65 myeloid-associated markers.30,35 Table 3 provides a list of those aberrant phenotypes frequently associated with different genetic subgroups of acute leukemias.

**Immunophenotypic characterization of myelodysplastic syndromes**

For many years, the use of single staining of mononuclear cell fractions from bone marrow samples has limited the utility of immunophenotyping in the diagnosis of myelodysplastic syndromes (MDS). Nevertheless, early studies identified abnormal patterns of antigen expression in the bone marrow of MDS patients.24,36-38 For example, decreased expression of CD35, CD11b, CD15, CD11a, CD54 and CD116 together with abnormally high percentages of CD35+, CD87+, CD14+, CD44+ and CD64+ cells, were reported on peripheral blood neutrophils in a variable proportion of MDS patients (between 18% and 80% of the cases).24 Similarly, increased expression of precursor and early myeloid markers such as CD117, HLADR, CD34+, CD33 and CD13, together with lower reactivity for more mature antigens (e.g.: CD11b, CD11c, CD16 and NAT-9), have also been described in the BM of MDS patients.24 More recent studies show that multiparameter flow cytometric characterization of specific bone marrow subpopulations according to their lineage and maturation stage, are of great clinical utility for the diagnosis of MDS in patients with inconclusive morphological and cytogenetic features as well as for prognostic stratification.24,36,38,40 However, it should be noted that most abnormalities are related to the leukocyte compartment whereas only small percentages of cases have abnormal antigen expression in the erythroid compartment (e.g.: altered reactivity for CD56, CD71, glycoporphin A and/or CD45). Remarkably, information about the antigen expression patterns of megakaryocytic precursors in MDS is scanty.

The most common patterns of altered antigen expression include: 1) an increased number of myeloblasts; 2) abnormal sideward light scatter (SSC) for neutrophil and monocytic lineage cells; 3) abnormal expression patterns of CD13/CD16 or CD11b/CD16, together with coexpression of CD56 on other lymphoid-associated markers in bone marrow neutrophil-lineage cells; 4) co-expression of CD56 on monocytes; 5) abnormal expression patterns of CD71, glycoporphin A and/or CD45 on nucleated red cells; 6) increased numbers of megakaryocytic cells; and 7) an altered myeloid/lymphoid cell ratio.24,38-40

**Other applications of flow cytometry in the diagnosis and management of patients with hematological malignancies**

Apart from immunophenotyping for diagnosis and classification of acute leukemias, B-CLPD and MDS, the specific identification and enumeration of leukemic cells based on their aberrant phenotype is currently used for disease staging and for monitoring minimal residual disease (MRD) levels during and after therapy. Accordingly, the detection of minimal numbers of neoplastic cells infiltrating peripheral blood, bone marrow, and the central nervous system, currently represents one of the most widely used applications of flow cytometry to evaluate the extent of the disease in both B- and T-cell lymphomas and, to a lesser degree, also in ALL patients with suspected central nervous system involvement.15,18-19 Furthermore, evaluation of MRD during and after therapy, has proven to be of great help for predicting impending relapses in acute leukaemia patients14 and for early evaluation of the efficacy of different treatment modalities in mature lymphoid neoplasias.1 Antigen expression patterns might also be used for predicting response to tumor cell-associated antigen-directed therapies41 and they may indicate potential new drug targets for inducing cell differentiation or
apoptosis pathways or for inhibiting cell proliferation.42

In addition to the above listed applications of immunophenotyping in the diagnosis of hematological malignancies, staining for one or more antigens can also be combined with analysis of DNA content.5 In this area, detection of DNA hyperdiploidy is of great utility for the genetic classification of both ALL and multiple myeloma. Moreover, the analysis of cell cycle distribution of neoplastic cells has proven to be relevant for the prognostic stratification of NHL and multiple myeloma, in which increased proliferation is usually associated with high-grade disease and a worse clinical outcome, respectively.5

Despite the fact that immunophenotypic approaches can also be combined with assessment of drug resistance at both antigenic and functional levels, the diagnostic and prognostic relevance of these assays at diagnosis remains to be established.5

New flow cytometry tools for the immunophenotypic diagnosis of hematological malignancies

It is expected that the application of flow cytometry in the diagnosis of hematological neoplasias will continue to expand. Apart from the development, identification and evaluation of new antigenic markers, future studies shall also take advantage of recent improvements in instrumentation, bead technology, multicolor staining and multiparameter analyses (Table 4). Accordingly, the development of new flow cytometry tools for the immunophenotypic diagnosis of hematological malignancies is an area of increasing interest.

### Table 4. Multiparameter flow cytometric immunophenotyping of haematological malignancies: currently available capabilities and required new tools

<table>
<thead>
<tr>
<th>Tools</th>
<th>Current availability</th>
<th>Further requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multicolor flow cytometry</td>
<td>4 to 17 colors</td>
<td>Required number of colors to be determined</td>
</tr>
<tr>
<td>Compatible fluorochromes</td>
<td>Up to 17 colors</td>
<td>Development of new high-quality fluorochromes (e.g. quantum dots)</td>
</tr>
<tr>
<td>New monoclonal antibody reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early myeloid markers</td>
<td>CD36/CD64a</td>
<td>Adapt and standardize existing reagents for their use in flow cytometry</td>
</tr>
<tr>
<td></td>
<td>IREM1a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kell grα</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EoPOα</td>
<td></td>
</tr>
<tr>
<td></td>
<td>van Willebrand factora</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD203cα</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tryptaseα</td>
<td></td>
</tr>
<tr>
<td>Fusion proteins</td>
<td>PML-RARα</td>
<td>Improve the quality of the performance of some of the existing reagents</td>
</tr>
<tr>
<td></td>
<td>TEL-AML-1α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2A-PBXα1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BKT-α</td>
<td></td>
</tr>
<tr>
<td>Mutated/dysregulated proteins</td>
<td>Bcl-2α</td>
<td>Develop new reagents for the detection of genetic markers (e.g. BCR/ABL fusion protein, mutated CD117, mutated JAK2, RAG2, etc.)</td>
</tr>
<tr>
<td></td>
<td>p53α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rbβ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bcl-6α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bcl-10β</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclin D1α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-mycα</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLLβ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2Aα</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FoxP1α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RAG1β</td>
<td></td>
</tr>
<tr>
<td>Other markers</td>
<td>ZAP70α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AKPβ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BLUMP-1β</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pax-5β</td>
<td></td>
</tr>
<tr>
<td>Software tools</td>
<td>Limited software programs for multicolor analysis</td>
<td>Advanced automated multicolor software tools</td>
</tr>
</tbody>
</table>

*Good results; *Poor results in patient samples; *Not available for flow cytometry or further testing needed
cytometers with increasing multicolor capabilities together with the availability of a greater number of high quality, compatible fluorochromes, has driven the use of eight- and more colors in routine diagnostic laboratories in the last two years. In parallel, the development of highly-sensitive four-way, high-speed sorting instruments has facilitated routine purification of neoplastic cells from patients' samples for further genetic and molecular diagnostic studies, which is of particular relevance once minimal disease levels are detected (e.g. bone marrow aspirate samples from patients with monoclonal gammopathies).

As in the past, the production, identification and evaluation of new monoclonal antibody reagents represents an area with a major impact on the future development of clinical flow cytometry in hematology. The availability and evaluation of new markers for the identification of early commitment of hematopoietic cells into the monocytic (e.g.: CD36 in combination with CD64), erythroid (e.g.: Kell gp), megakaryocytic (e.g.: von Willebrand factor), basophil/mast cell (e.g.: CD205c, tryptase), eosinophil (e.g.: EoPO) and dendritic cell lineages, will certainly contribute to improving the classification of ANLL.

Similarly, the development of high quality monoclonal antibody reagents directed against transcription factors would be of great utility to dissect the pathways involved in leukemogenesis. Even more interesting are the recent efforts to produce pairs of monoclonal antibodies directed against fusion proteins derived from gene rearrangements occurring in specific chromosomal translocations (e.g.: BCR-ABL protein): these could be used for the identification of diagnostic genetic markers by flow cytometry both in cell lysates and in single cell suspensions. In this area, the parallel development of multiplexed-bead arrays for the quantitative evaluation of soluble proteins in both serum and cell lysates, will certainly facilitate the rapid introduction of newly developed antibody reagents for the identification of fusion proteins, mutated proteins and/or phosphorylated proteins, as well as for the quantitative evaluation of proteins cleaved from the surface of neoplastic cells (e.g.: β2-microglobulin in plasma cell disorders).

Despite the great clinical utility of flow cytometric immunophenotyping of hematological malignancies and the promising technological advances which have occurred in the past few years, standardization of technical procedures, data analysis, interpretation and reporting still remains a major challenge. In line with this, different, automated and semi-automated sample preparation devices have been produced and commercialized marketed in the last decade. In addition, recent reports show that new software tools based on vector quantification approaches can be produced and applied to the automated analysis of flow cytometry data files. In line with this, we have recently shown that screening patients with peripheral blood absolute lymphocytosis for mature B-cell neoplasias, could be fully automated, providing an extremely high specificity and sensitivity in the diagnosis of B-cell malignancies.

Altogether these new advances and tools make the future of leukemia/lymphoma immunophenotyping particularly attractive and promising, a coordinated effort between industry, academic and clinical hematologists as recently started in Europe by the EuroFlow consortium being mostly welcome to speed up their clinical application.

Acknowledgments
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| 12 | hematology - the european hematology association education program | 2006; 2(1) |


Establishing a regional hemato-oncology diagnostic service

The diagnosis of hematological malignancies has become increasingly complex. This is due to the range and sophistication of tests, the extent of data needed to confirm a diagnosis and the detailed information required by clinicians regarding disease status and prognosis. This necessitates high levels of medical and scientific expertise and is ideally performed in a large centralized regional referral center. This maximizes efficiency and quality and ensures that patients and clinicians gain maximum benefit from scientific and clinical developments. Specialized regional hemato-oncology diagnostic laboratories have now been established in a number of countries. The advantages and the requirements of this approach are discussed and listed in Tables 1 and 2.

Over the past decade there have been significant advances in technologies available for the scientific assessment and classification of hematological malignancies. With the increasing number of test options it is neither technically feasible nor cost-effective for these complicated and expensive analyses to be performed in all routine laboratories. However it is important that these tests be performed as they are required to make a diagnosis according to the WHO classification. This can be achieved by performing traditional tests in-house (e.g. morphology and basic phenotyping) and out-sourcing the difficult, new expensive tests. Alternatively samples can be sent to a central facility for one or more tests. The latter approach enables a complete diagnostic work-up if all testing is performed and interpreted in one location. A WHO diagnosis can then be made by integrating morphology, phenotype and genotypic (cytogenetics and molecular) data. This is the principle behind regional haemato-oncology diagnostic services.

Modern clinical hemato-oncology practice, with its use of intensified therapeutic regimens and transplantation, is also driving change in diagnostic laboratories. Clinicians are now requiring more detailed information regarding the disease and its status. Diagnostic laboratories must, therefore, have a complete range of tests in order to be able to support increasingly complex therapeutic approaches. This is not only for precise diagnosis, but also for the identification of markers of prognosis, potential therapeutic targets and methods for monitoring low levels of disease. The results need to be generated in a timely fashion, be interpreted in the clinical context and be able to be communicated to clinicians. Again, this can be achieved in a quality regional diagnostic service.

Some countries are now supporting, recommending or legislating that diagnostic hemato-oncology services be centralized. In the United Kingdom (UK) national guidelines (NICE Guidelines) require regional diagnostic services. These must have appropriately qualified medical and scientific staff and use a range of diagnostic tests to establish accurate and precise diagnoses of hematopoietic malignancies. Results are to be integrated and interpreted by experts who work together with the referring clinical hematologists and care teams. The aim of this approach is to make the diagnostic excellence available to as large a patient population as possible thereby ensuring equity of access and improvement in the quality of patient care (Table 1). In the UK, regional hemato-oncology diagnostic services have been established in Cambridge and Leeds. These diagnostic laboratories, which serve geographical regions with populations of 3-4 million and between 10 and 20 referring clinical hematology units, are a model for other UK centers. In Germany the model is different; competence networks, which are mainly national, have been formed in response to a directive from the Federal Ministry for Education and Research in 1997. The German Acute and Chronic Leukemias competence network
Table 1: Advantages of regional haemato-oncology diagnostic services.

<table>
<thead>
<tr>
<th>Advantage</th>
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<tbody>
<tr>
<td>Regional diagnostic centre of excellence</td>
</tr>
<tr>
<td>Diagnostic standardisation</td>
</tr>
<tr>
<td>Uniform disease definitions</td>
</tr>
<tr>
<td>Uniform approaches to disease monitoring</td>
</tr>
<tr>
<td>Flexibility to enable rapid response to scientific and technical developments with the rapid introduction of new diagnostic tests</td>
</tr>
<tr>
<td>Database of cases</td>
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<tr>
<td>Audit of laboratory data</td>
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<tr>
<td>Retrospective case review</td>
</tr>
<tr>
<td>Epidemiology</td>
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<tr>
<td>Sample banking</td>
</tr>
<tr>
<td>Participation in research</td>
</tr>
<tr>
<td>Close ties with multiple clinical service units, including national trials</td>
</tr>
<tr>
<td>Centre of expertise for teaching and training</td>
</tr>
<tr>
<td>Equity of patient access</td>
</tr>
<tr>
<td>Supports clinical excellence</td>
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</table>

Table 2: Requirements for a regional haemato-oncology diagnostic service.

<table>
<thead>
<tr>
<th>Requirement</th>
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<tbody>
<tr>
<td>Commitment from users and funders to centralise testing and integrate data</td>
</tr>
<tr>
<td>Excellent communication with referring clinicians</td>
</tr>
<tr>
<td>Regional cooperation and coordination</td>
</tr>
<tr>
<td>Medical and scientific expertise in haemato-oncology diagnosis</td>
</tr>
<tr>
<td>Transport systems for specimen delivery</td>
</tr>
<tr>
<td>Appropriate equipment and other technical resources</td>
</tr>
<tr>
<td>Financial support</td>
</tr>
<tr>
<td>Excellent communication with referring centres, including regular team meetings</td>
</tr>
<tr>
<td>Information technology systems and support for rapid provision of results</td>
</tr>
<tr>
<td>Commitment to education and training</td>
</tr>
</tbody>
</table>

comprises over 300 centers and has worked towards standardization of diagnostic procedures in leukaemia. Across Europe there are initiatives to integrate all major diagnostic groups into a European Platform on Leukemia Diagnostics and European Networks of Excellence. Standardization of testing and disease monitoring should be achievable through these networks.

Structure of a regional haemato-oncology diagnostic service

The laboratory assessment of neoplastic hematopoietic cells requires investigation of cell morphology, determination of cell lineage and stage of differentiation, and identification of disease-specific features (phenotypic or genetic) which may be used for targeted therapies or for disease monitoring. The service may be for hematological samples only or could be integrated with histopathology and thereby include tissue biopsy specimens. Centralization of laboratory services facilitates a standardized and integrated approach to diagnostic testing, data interpretation and definition of prognostic factors.

As there are many indications for a sample to be referred to a regional hemato-oncology diagnostic laboratory it is important that specimens be triaged upon arrival to determine the most appropriate tests to be performed. The decision will be based on the clinical information, the sample type (e.g. blood, bone marrow aspirate or trephine, body fluid, biopsy), stage of disease (diagnosis or follow-up) and an initial screen of the morphology. Further investigations should be in accordance with documented test pathways developed by the laboratory and in collaboration with referring hematologists. These standardized protocols, which should be based on published literature, will assist in ensuring reproducibility, avoid unnecessary test duplication and be of value for case comparisons and auditing.

Testing is required both at diagnosis and throughout the patient’s clinical course. The initial diagnostic sample should have a complete work-up with multiple test modalities, for the following reasons:

1. accuracy of diagnosis and disease classification by WHO criteria. There is enhanced accuracy with complete disease definition by as many relevant test modalities as possible;
2. to identify markers of prognosis (phenotypic or genotypic). Examples include FLI3 or NPM in acute myeloid leukemia, ploidy in acute lymphoblastic leukemia, complex karyotypes and CD38, ZAP70, p53 or IgVH mutational status in chronic lymphocytic leukemia;
3. to identify potential therapeutic targets. Examples include CD20 or CD22 expression in B-cell malignancies and CD52 antigen expression in chronic lymphoid disorders;
4. to identify disease-specific markers for disease monitoring. This may require cross-checking data from different test modalities that can detect the same abnormality but with differing sensitivities (e.g. BCR-ABL by fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR)).

Follow-up samples received by the laboratory during or after completion of therapy are to assess persistence of disease. Tests should be selected to target the known cellular characteristics, as determined on the diagnostic sample, and at the required sensitivity. This tailored approach minimizes unnecessary testing and maximises the quality of information available to the clinician. The role of each type of test will be described.

Morphology

Morphological assessment of the blood, bone marrow and other diagnostic material (e.g. body fluids, tissue biopsies) remains the cornerstone for the diag
nosis of many hematological malignancies. This morphological assessment may have been performed at the referring laboratory but must be repeated upon receipt at the regional diagnostic laboratory. Morphology will determine which ancillary tests are required to confirm or validate the diagnosis, classify the malignancy by WHO criteria or monitor residual disease. A provisional diagnosis can, however, be made in many cases, on morphology alone and communicated to the referring clinician.

**Phenotyping**

Immunophenotyping was introduced into diagnostic pathology over 30 years ago to assist in the diagnosis and classification of hematopoietic malignancies. It remains an essential component in the laboratory work-up of many hematological malignancies, particularly acute leukemias and chronic lymphoid malignancies. The role of immunophenotyping has expanded beyond this to include the detection of markers of prognosis (e.g. ZAP70), determination of disease phenotypes associated with specific chromosomal abnormalities, detection of targets for immunotherapy (e.g. CD20) and to monitor residual disease.7,8 Specifically, phenotypic monitoring of residual disease is of value in acute leukemias, chronic lymphoproliferative disorders and in multiple myeloma. The majority of phenotyping is performed by multi-parameter flow cytometry. This can be applied to haematological samples (blood and bone marrow), body fluids and disaggregated tissue samples.9 Highly skilled scientific personnel, who are able to perform, interpret and understand the limitations of multi-parameter flow cytometry, are required. The laboratory should also have the ability to perform fluorescence microscopy (e.g. for PML protein) and immunocytochemistry (e.g. of tissue sections or for rare cells that require simultaneous assessment of morphology and antigen expression). The expanded repertoire of phenotyping applications, the continually expanding number of antibodies and the sophistication of technology demonstrates how important it is for phenotyping to be performed in a specialist laboratory. European centers are now collaborating to develop uniform immunophenotyping strategies (e.g. European Leukemia Network).6

**Cytogenetics**

The detection of chromosomal abnormalities is another critical element in the assessment of hematological malignancies as many genetic abnormalities are associated with distinct clinical entities. The demonstration of genetic abnormalities may be relevant for disease definition and classification, determining prognosis (e.g. the International Prognostic Score System in myelodysplastic syndromes) and monitoring therapy (e.g. chronic myeloid leukemia). As for phenotyping, scientific skill is required, particularly for conventional karyotyping of metaphases (the gold standard) and must be performed in a regional center. FISH is increasingly being utilized for assessment of specific chromosomal abnormalities. It can be performed on interphase and metaphase cells on cytogenetic preparations, nuclei extracted from paraffin-embedded tissue or on paraffin-embedded tissue sections. By targeting specific genetic lesions (e.g. the PML-RARα of t(15;17) in acute promyelocytic leukemia; 11q23 abnormalities in MLL leukemias), it can be used to provide rapid diagnostic and prognostic genetic information. The decision as to which specific genetic abnormalities should be assessed by FISH will be guided by morphology and phenotyping data, thereby emphasizing the importance of an integrated laboratory. Genetic testing of hematological malignancies is a rapidly evolving field and regional or centralized services should have the flexibility and skill to adopt new, complex and expensive technologies and applications (e.g. multi-color FISH or spectral karyotyping and comparative genomic hybridization).

**Molecular genetics**

Molecular genetic analysis requires sensitive technology for diagnosis, confirming clonality, providing prognostic information and disease monitoring for a range of hematological malignancies. PCR-based techniques (e.g. PCR, reverse transcriptase-PCR, quantitative reverse transcriptase-PCR and sequencing) can only detect a subset of molecular abnormalities that occur in hematological malignancies. These tests therefore complement and add value to results obtained by conventional karyotyping and FISH.3 Genetic rearrangements that can currently be detected using PCR include fusion genes as a consequence of translocations and occasionally deletions, over-expression of oncogenes, and point mutations which are cryptic by cytogenetic methods. Many of these changes carry prognostic significance and may be useful in targeting treatment aimed at controlling specific genetic defects. For example, the success of all-trans retinoic acid and imatinib mesylate demonstrate the importance of identifying molecular defects and in chronic lymphocytic leukemia, determination of IgVH mutational status aids prognosis. The increased sensitivity of PCR and other novel quantitative assays permits detection of low levels of minimal residual disease below the threshold detectable by morphological examination and cytogenetic analysis. This allows continued monitoring of patients and the opportunity to treat patients prior to clinical relapse while the leukaemia burden is still relatively low. Quantitative methods for disease-
associated translocations (e.g. BCR-ABL quantification in chronic myeloid leukemia) and detection of tumor-specific DNA sequences (e.g. lymphoblastic leukemias) are the methods of choice for the molecular monitoring of residual disease.

Testing for the molecular consequence of an acquired genetic change is therefore of increasing importance in the assessment of hematological malignancies. Due to the complexity of the analyses, the range of tests and applications, rapid changes in technology and the scientific expertise required, molecular genetic studies must be performed in a specialized, centralized service. Standardized approaches to testing, including test selection, timing and utilization of international protocols (e.g. BIONET) should be used. Changes in technology (e.g. multiplex PCR, large scale mutation detection and gene expression profiling) will improve the repertoire of diagnostic and prognostic information and other applications available to the clinician.

Communication and information technology

Communication within the diagnostic laboratory and between the clinician and the diagnostic service is critical (Table 2). The diagnostic hematologist must be aware of the clinical situation of the patient whose sample is being analyzed (i.e. stage of disease; clinical protocols; proposed management plan). The clinician must be provided with the diagnostic information in an interpretable and timely fashion. The diagnosis must be classified according to agreed standardized criteria with internationally accepted criteria being utilized wherever possible. For most entities this is likely to be based on the WHO classification though other criteria (e.g. those of the Polycythemia Vera Study Group) may also be used; it must however be clear to users which classification is being used. Clinicians and diagnostic hematologists should meet regularly to discuss and review clinical and diagnostic information (including morphological images, flow cytometry and genetic data). In the UK this is achieved in the form of Multidisciplinary Team meetings. This forum ensures that results for all patients are discussed in context and used in clinical decision making.

A quality information technology system is essential for a regional diagnostic service (Table 2) in order to integrate laboratory data, communicate results to referring clinicians and to maintain a sample database. The information technology system must support the integration of all test results. This integrated report should summarize results of all test modalities, provide a diagnosis and disease classification, give prognostic information and offer recommendations for further testing. It should also support the inclusion of morphological and other images (e.g. karyotype; FISH; PCR gels). This report should be accessible electronically to all referring clinicians irrespective of their geographical location via a secure web-based system. This approach is far superior to paper-based or standard laboratory information systems for dissemination of complex diagnostic information. All integrated reports should be incorporated into a comprehensive institutional leukemia database. This is useful not only for the individual patient (e.g. comparisons between presentation and relapse), but also for audits, epidemiological studies, teaching and research. A large searchable database will enable new disease entities to be discovered and epidemiological and statistical data to be extracted.

Other functions of a regional diagnostic service

A regional integrated hemato-oncology diagnostic service should have the flexibility and scientific expertise to be able to introduce new tests and technologies rapidly. Being centralized these new tests will be widely available to a large number of patients in a shorter period of time. The advantages of this have recently been demonstrated with the introduction of routine molecular genetic testing for JAK2 mutations in the investigation of myeloproliferative disorders. Other tests which could soon be adopted as a result of research developments include proteomic screening of cells and serum and gene expression analyses in malignancies.

A regional hemato-oncology diagnostic service should be an active participant in research and development. This may be in isolation (e.g. epidemiological; new disease entities; new test development) or in collaboration with academic centers or national bodies (e.g. clinical trials). Research should utilize the strengths of the service and the availability of clinical samples. Sample banking is encouraged but is dependent on having institutional ethical approval, being compliant with national regulations, having patients’ consent and a secure database. Samples that could be banked include cells, DNA, RNA, protein and plasma from patients with a range of hematological disorders.

Education and training are other important activities of a regional diagnostic service. The laboratory should provide regular educational material to stakeholders detailing the range of services provided, samples required, anticipated turn-around-times and any new developments or tests available. As one of the requirements for a regional facility is scientific and medical expertise in the pathology, biology and investigation of clonal hematological disorders, these centers are the ideal location for postgraduate medical and scientific training and subspecialization.
Conclusions

Regional centers for hemato-oncology diagnostic services have many advantages for patients and clinicians over traditional in-house testing with outsourcing of some tests. This approach supports excellence in patient care, teaching and research and is efficient. A regional service provides a critical mass of scientific and medical expertise which is available to a larger number of patients than would otherwise be possible. Inter-regional collaboration is feasible and will support national and European networks. Centralized diagnostic facilities are better able to respond to new demands, be they clinical, scientific (e.g. introduction of new investigations), political or economic and should be sustainable. However, the major gains from a regional service are the ability to integrate results of all tests performed, generate a pathology report of clinical relevance and communicate information to clinicians. This is a patient-focused approach which will inevitably be followed by clinical benefits.

References

Cancer is a genetic disease of the somatic cell. The accumulation of multiple genetic abnormalities, or hits, characterizes the evolution of the cancer cell. Thus genomic instability is one of the hallmarks of cancer. This genomic instability is commonly manifested by structural or numerical chromosomal aberrations. Structural genomic aberrations leading to activation of oncogenes or elimination of tumor suppression genes have been studied extensively. However, very little is known about the oncogenic role of numerical chromosomal aberrations, aneuploidy, which are the most common abnormalities in cancer.

Three (trisomy) or four (tetrasomy) copies of chromosome 21 are characteristic of many cases of childhood acute lymphoblastic leukemia (ALL). Children with Down syndrome (DS) who carry trisomy 21 in all their cells have a 20 fold increased risk of childhood ALL and 600-fold increased risk of acute megakaryocytic leukemia (AMKL). Down syndrome is not a classic genomic instability syndrome as the general risk for cancer is lower in DS patients. Rather, the high incidence of childhood leukemia in children with DS strongly suggests that additional copies of chromosome 21 are leukemogenic. Hence, studies of the pathogenesis of leukemia in patients with DS may clarify the role of acquired trisomy 21 in sporadic leukemias.

The genetics of transient myeloproliferative disorder and acute megakaryocytic leukemia

Approximately 10% of children with DS are born with a syndrome of clonal megakaryocytosis commonly called transient myeloproliferative disorder (TMD), transient abnormal myelopoesis (TAM) or transient leukemia. As suggested by the different names the disorder is usually transient and resolves spontaneously within up to several months. The biological mechanism of the spontaneous resolution is unclear. About 1% of DS patients will, however, develop a full blown malignant AMKL during their first four years of life that will not regress without chemotherapy. In fact, the risk of AMKL is about 600 times higher in children with DS. The factors underlying the transformation from benign TMD into malignant AMKL are largely unknown.

Thus the megakaryocytic malignancies of DS provide a natural genetic model of multistep lineage-specific leukemogenesis. Both the congenital disorder and the full blown AMKL are characterized by arrested differentiation of the megakaryocytic lineage. The peculiar association between DS and childhood megakaryoblastic disorders has led to intensive search for gene or genes on chromosome 21 that may cause the differentiation arrest and initiate the leukemia. A surprising twist in this story came with the discovery that a gene on chromosome X, GATA1, was mutated in the megakaryoblasts from all patients with DS and either TMD or AMKL. The mutations were also found in fetal liver of aborted DS fetuses. The mutations are acquired as they are not found in remission samples, and are specific to the megakaryoblastic disorders associated with trisomy 21. Thus a clear model for multistep leukemogenesis in DS emerges: in a relatively high proportion of DS patients, acquired mutations in GATA1 are selected in utero and are probably responsible for the differentiation arrest and the initiation of clonal proliferation of immature megakaryoblasts. These mutations are necessary but insufficient for the development of the full blown AMKL that affect some of these patients during early childhood.

GATA1 encodes a zinc-finger transcription factor that regulates the normal development of erythroid, megakaryocytic and basophilic/mast cell lineages. Mice lacking GATA1 expression in the megakaryocytic lineage have thrombocytopenia and extensive proliferation of immature megakaryoblasts. Inherited inactivating mutations in GATA1 in humans cause a familial dyserythropoietic anemia and thrombocytopenia.

Congenital preleukemic syndromes

Down’s syndrome as a model for aneuploidy in leukemia
nia. Thus GATA1 normally suppresses the proliferation of megakaryocytic and erythroid precursors while promoting their differentiation.

Two isoforms of GATA1 are usually detected: a full length GATA1 translated from the first ATG on exon 2, and a shorter form (GATA1s) that is initiated from an ATG on exon 3. The normal function of GATA1s is unknown. Presumably the balance between these two products serves a regulatory function in normal megakaryocytic development. All the acquired mutations in the megakaryoblastic disorders of DS result in elimination of the full length GATA1 and the preservation of GATA1s.

The collaboration between gene(s) on chromosome 21 and mutated GATA1 in megakaryocytic malignancies of DS is unique in its intrauterine occurrence and in its putative initiating role of a common and generally reversible clonal hematopoietic proliferation syndrome. At least three fascinating questions are raised:

a) Why do all the selected mutations result in the formation of the short isoform of GATA1s? Does this isoform have a dominant pro-leukemogenic effect?

b) Why do GATA1 mutations and the megakaryoblastic proliferation occur only in utero? In DS germline trisomy 21 exists in all hematopoietic progenitors throughout life. GATA1-dependent megakaryocytopoiesis in the bone marrow also continues throughout post-natal life. So why does the selection of mutations in GATA1 in DS patients occur only in the fetal liver?

c) What is/are the gene (or genes) on chromosome 21 that, when existing in one additional copy, selects for the cells carrying the GATA1s mutation? What is the mechanism of this selection?

A clue to the answer to the first two questions comes from a recent study from the laboratory of Stuart Orkin. Knock-in of the mutated GATA1s into the GATA1 locus surprisingly resulted in normal adult megakaryocytopoiesis. However, examination of the fetal liver revealed abundant proliferation of megakaryocytic progenitors. Orkin’s group propose the existence of a fetal hematopoietic progenitor that is sensitive to a dominant pro-proliferative effect of GATA1s. The presence of trisomy 21 enhances the survival and proliferation of these fetal cells resulting in a congenital leukemia syndrome. A dominant leukemogenic role for GATA1s emerges from a recent analysis of gene expression of DS patients that revealed that GATA1s is not a simple loss-of-function mutation.

The third question is what gene (or genes) on chromosome 21 promotes proliferation and provides a survival advantage to cells that have acquired mutations in GATA1, a gene on chromosome X? Because the trisomy 21 in DS is constitutional, i.e. it is present in every cell, it may promote leukemia in a non-autonomous manner. Possibly, the presence of trisomy 21 in non-hematopoietic stromal cells in the fetal liver may change the micro-environment and support the proliferation of the special fetal hematopoietic progenitors that are sensitive to GATA1s. This hypothesis may also explain why the transient megakaryoblastic proliferation resolves after birth. However, since very little is known about the regulation of fetal liver hematopoiesis, this hypothesis is currently difficult to study.

Alternatively, increased expression of certain chromosome 21 genes in the same cell that carries the GATA1s mutation may enhance its proliferation or survival (a cell autonomous hypothesis). The strongest candidate has been RUNX1 (also known as AML1 or CBFA2) (reviewed in ref. 9). RUNX1 is a transcription factor that is required for normal hematopoiesis. It is commonly mutated and involved in various translations in both myeloid and lymphoid leukemias. However, RUNX1 abnormalities have generally not been detected in AMKL and, except for a single case report, mutations in RUNX1 have not been found in AMKL associated with DS. RUNX1 activity is very sensitive to gene dosage. Inherited mutations in RUNX1 causing haplo-insufficiency with low level of expression in hematopoietic stem cells give rise to a syndrome of familial thrombocytopenia and increased susceptibility to leukemia. This rare human syndrome, and other functional studies suggest that RUNX1 regulates megakaryocytopoiesis. It is therefore reasonable to hypothesize that an extra copy of RUNX1 may enhance megakaryocytopoiesis.

We have recently demonstrated the potential involvement of ERG, an ets transcription factor on chromosome 21q, in the DS megakaryocytic leukemias. ERG is a proto-oncogene that is rarely involved in AMKL caused by the ERG-TLS translocations. Recently, overexpression of ERG was identified as an independent bad prognostic factor in AML with normal karyotype. We have shown that it is expressed in CD34 cells, in normal megakaryocytes and platelets, in megakaryocytic leukemias (whether or not associated with DS), but not in normal or malignant erythoblasts. ERG is induced upon megakaryocytic differentiation of erythroleukemia cells. Forced expression of ERG in the erythroleukemia cell line causes a phenotypic shift from the erythroid into the megakaryocytic lineage. Together these observations suggest that ERG is a positive regulator of normal and malignant megakaryopoiesis.

We propose a developmental rush hour model for the occurrence of megakaryocytic leukemias in DS (Figure 1). Extra copies of several genes on chromo-
some 21 (including ERG, RUNX1, probably ETS2 and possibly some others) create a positive pressure towards megakaryocytogeneses, similarly to the traffic pressure towards downtown during rush hour. The observation that normal DS infants have significantly higher platelets counts during the first six months of life supports this suggested enhanced fetal megakaryocytogenesis. The GATA1s mutation is similar to a traffic accident in preventing megakaryocytogenesis from reaching the target -platelet formation, and further enhances the proliferation of a putative fetal megakaryocytic precursor, as shown by Orkin et al. The mutation in GATA1 downregulates the expression of RUNX1 and increases the expression of BACH1 leading to a further block of megakaryocytic differentiation. The consequence is a pile-up of megakaryocytic precursors. In contrast to the car traffic-jam, only megakaryocytic progenitors with the GATA1s accumulate. This clonal accumulation results in the congenital leukemic phenotype.

A major obstacle to the identification of leukemias-promoting genes on chromosome 21 is the lack of an appropriate mouse models. Furthermore, no contribution to this mystery has been obtained from recent gene expression studies of DS AMKL. An alternative hypothesis to the model presented above is that other genetic elements on chromosome 21, which are not represented in the mouse models or on the DNA microarrays (e.g. genes coding microRNA) are the critical oncogenes of the AMHL occurring in patients with DS.

Down syndrome and acute lymphoblastic leukemia

The megakaryocytic leukemia of DS is a unique disease. However patients with DS are also at a markedly increased risk of developing childhood acute lymphoblastic leukemias (ALL). Because trisomy and tetrasomy of chromosome 21 are the most common acquired chromosomal abnormalities in ALL, the study of DS ALL may have direct implications for understanding sporadic childhood ALL. In most published multi-institutional ALL protocols, DS ALL patients account for about 1-3% of all patients. The age distribution and the immunophenotype are similar to those of common ALL. Common ALL is a B-cell precursor leukemia that occurs most commonly in young preschool children.

ALL may be caused by a direct oncogenic effect of trisomy 21, similarly to the role of additional chromosomes 21 in sporadic leukemias. Alternatively, the effect of trisomy 21 may be developmental. Similarly to the suggested model for the megakaryocytic leukemias of DS, constitutional trisomy 21 may enhance the proliferation of a normal fetal lymphoid progenitor. This excess proliferation could evolve into leukemia if additional genetic events occur. Viral infections and immunological responses have long been suggested to have a role in the pathogenesis of childhood common ALL. The markedly increased risk of ALL in DS could also be caused by the altered immunological environment and the increased infection rate that characterize DS.

Molecular epidemiology studies may clarify the leukemogenic role of constitutional trisomy 21. Common sporadic childhood ALL is usually associated with one of two genetic abnormalities: a structural chromosomal anomaly - fusing the AML1 (RUNX1) gene on chromosome 21 with the TEL (ETV6) gene on chromosome 12, or a numerical abnormality, hyperdiploidy. These two genetic aberrations are mutually exclusive suggesting that each activates an oncogenic pathway leading to B-cell precursor leukemia. If trisomy 21 enhances the risk for childhood ALL indirectly we could expect a similar rate of secondary aberrations (hyperdiploid or TEL/AML1 translocation) to that in sporadic common ALL. If, on the other hand, constitutional trisomy 21 has a direct leukemogenic effect, like the role of the acquired extra copies of chromosome 21 in hyperdiploid ALL, then we would expect a lower prevalence of TEL/AML1 or hyperdiploid genotypes in the ALL of DS.
There have been several studies summarizing the epidemiology of DS ALL during ALL protocols in the 1970s and 1980s as well as more recent reports by the COG and the NOPHO groups. However, the number of DS ALL patients is still relatively small for any definite conclusions or subgroup analysis. For example, several studies demonstrated a very low prevalence of TEL/AML1 translocations (consistent with the direct role hypothesis), while another demonstrated the reverse. Clearly an international molecular epidemiological study of DS ALL is warranted.

Conclusions
What are the general implications of DS leukemias for leukemogenesis in normal children?
Recent studies have clarified that most, if not all, childhood leukemias arise during fetal hematopoiesis. Thus, the sporadic childhood leukemias, like DS leukemias, evolve in a multistep process. A primary genetic event (first hit) is acquired in utero (in the case of DS the GATA1 mutation is the second event, the first being the germline addition of an extra chromosome 21). This event results in the formation of a large preleukemic clone that can be detected at birth by molecular techniques. As in TMD this clone regresses spontaneously in almost all children. Additional postnatal genetic events in the residual preleukemic cells are necessary for the generation of acute leukemia, which occurs in a small fraction of these children. Thus, studies on the mechanism of TMD regression and on the nature of events leading to full blown AMKL in DS are relevant for the general understanding of the leukemogenesis process (Figure 2).

DS leukemias are also a prime example of collaborating pro-proliferation and differentiation-arresting mutations in leukemia. Such collaboration emerges as a general paradigm in leukemias. Finally, DS leukemias are a prime model to study the role of aneuploidy – one of the fundamental issues in carcinogenesis research.

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Figure 2. A multi-step model of leukemogenesis. Comparison between DS and sporadic childhood leukemia. A. The leukemia of DS - acquired GATA1 mutation during fetal liver hematopoiesis in cells carrying a germline trisomy 21 results in transient congenital leukemic proliferation (TMD). An additional postnatal event is required for the development of full blown acute megakaryocytic leukemia (AMKL) B. Sporadic childhood leukemia - A prenatal acquired genetic event (e.g. chromosomal translocation, hyperdiploidy) creates a large submicroscopic prenatal clone evident at birth. In most instances this clone will disappear, unless a postnatal genetic hit causes its progression to full blown leukemia.


Fanconi anemia: genomic instability leading to aplastic anemia and cancer predisposition

Fanconi’s anemia (FA), first described by the Swiss physician Guido Fanconi in 1927, is a recessive condition characterized by nearly universal progressive bone marrow failure, multiple congenital anomalies and a predisposition to leukemia and cancer, particularly squamous cell carcinoma and hepatocellular carcinoma.1,2 The rate of heterozygosity may be as high as 1 in 300 with a prevalence of 1-2 affected individuals per 100,000 births. At the cellular level the condition encompasses hypersensitivity to DNA cross-linking agents, oxidative damage and other DNA-damaging agents. The complexity of the disease phenotype is increasingly understood to be due to the fact that mutations in any one of multiple genes leads to the common cellular phenotype with broad syndromic features. There are currently 11 genes implicated in FA and at least 12 corresponding complementation groups known by somatic cell fusion or retrovirus typing. (Figure 1) Nearly all patients with FA develop bone marrow failure and complications of pancytopenia are the leading causes of death, although improved clinical support has led to an increased awareness of the risk of leukemia or carcinoma, which are almost universally fatal complications. While the molecular genetic basis of the disease is increasingly characterized, understanding of the pathophysiology is still largely incomplete. Future applications of new therapeutics will rely on advances in stem cell biology, gene therapy and the development of small molecules modeled on new targets identified as the molecular pathobiology is defined.

Bone marrow failure and hematologic abnormalities in Fanconi anemia

The cumulative risk of developing bone marrow failure in FA patients is ~90% with a median age for the onset of aplastic anemia of 8 years old.3 Thus the current treatment for FA focuses on hematologic support (see below) including red blood cell and platelet transfusions and corrective stem cell transplantation. Although improved supportive care has prolonged the survival of patients from only a few years after the diagnosis of bone marrow failure, the median age of death is still 24 years old.7 Most patients die from complications of bone marrow failure including bleeding and infection and have a prolonged period of transfusion-dependence during the aplastic phase of their illness. However, due to hematopoietic mosaicism and possibly inter-genic variability in phenotype, the course of marrow failure is highly variable.

The diagnosis of FA should be considered in children with congenital anomalies or growth failure associated with blood cytopenias or macrocytosis indicative of stress erythropoiesis and in adults with early onset of head and neck or ano-genital tumors with unusual responses to chemotherapy or radiation treatment. The timing of the onset of pancytopenia varies widely and the initial lineage involved can be erythroid, myeloid or platelets. Most patients show signs of stress erythropoiesis (macrocytosis, elevated hemoglobin F) before the onset of clinically symptomatic anemia. Attempts to culture bone marrow progenitors in vitro from patients with FA demonstrate decreased numbers of myeloid and erythroid colonies consistent with bone marrow failure. For most complement types, the onset of pancytopenia occurs between 6-8 years of age. Although the time to progression of severe aplastic anemia varies, the need for therapeutic intervention and/or hematologic support generally becomes necessary within 5 years after the onset of clinically evident cytopenia. During the progression of the pancytopenia, the patients show the expected complications of bleeding, infection and fatigue. Since the progression of the pancytopenia is slow, the anemia is well-tolerated and bleeding tends not to be a major problem until platelet counts are consistently very low. Because
the outcome of stem cell transplantation may be adversely affected by multiple transfusions of red cells or platelets, a conservative approach to the use of cellular products is warranted. In our large clinic the Fanconi Anemia Comprehensive Care Center at Cincinnati Children’s Hospital, we generally reserve platelets for clinically relevant bleeding and red cell transfusions for symptomatic anemia such as the presence of tachycardia, shortness of breath or headache.

To date, the only definitive treatment for FA-associated aplastic anemia is stem cell transplantation. Interestingly, FA was the first disease in which umbilical cord blood was successfully utilized as a source of stem cells for allogeneic transplantation. Early studies were characterized by high transplant-related mortality rates attributable to both the enhanced toxicity concomitant with the FA phenotype and to poor engraftment. Subsequent modifications of the ablative preparative regimen, which included reducing the total irradiation dose and reducing the chemotherapy dosage, have significantly improved the outcome of allogeneic stem cell transplants from HLA-matched sibling donors. Several large series have shown survival rates of 70-80% in this setting. However, in a 20-year study by the International Fanconi Anemia Registry (IFAR), only 29% of FA patients underwent hematopoietic stem cell transplantation. Matched unrelated donor transplants remains experimental in this disease. The survival rate after a transplant from an unrelated donor has been reported low as 30%. Using more recent protocols incorporating fludarabine, several leading transplant centers report survival rates varying between 30-60% after short-term follow-up and small series. Thus newer unrelated donor transplant protocols appear promising, while identification of donors, graft failure and graft-versus-host disease remain significant obstacles.

During the period prior to stem cell transplantation, supportive care therapies are clearly effective and warranted. Nearly 50% of children respond to androgen therapy. While this therapy can cause serious side effects, judicial dosing and attention to potential complications can lead to successful long-term postponement of the need for stem cell transplantation. The results of a recent dose-escalation study of oxandrolone, were encouraging, with the majority of patients developing a clinical response with no signs of masculinization (F. Smith, personal communication). There is contradictory evidence in the literature suggesting that androgen therapy may adversely alter the outcome of stem cell transplantation. These data are difficult to interpret, since they were generated from single institution series and the patients enrolled received variable dosages, schedules and durations of androgen therapy.

In addition to androgen therapy, the use of growth factors has also been demonstrated to be successful in treating cytopenias. In the only prospective trial examining the use of granulocyte colony-stimulating factor (G-CSF), Rackoff et al. reported that 50% of patients responded to treatment with G-CSF with minimal side effects. There are anecdotal reports of responses to erythropoietin. In spite of attempts to enhance platelet numbers with growth factors, such as interleukin (IL)-11, IL-3 and others, no satisfactory growth factor treatment for thrombocytopenia has been reported; patients treated with androgens or G-CSF do show responses in the platelet lineage at some frequency.

The decision to move to stem cell transplantation is complicated and influenced by patient-centered and family considerations, the type and quality of match of the stem cell donor and the frequency of side effects of pancytopenia or treatments of pancytopenia. Since prior infections and transfusion history can have negative impacts on transplant outcomes, it seems prudent to move to stem cell transplantation once these complications seem inevitable in spite of supportive care measures. At the time of stem cell transplant, strong consideration should be given to referring the patient to a center with significant experience in transplants for this disease.

There is a growing appreciation that phenotypic variation of the clinical features may be related to specific mutations. Recent studies have demonstrated that clinical progression of the disease may be influenced by inter- and intra-genic variations. A study of 754 subjects registered with the IFAR demonstrated that patients belonging to complement group C had significantly earlier onset of bone marrow failure and poorer overall survival as compared to patients within groups A and G. Interestingly, in group C, the overall survival of patients with at least one intron 4 (IVS4+4A>T) or one exon 14 (R548X and L554F) mutation was poorer than that of patients with mutations elsewhere in the gene.
Non-hematologic abnormalities in Fanconi anemia

The spectrum of non-hematologic abnormalities in FA is large. Some FA patients may have no obvious congenital malformations. Growth retardation and skin abnormalities are the most common non-hematologic features of FA. Skin abnormalities include café-au-lait spots and/or hypopigmentation of the skin. In addition, patients can be recognized by their microcephaly and characteristic facies with a broad nasal base, epicanthal folds, and micrognathia. Upper limb abnormalities are also common and vary in severity from hypoplasia of the thenar eminence to complete absence of the thumb. Abnormalities of the thumb require intervention before the age of 2 years in order to allow adequate central adjustment to use of the transplanted appendage as a thumb. Other manifestations include genito-urinary, gastro-intestinal, cardiac, renal, and tracheo-esophageal malformations. Hearing loss due to middle ear bony abnormalities, otic canal malformations and even complete otic aplasia are seen. Children with FA are increasingly recognized to have complex endocrinopathies that include insulin-resistance, growth hormone deficiency, reduced fertility and/or thyroid hormone abnormalities. Taken together, the myriad of organ systems that are affected suggests that multi-disciplinary care offers the optimal treatment for the child and family with FA.

Genomic instability and cancer predisposition in Fanconi anemia

As noted above, the cellular phenotype of FA includes hypersensitivity to DNA cross-linking agents, oxidative damage and other DNA-damaging agents. The cellular responses to these injurious agents include exaggerated G2 cell cycle arrest, a large increase in chromosomal abnormalities such as chromatid and interchanges breaks, enhanced susceptibility to pro-inflammatory cytokines, defective p53 induction and increased apoptosis. The increased chromosomal breakage in response to diepoxybutane (DEB) or mitomycin-C (MMC) is currently the biochemical test used for the diagnosis of FA. Although not formally linked, these cellular phenotypes are thought to be involved in the progressive stem cell loss accompanying pancytopenia, the increased sensitivity to radiation and chemotherapy seen in patients treated for cancer or in preparation of stem cell transplantation and the increased susceptibility of FA patients to leukemia and cancer. In a large, 20-year follow-up of over 600 patients, Kutler et al. reported a leukemia risk of ~33% by the age of 40 and a similar risk of squamous cell carcinoma of the head and neck or ano-genital region. Other leukemias and cancers have been reported in smaller series, including hepatocellular carcinoma, neuroblastoma, Wilms’ tumor and brain tumors. One particularly notable phenotype is the association of early acute myeloid leukemia with FANCD1/BRCA2 mutations. FA patients with biallelic mutations in FANCD1/BRCA2 are at a higher risk of developing early onset acute leukemia. In these patients, leukemia has been diagnosed at a median of 2.2 years of age in contrast to a median onset of 13.4 years in all other FA patients. These children also have a high risk of brain tumors. The majority of solid tumors reported in FA occur in young adulthood.

Molecular pathophysiology of Fanconi anemia

An increasing number of genes involved in FA have been identified and cloned and disease-causing mutations have been catalogued. To date, mutations in any one of 11 genes can cause the FA disease phenotype. All but FANCB are located on autosomal chromosomes. Approximately 65% of patients with FA are in complementation group A, 15% in complementation group C and 10% in complementation group G. However, there are still cell lines derived from FA patients which do not carry mutations in any of the known FA genes or do not fall into the known complementation groups. These indicate that additional FA-associated genes are yet to be identified. Transgenic expression of these FA genes in the respective FA cells in vitro corrects the increased chromosomal breakage caused by DEB and the increased sensitivity to MMC. In addition, expression of these genes in bone marrow progenitors from patients with FA increases hematopoietic cell survival in in vitro assays.

In spite of the fact that the first FA gene was identified and cloned by Buchwald and colleagues over a decade ago, the pathophysiology of FA and the function of FA genes remains unknown (for a recent
motifs and therefore may provide the first solid link between the FA core complex and DNA damage repair by nuclear localization and the mono-ubiquitination of FANCD2. Once monoubiquitinated, FANCD2 associates with BRCA2, Rad51 and other proteins that have been implicated in DNA repair in DNA damage foci in the nucleus. However, the monoubiquitination of FANCD2 occurs by yet unknown biochemical mechanisms and the link between the FA core complex and DNA damage sensing and DNA repair remains unclear. The most recently cloned FA genes, FANCI and FANCM, encode proteins that appear to have DNA interacting motifs and therefore may provide the first solid link between FA proteins and DNA repair. FANCI was identified as a previously studied protein called BRIP1 (or BACH1) which possesses DNA helicase activity. FANCM was identified as a large protein with both helicase and endonuclease domains. While the C-terminal endonuclease domain of FANCM has diverged from orthologs to the point of potential non-function, the helicase domain is highly conserved compared to the yeast MPH1 helicase and archaea Hef protein. It remains to be determined how these new FA proteins with DNA-interacting domains participate in the process of DNA repair.

New therapies in Fanconi anemia

The isolation and characterization of the cDNA of the FA complementation groups has enabled attempts to correct the genetic defect in the bone marrow of affected individuals (for a more detailed review, see ref. 17). In vitro expression of these cDNA in cells from patients with FA corrects each of the defects that can be measured – chromosomal breakage, MMC sensitivity in vitro and bone marrow progenitor cell growth.19 In addition, gene transfer studies have successfully shown that correction of defects in mice with gene-targeted deficiency of Fanca or Fancc is feasible.14 Since the genetic defects that cause the Fanconi phenotype appear to affect hematopoietic stem cells or cells with long-term multilineage potential, it is possible that even correction of a limited number of these cells, if accompanied by a selective growth advantage over uncorrected cells could result in oligoclonal repopulation of the bone marrow compartment by gene-corrected cells. In this regard, rare FA patients have been diagnosed with a FA germline defect (disease-associated mutation in an FA gene with chromosomal fragility in fibroblasts) but normal hematopoiesis and normal blood/marrow MMC/DEB tests. This clinical phenotype is hypothesized to be due to spontaneous gene correction in the hematopoietic cell compartment, suggesting selection and expansion of gene-corrected stem cell clones could be very feasible.21,22 However, in one report23 genetic reversion was not accompanied by a return of blood counts to normal values, calling into question a proliferative advantage for the reverted cells. Thus, a key scientific question to be answered in the human setting is whether FA stem cells reinfused after gene correction using current gene transfer methods do, in fact, have a proliferative advantage in vitro.

In addition to the usual difficulties of gene transfer into human hematopoietic stem cells, FA presents disease-specific challenges which make it a particularly difficult disease to approach with this technology. Perhaps the most significant of these challenges is that by the time gene correction is considered, the number of target stem cells is critically depleted due to disease progression. Indeed, we and others have found significant depletion of CD34 cells in the bone marrow of very young patients even prior to the onset of severe peripheral cytopenias (J. Beuren, personal communication; P. Kelly and DAW, unpublished data). These findings imply that hematopoietic stem cells must be collected very early in the disease process before significant hematologic problems have arisen. Even when adequate numbers of CD34 cells can be harvested, these cells appear prone to excessive apoptosis during the in vitro manipulations required for gene transfer.

At least in one animal model, the Fancc knockout mouse, in vitro manipulation of hematopoietic stem cells for an extended period of time is associated with development of genomic instability and leukemia in uncorrected cells reinfused into lethally-irradiated recipients.24 The relevance of this observation to human FA stem cell manipulation is unknown. However, given the natural history of FA, which includes a significant risk of developing de novo leukemia, a clinical hematopoietic stem cells gene transfer trial in FA will have some likelihood of encountering hematologic malignancy. With this in mind, a FA gene therapy trial will require very extensive safety monitoring in order to identify gene therapy-related leukemogenesis that is distinct from the natural history of the disease.

Finally, as it relates to the use of mild marrow cytoreduction successfully employed in European trials for therapy of adenosine deaminase severe combined immunodeficiency disease (SCID), X-linked SCID and chronic granulomatous disease, the use of alkylating agents in FA patients, even at low doses, may be difficult given the hypersensitivity to DNA-damaging agents and the possibility of deficiencies in DNA repair pathways that are part of the FA disease phenotype. Thus, if transfer and expression of the
appropriate FA cDNA into hematopoietic stem cells does not provide a selective \emph{in vivo} advantage when reinfused into the recipient, alternative methods of \emph{in vivo} selection may be necessary.

Given these challenges of gene transfer in FA, numerous groups are in the process of developing new approaches to the treatment of FA with gene therapy. These include the use of newer cytokine combinations, reducing \emph{ex vivo} manipulation to a minimum while maintaining gene transfer efficiency with higher titer vector preparations or different vector backbones, development of lentivirus vectors that may allow transduction of quiescent hematopoietic stem cells with little \emph{ex vivo} culture and minimal cytokine exposure and the use of non-integrating vectors or protein transfer methods to provide complementation immediately after removal of cells from the bone marrow microenvironment. Indeed, significant recent progress has been reported in the development of lentivirus vectors for use in other hematologic diseases. However, it is very clear that early collection of cells to assure reasonable numbers of CD34+ target cells will be critical for any future success of gene therapy applications. The use of human embryonic stem cells to derive CD34 blood precursors may, in the future, be a strategy for generating large numbers of clonally-derived, gene-corrected that are molecularly characterized cells for therapeutic use in this disease. This strategy could, theoretically, provide solutions to many of the above mentioned problematic issues.

**Future prospects**

While the potential therapeutic application of the increasing molecular understanding of FA proteins is not yet clear, it is likely that full knowledge of the structure and function of these proteins will provide a wealth of new targets for the development of small molecules and other molecular interventions. Although FA is currently an orphan disease, it is likely that the importance of this pathway in cancer will lead to the involvement of pharmaceutical companies as well as academic institutions to further these approaches.

**Acknowledgments**

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**References**

Dyskeratosis congenita (DC) is an inherited bone marrow failure syndrome exhibiting considerable clinical and genetic heterogeneity. X-linked recessive, autosomal dominant and autosomal recessive subtypes are recognized. The gene mutated in X-linked DC (DKC1) encodes a highly conserved nucleolar protein called dyskerin. Dyskerin associates with the H/ACA class of small nucleolar RNA in small nucleolar ribonucleoprotein particles that are predicted to be important in guiding the conversion of uracil to pseudouracil during the maturation of ribosomal RNA. Dyskerin also associates with the RNA component of telomerase (TERC), which is important in the maintenance of telomeres. Mutations in TERC have been identified in patients with autosomal dominant DC and in a subset of patients with aplastic anemia and myelodysplasia. Recently heterozygous mutations of telomerase reverse transcriptase (TERT) were found in some patients with autosomal dominant DC and aplastic anemia. Additionally, patients with a severe multi-system disorder, Hoyeraal-Hreidarsson syndrome have been found to have DKC1 mutations. Collectively, these findings have demonstrated that classical DC, the Hoyeraal-Hreidarsson syndrome, and a subset of aplastic anemia are due to a primary defect in telomerase. The multi-system abnormalities seen in these patients, including the increased incidence of malignancy, have highlighted the critical role of telomeres and telomerase in humans. From a clinical perspective the link between DC and aplastic anemia and in turn to defective telomerase suggests that treatments directed at correcting telomerase activity might benefit patients with DC/aplastic anemia who do not respond to conventional therapy. They also demonstrate that telomerase deficiency due to constitutional mutations in DKC1, TERC and TERT leads to telomere shortening, chromosome instability and an increased risk of cancer.

Introduction

During the last decade there have been significant advances in our understanding of dyskeratosis congenita (DC), a disease that was first described 100 years ago. These advances have demonstrated that DC is principally a disease of defective telomere maintenance and that its atypical/cryptic manifestations include clinical presentation as aplastic anemia and myelodysplasia. These new observations have implications for the management of patients with DC and related disorders. They also provide new opportunities for exploring the role of telomeres and telomerase in more common processes such as aging and cancer.

Classical dyskeratosis congenita

Clinical aspects

Classical dyskeratosis congenita (DC, also known as Zinsser-Engman-Cole syndrome) is a bone marrow failure syndrome characterized by the mucocutaneous triad of abnormal skin pigmentation, nail dystrophy and mucosal leukoplakia. A variety of other abnormalities (dental, gastrointestinal, genitourinary, hair graying/loss, immunological, neurological, ophthalmic, pulmonary and skeletal) have also been observed (Table 1). Bone marrow failure develops in many patients and there is an increased predisposition to malignancy and fatal pulmonary complications.

Clinical features of DC often appear during childhood although there is a wide age range. The mucocutaneous abnormalities appear first, usually by the age of 10 years. Bone marrow failure frequently develops before the age of 20 years; 80-90% of patients will have developed bone marrow abnormalities by the age of 30 years. In some patients the bone marrow abnormalities may appear before the mucocutaneous manifestations (occasionally in the first year of life) and can lead to an initial diagnosis of idiopathic aplastic anemia. There is considerable clinical variabili-
Table 1. Abnormalities/complications in patients with classical DC.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>% of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classical features</strong></td>
<td></td>
</tr>
<tr>
<td>Abnormal skin pigmentation</td>
<td>89</td>
</tr>
<tr>
<td>Nail dystrophy</td>
<td>88</td>
</tr>
<tr>
<td>Bone marrow failure</td>
<td>85.5</td>
</tr>
<tr>
<td>Leucoplakia</td>
<td>78</td>
</tr>
<tr>
<td><strong>Other features</strong></td>
<td></td>
</tr>
<tr>
<td>Epiphora</td>
<td>30.5</td>
</tr>
<tr>
<td>Learning difficulties/developmental delay/mental retardation</td>
<td>25.4</td>
</tr>
<tr>
<td>Pulmonary disease</td>
<td>20.3</td>
</tr>
<tr>
<td>Short stature</td>
<td>19.5</td>
</tr>
<tr>
<td>Extensive dental caries/loss</td>
<td>16.9</td>
</tr>
<tr>
<td>Esophageal stricture</td>
<td>16.9</td>
</tr>
<tr>
<td>Premature hair loss/graying/sparse eyelashes</td>
<td>16.1</td>
</tr>
<tr>
<td>Hyperhidrosis</td>
<td>15.3</td>
</tr>
<tr>
<td>Malignancy</td>
<td>9.8</td>
</tr>
<tr>
<td>Intrauterine growth retardation</td>
<td>7.6</td>
</tr>
<tr>
<td>Liver disease/peptic ulceration/enteropathy</td>
<td>7.3</td>
</tr>
<tr>
<td>Ataxia/cerebellar hypoplasia</td>
<td>6.8</td>
</tr>
<tr>
<td>Hypogonadism/undescended testes</td>
<td>5.9</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>5.9</td>
</tr>
<tr>
<td>Urethral stricture/phimosis</td>
<td>5.1</td>
</tr>
<tr>
<td>Osteoporosis/aseptic necrosis/scoliosis</td>
<td>5.1</td>
</tr>
<tr>
<td>Deafness</td>
<td>0.8</td>
</tr>
</tbody>
</table>

NB. As patients identified as having DC on the basis of mutation analysis are included, these percentages will vary.


diversity between patients, sometimes even within the same family. A given patient may have diverse combinations of the abnormalities listed in Table 1. Although it is difficult to make generalizations, the X-linked recessive form appears to be associated with a more severe phenotype than the autosomal dominant form. The autosomal recessive families also show considerable heterogeneity, with some patients having severe bone marrow failure by the age of 10 years and others having no hematologic abnormalities even by the age of 40 years. The main causes of death are bone marrow failure/immunodeficiency (~60-70%), pulmonary complications (~10-15%) and malignancy (~5-10%). Malignancies usually develop in the third decade and include carcinomas (bronchus, colon, larynx, esophagus, pancreas, skin, and tongue), leukemias and lymphomas.

Bone marrow failure/immunodeficiency is the principal cause of premature mortality in DC patients. Oxymetholone can produce an improvement in hematopoietic function in some patients for a variable period of time. The curative treatment for severe bone marrow failure is allogeneic hematopoietic stem cell transplantation. Unfortunately because of pulmonary/vascular complications, the results of allogeneic stem cell transplantation have been less successful. The presence of pulmonary disease in a significant proportion of DC patients explains the high incidence of fatal pulmonary complications in the setting of stem cell transplantation. These observations of high transplant-related toxicity in DC patients highlights that many tissues (such as the gastrointestinal tract, liver, lungs and skin) have difficulties in repair following chemical or radiation stress.

**Genetics and link to telomerase**

DC is genetically heterogeneous. X-linked recessive, autosomal dominant, and autosomal recessive subtypes are recognized.

**X-linked DC**

**DKC1** and dyskerin. Positional cloning resulted in identification of the **DKC1** gene that is mutated in X-linked DC. In X-linked DC patients mutations are spread throughout the **DKC1** gene (Figure 1), which encodes a 514 amino acid protein, referred to as dyskerin. Dyskerin is a highly conserved nucleolar protein with many homologs in other species including yeast (Cbf5p), rat (NAP57), drosophila (mfl) and mouse (**Dkc1**). **Dkc1** has a number of domains including a TruB domain at amino acid 107-247 (named after an *E. coli* pseudouridine synthase), a PUA domain at amino acid 296-371 (pseudouridine synthase and archaeosine-specific transglycosylases) and two nuclear localization signals at amino acid 11-20 and aa446-458 (Figure 1). The TruB domain is the catalytic domain of pseudouridine synthases, which is shared with bacterial TruB proteins, yeast Pus4p and the Cbf5p family.

The PUA domain is a putative RNA binding domain. Dyskerin interacts with three proteins (GAR1, NHP2 and NOP10) to form the core of the H/ACA ribonucleoprotein particles. In yeast, the dyskerin homolog Cbf5p interacts with similar nucleolar proteins (GAR1, Nhp2, and Nop10) and these associate with the H/ACA small nucleolar RNA to form small nucleolar ribonucleoprotein particles. H/ACA small nucleolar RNA are characterized by having a hairpin-hinge-hairpin-ACA (H/ACA) motif. As a part of these H/ACA ribonucleoprotein particles, the yeast Cbf5p has been shown to be a pseudouridine synthase guiding the conversion of uridine to pseudouridine at specific sites of ribosomal RNA (pseudouridylation). Yeast Cbf5 mutants demonstrate a pronounced defect in ribosomal RNA biosynthesis and are deficient in cytoplasmic ribosomal subunits. As dyskerin also has a TruB domain, dyskerin has been predicted to have pseudouridylation activity like its homologs. This is an essential step for ribosome biogenesis and therefore it has been suggested that part of the pathology in X-linked DC probably relates to defective ribosome biogenesis. Recent studies on the mouse dyskerin (**Dkc1**) gene provide additional support for this. Studies in human cells to date (see below) have not substantiated this role for dyskerin but further studies in human cells might be useful to clarify this issue.
Several DKC1 mutations have been identified and these are shown in Figure 1. The majority of mutations in DKC1 cause single amino acid substitutions, one of which (A353V) accounts for approximately 40% of cases of X-linked DC cases. The phenotype in these patients with the same dyskerin mutation can vary considerably and suggests other genetic/environmental factors influence the DC phenotype. Although the mutations are spread throughout the DKC1 gene there are two prominent clusters involving amino acids 31-72 and 314-420, encoded in exons 3-6 and exons 9-12. It is noteworthy that these two clusters of mutations lie outside the predicted TruB domain. Presently there are few data regarding dyskerin and other protein changes in X-linked DC. Interestingly modeling on the crystal structure of the archaeal Cbf5-Nop10-Gar1 complex suggests that most of the mutations found in X-linked DC are clustered on one side of the PUA domain of dyskerin which is predicted to play a specific role in binding H/ACA and telomerase RNAs.

**Link to telomerase**

It has been shown that dyskerin and the other three proteins (GAR1, NHP2 and NPO10) that form the core of the ribonucleoprotein particles also associate with the RNA component of telomerase (TERC) which too contains an H/ACA motif. Together with telomerase reverse transcriptase (TERT), TERC forms the core of the active telomerase complex that is important in the maintenance of the 6bp repeat sequences (telomeres) that cap the ends of chromosomes. Mitchell et al. found that in one fibroblast and four lymphoblast cell lines from patients with X-linked DC the level of TERC was reduced while no significant defect in ribosomal RNA processing or site-specific pseudouridylation was detected. It now appears that telomerase RNA processing and stable RNA accumulation require dyskerin binding to the H/ACA motif of TERC and dyskerin mutations therefore lead to reduction in TERC. Furthermore, telomere lengths in the lymphoblast lines were shorter than expected for age-matched normal individuals. Telomerase activity, induced by overexpression of TERT in the X-linked DC fibroblast cell line, was shown to be reduced compared to similarly treated lines from DC carriers. It has also been shown that telomeres are shorter in blood cells from patients with autosomal forms of DC. The combination of these findings suggested that DC might be principally a disease of telomere maintenance. Further clarification has come from the...
elucidation of the genetic basis of autosomal dominant DC (see below).

**Autosomal dominant DC: TERC and DC pathology.** Linkage analysis in a large DC family showed that the gene for autosomal dominant DC is on chromosome 3q, in the same interval where the gene for TERC had been previously mapped. This led to TERC mutation analysis in this and other DC families and the demonstration that autosomal dominant DC is due to mutations in the TERC gene. TERC is a 451 nucleotide RNA. Figure 2 shows the position of these mutations on a schematic representation of TERC. TERC consists of four structural domains: the pseudoknot domain, CR4-CR5 domain, the H/ACA domain and the CR7 domain. Structure-function analysis reveals that the pseudoknot and CR4-CR5 domains (together with TERT) are required for the catalytic function while the H/ACA and CR7 domains are necessary for TERC RNA accumulation.

Collectively, these studies have demonstrated that heterozygous TERC mutations in autosomal dominant DC patients result in reduced telomerase activity either through impaired RNA accumulation/stability or a catalytic defect. Experiments reconstituting telomerase with both normal and mutant TERC molecules showed no evidence for a dominant negative effect. Instead the data suggests that the TERC mutations act via haplo-insufficiency. Additionally, it has been established for some of these mutations (e.g. 72C->G), which are located within the conserved stem cell structure that it is their effect on the secondary structure, rather than the primary sequence which determines the functional consequences.

Since the DKC1-encoded protein dyskerin and TERC are both components of the telomerase complex and all DC patients have very short telomeres it is currently believed that DC arises principally from an abnormality in telomerase activity. This telomerase deficiency results in accelerated telomere shortening in DC cells and is associated with increased loss of cells particularly from tissues which need constant renewal, such as the hematopoietic and dermatological systems that bear the brunt of DC. Evidence for a hematopoietic stem/progenitor cell defect in DC has been established in several studies and is there-
fore consistent with a basic defect in telomerase.

The effect of mutation of one allele of human telomerase RNA in families with autosomal dominant DC can be compared with the effect on laboratory mice of knocking out both alleles of the gene coding for telomerase RNA (Terc). Such mice showed no significant abnormalities in early generations but later generations showed progressive telomere shortening and a variety of defects including reduced proliferative capacity of hematopoietic cells.15,16 Its noteworthy, targeted disruption of Dkc1 causes embryonic death in mice and X-linked DC in humans generally tend to be more severe than autosomal dominant DC, particularly with regard to the mucocutaneous features. This suggests that dyskerin mutations in humans, as well as producing a major defect in telomerase might also have other functional consequences.

It is noteworthy that in families with autosomal dominant DC, there is an earlier age of onset and a greater number of disease features in succeeding generations. This increase in severity of the disease (anticipation) in successive generations has been associated with progressive telomere shortening17 as is the case in the Terc-/- mice. DC patients and Terc-/- mice also have an increased incidence of cancer. It is likely that in both cases the increased incidence of malignancy is caused by chromosome instability, which in turn is a result of critically, shortened telomeres. Indeed end-to-end chromosome fusions have been observed in both Terc-/- mice16 and in DC patients.1

Autosomal dominant DC due to heterozygous mutations in TERT. Recently heterozygous TERT mutations have been identified in a small number of patients with autosomal dominant DC18,19 (Figure 3). These observations further strengthen the model that DC is a disorder of telomere maintenance.

Autosomal recessive DC. The genetic basis of the autosomal recessive form(s) of DC is presently unknown. Since dyskerin, TERC and TERT are key components of the telomerase complex other molecules associated with this complex or involved in ribosomal RNA biosynthesis would seem obvious candidates. Further studies are necessary to determine the genetic basis of autosomal recessive DC.

Implications for DC patients. The demonstration of DKC1, TERC and TERT mutations in DC families provides an accurate diagnostic test in approximately 40% of cases. It also provides the basis for designing the much needed new treatments. Since in any given patient DC is a single gene disorder and the cells that need to be targeted (hematopoietic stem cells) are accessible, DC is a good candidate for hematopoietic gene therapy. Furthermore there is evidence from fibroblast culture studies and from the skewed patterns of X-chromosome inactivation seen in carriers of X-linked DC1 that cells transfected with the normal gene would have a survival advantage compared to the uncorrected cells. Such an advantage would also be predicted from the role of dyskerin, TERC and TERT in telomere maintenance.
Other blood disorders that have been linked to DC and telomerase deficiency

Hoyeraal-Hreidarsson syndrome

Analysis of patients on the DC registry has identified considerable clinical heterogeneity. It has also revealed that some DC patients have features that overlap with those observed in patients with the Hoyeraal-Hreidarsson syndrome. This is a severe multi-system disorder that can present in the neonatal period and infancy. It is characterized by severe growth retardation, bone marrow failure, immunodeficiency and neurological abnormalities. The overlap of these features of Hoyeraal-Hreidarsson syndrome with those of some DC patients led to analysis of the DKC1 gene in Hoyeraal-Hreidarsson patients. These studies demonstrated that some male Hoyeraal-Hreidarsson cases have a severe variant of DC in which death from bone marrow failure/immunodeficiency occurs before the appearance of the diagnostic features of DC. The studies also highlighted the immunological defects that can be seen in DC, ranging from the severe T+B+NK- immunodeficiency in some patients to the more variable immunological abnormalities observed in others. Several mutations in dyskerin have now been identified in Hoyeraal-Hreidarsson patients (Figure 1).

Female cases of Hoyeraal-Hreidarsson syndrome are also recognized and it is likely that they represent a severe variant of the autosomal recessive form (s) of DC, the genetic basis of which presently remains unknown.

Aplastic anemia and myelodysplasia

In some of the families in the DC registry, affected members have died of severe aplastic anemia before the age of 10 years and a diagnosis of DC was made subsequently, only when other members of the family survived long enough to develop the classical mucocutaneous features. If it were not for the presence of subsequent members, these patients would have been characterized as having idiopathic aplastic anemia. The primary defect in idiopathic aplastic anemia is believed to be at the stem cell level but its precise cause is not known. Like patients with DC, patients with idiopathic aplastic anemia also have short telomeres compared to age-matched controls. These observations led us to analyze the DKC1 and TERC genes in patients with idiopathic aplastic anemia. Although the DKC1 gene screen was found to be normal, mutations in TERC have been found in some cases of aplastic anemia (including paroxysmal nocturnal hemoglobinuria) and myelodysplasia. It is noteworthy that patients with myelodysplasia also have short telomeres. Patients with myelodysplasia, like patients with aplastic anemia, are believed to have a defect at the level of the stem cell but the primary pathology again remains unknown in the majority of cases. Patients with TERC mutations who present predominantly with features of aplastic anemia or myelodysplasia can be regarded as having atypical/cryptic DC. It is possible that TERC mutations in this group of patients are less severe and have their main clinical impact only on the most proliferative tissue in the body, the hematopoietic tissue. An alternative possibility is that in these patients there may be an interaction with an environmental insult such as infection, which results in maximal cumulative damage to the hematopoietic tissue.

These findings, together with the recent identification of heterozygous TERT mutations (Figure 5) in some cases of aplastic anemia show that in a subset of patients with aplastic anemia/myelodysplasia the primary defect is in the maintenance of telomeres. This has implications for the management of patients in whom conventional therapies fail. They also highlight the clinical and genetic heterogeneity of DC and a possible rationale for screening the DC genes in uncharacterized patients (e.g. patients with unexplained pulmonary or liver disease) who have clinical features that overlap with those of DC.
Conclusions and future perspectives

The study of DC has demonstrated that constitutional mutations in genes (DKC1, TERC and TERT) encoding three key components of telomerase are associated with telomerase deficiency and dramatically compromise the proliferative resumption of hematopoietic and epithelial tissue resulting in multi-system abnormalities. It has thus highlighted the critical role of telomerase in human growth and development. From the perspective of bone marrow failure syndromes these studies have shown that in some cases of aplastic anemia and myelodysplasia the primary defect relates to telomere maintenance. This has implications for the diagnosis of patients with atypical features and for the development of future therapies for those in whom conventional treatments fail.

The precise contribution of defects in pseudouridylation in the pathogenesis of X-linked DC remains unclear. Further studies are necessary on human cells to clarify this. A subset of patients with DC presently remains uncharacterized. The elucidation of the genetic basis of the disease in these patients may help to clarify the relative importance of telomere maintenance versus ribosomal biogenesis in the pathogenesis of X-linked DC. In turn these studies may identify new molecules with a role in the maintenance of telomeres and/or ribosomal biogenesis.

As the phenotype of DC includes an increased occurrence of malignancy, these diseases have highlighted that telomerase deficiency is a risk factor for the development of cancer. Further studies that result in improving our understanding of DC might have important implications for our understanding and management of cancer in general.

Strategies aimed at correcting the molecular defect in DC could pave the way for developing the much-needed new therapies for this group of patients. From the perspective of telomere biology such an approach represents on exciting opportunity to determine whether telomere rejuvenation is possible in human cells. If successful this could have implications for the treatment of pathologies outside the field of DC.

Acknowledgments

I would like to thank my current (Richard Beswick, Michael Kirwan, Anna Marrone, Amanda Wulfe and Tom Vulliamy) and past colleagues (Stuart Knight, Philip Mason, and David Stevens) whose contribution has been critical over the years to DC research. I am also grateful to the DC families and all our colleagues (doctors and nurses) for their support in establishing the Dyskeratosis Congenita Registry and to the MRC and Wellcome Trust for financial support.

References

Hepcidin is the long-anticipated hormone responsible for the regulation of iron recycling and body iron balance. Hepcidin acts by blocking the influx of iron into plasma. This is achieved by hepcidin binding to and inducing the degradation of the cellular iron exporter, ferroportin, found in sites of major iron flows: duodenal enterocytes involved in iron absorption, macrophages that recycle iron from senescent erythrocytes, and hepatocytes that store iron. Hepcidin synthesis is in turn controlled by iron concentration, hypoxia, anemia and inflammatory cytokines. Dysregulation of hepcidin is involved in the pathogenesis of a spectrum of iron disorders. Deficiency of hepcidin is the unifying cause of hereditary hemochromatosis, and excessive cytokine-stimulated hepcidin production causes hypoferremia and contributes to anemia of inflammation. The development of pharmacological hepcidin agonists and antagonists should be useful in the treatment of these conditions.

Systemic iron homeostasis
Iron is an essential element which functions as a component of proteins and enzymes involved in oxygen transport and storage (hemoglobin, myoglobin), electron transport and energy metabolism (cytochromes, NADH dehydrogenase, succinate dehydrogenase), DNA synthesis (ribonucleotide reductase) and protection against oxygen radicals (catalase and peroxidases). However, excess free iron promotes the generation of highly reactive oxygen radicals which can damage lipid membranes, proteins and nucleic acids, and result in organ dysfunction. Normal iron homeostasis ensures that cellular iron needs are met without excessive iron accumulation.

An adult human body contains approximately 3-4 g of iron, with more than two-thirds incorporated in the hemoglobin of erythrocytes and erythroid precursors. The amount of iron entering and exiting the body each day is comparatively small, only 1-2 mg. Nonetheless, the iron absorption, which takes place in the duodenum, is tightly controlled and relevant in the long term. Inadequate dietary iron supply eventually leads to iron deficiency anemia, whereas genetic lesions that increase iron absorption result in iron overload disorders. In contrast to iron absorption, the excretion of iron appears to be unregulated and occurs through sloughing of mucosa and skin, and bleeding.

Daily iron requirements (about 20 mg, mostly for the production of hemoglobin for new erythrocytes) far surpass dietary iron supply, and are derived from the recycling of iron from senescent erythrocytes. Iron recycling takes place in splenic and other reticuloendothelial macrophages which phagocytose old and damaged red blood cells, recover iron from heme and export it back to plasma.

About a quarter of the total body iron is stored in hepatocytes and in the macrophages of the spleen, liver and bone marrow. The stores can be mobilized during periods of negative iron balance, such as during decreased dietary iron uptake or excessive blood loss. However, when the stores are depleted due to accumulated iron deficit, erythropoiesis becomes iron-restricted.

Complex homeostatic and transport mechanisms have evolved to maintain the dynamic balance between iron utilization (mainly by the bone marrow) and iron supply (absorption, recycling and mobilization from stores), and this balance is reflected in the relatively narrow range of extracellular iron concentration (10-30 µM in humans).

Molecular mechanisms of iron transport
From acquisition to utilization, iron is transported in and out of multiple cell types with the aid of specialized proteins. Several pathways for cellular iron import have been described. In the duodenum, non-heme dietary iron is reduced from...
Fe³⁺ to Fe²⁺ by a ferric reductase and transported across the membrane by divalent metal transporter-1 (DMT1). Heme iron is also absorbed and one candidate transporter has recently been described (heme carrier protein 1, HCP1). Inside the cell, the heme is most likely broken down by a heme oxygenase and free iron exported out of the cell together with non-heme iron.

Most cells, including erythroid precursors, import iron from plasma and extracellular fluid, where iron circulates bound to transferrin (Tf). Transferrin-bound iron is internalized through transferrin receptor 1 (TfR1), and is subsequently transported across the endosomal membrane into the cytoplasm by DMT1. Certain cell types can also import iron in the form of non-transferrin-bound iron, ferritin, hemoglobin/haptoglobin and heme/hemopexin complexes, but these mechanisms are more prominent in pathological conditions.

Finally, reticuloendothelial macrophages import iron indirectly through phagocytosis of old erythrocytes which are then lysed and iron is extracted from heme by heme oxygenase.

In contrast with multiple iron uptake pathways, a single iron export mechanism dependent on the membrane protein ferroportin is used by all cell types that export iron into plasma. Ferroportin is expressed in duodenal enterocytes that absorb iron, macrophages that recycle iron and hepatocytes that store iron. Ferroportin is also expressed in the placenta, where it participates in transfer of iron from mother to fetus, and total ferroportin deficiency in mice causes embryonic death. When ferroportin expression is preserved in the placenta to allow prenatal development but is inactivated in all other tissues, mice became severely iron-deficient after birth due to iron trapping in enterocytes, hepatocytes and macrophages, confirming the non-redundant function of ferroportin in cellular iron export.

In addition to ferroportin, iron export into plasma and subsequent loading onto transferrin also requires the presence of multicopper oxidases which convert Fe²⁺ to Fe³⁺. Ceruloplasmin is the ferroxidase involved in iron export from hepatocytes and macrophages, and its homolog hephaestin has a similar role in intestinal cells.

Hepcidin is the principal regulator of extracellular iron concentration

Hepcidin, a recently discovered peptide hormone, is the key regulator of systemic iron homeostasis. Hepcidin is produced in the liver, circulates in plasma and is excreted in urine. It is synthesized as prohepcidin, which undergoes furin cleavage to generate the mature hepcidin. The bioactive form is the 25 amino acid peptide stabilized by four disulfide bonds (Figure 1A) and is highly conserved across vertebrate species (Figure 1B).

The studies on mouse models and humans indicate that hepcidin is the negative regulator of iron absorption, recycling and release from stores. The first clue came from mice with incidental hepcidin deficiency due to the disruption of a neighboring gene. The hepcidin-deficient mice developed iron overload similar to that occurring in human hereditary hemochromatosis. Conversely, overexpression of hepcidin in transgenic mice resulted in severe iron-deficiency anemia. In humans, homozygous disruption of the hepcidin gene caused the most severe form of iron overload, juvenile hemochromatosis, whereas overproduction of hepcidin by liver tumors in patients with type 1a glycogen storage disease caused iron-refractory anemia which resolved only after the resection of the tumor, or after liver transplantation.

Mechanism of hepcidin action

Hepcidin acts by blocking cellular iron efflux into plasma from macrophages recycling iron, from stores in the liver and from absorptive enterocytes. Iron uptake by erythrocyte precursors uses up the limited plasma iron pool, rapidly causing hypoferremia. On the molecular level, cellular iron efflux is inhibited by hepcidin binding to the cellular iron exporter ferroportin, causing the internalization of ferroportin and
its degradation in lysosomes (Figure 2).\textsuperscript{8} Injection of a single dose of synthetic hepcidin in mice led to a dramatic drop in serum iron already within 1 hour,\textsuperscript{9} and a similar effect was seen with acute induction of hepcidin expression in tetracycline-inducible transgenic mice.\textsuperscript{10}

The hepcidin-ferroportin interaction maintains normal extracellular iron concentrations and in turn, the production of hepcidin is homeostatically increased by iron loading and decreased by anemia and hypoxia. As an illustration, when hepcidin concentration increases in plasma as a result of dietary iron intake, hepcidin causes increased internalization of ferroportin from the cell membrane and its subsequent degradation, resulting in inhibition of the iron efflux from the ferroportin-rich tissues into plasma. With decreased supply of iron into plasma but continued utilization of iron (mostly for erythropoiesis), the plasma iron concentrations are restored to normal. Conversely, in iron deficiency, hepcidin production decreases, resulting in greater concentrations of ferroportin molecules on the cell membranes and increased export of iron into plasma.

**Regulation of hepcidin by iron and the pathogenesis of hereditary hemochromatosis**

Hepcidin is produced rapidly in response to dietary iron intake: ingestion of a single dose of 65 mg of iron increased urinary hepcidin in human volunteers within several hours.\textsuperscript{3} Chronic dietary or parenteral iron loading also induced hepcidin mRNA in mice.\textsuperscript{11} The mechanism of hepcidin regulation by iron is still unknown. Isolated hepatocytes fail to upregulate hepcidin in response to iron,\textsuperscript{12} suggesting that the iron sensor may be distant from the liver or that the iron sensing/signaling complex is disrupted by hepatocyte isolation.

Some clues about molecules involved in the pathway of hepcidin regulation by iron come from mutations causing hereditary hemochromatosis in humans and animal models. Hereditary hemochromatosis due to homozygous disruption of HFE, transferrin receptor 2 (TfR2) and hemojuvelin (HJV), is characterized by hepcidin deficiency in spite of massive iron overload indicating that these molecules act as direct or indirect regulators of hepcidin synthesis. Of the three, HJV appears to be the key regulator of hepcidin as disruption of hemojuvelin results in juvenile hemochromatosis, which is phenotypically undistinguishable from disease caused by homozygous disruption of the hepcidin gene itself.\textsuperscript{13} Indeed, patients with HJV hemochromatosis had undetectable levels of urinary hepcidin.\textsuperscript{13}

Humans and mice with homozygous HFE or TfR2 disruption have milder forms of hemochromatosis and their hepcidin urinary or mRNA levels, although inappropriately low for the degree of iron loading, are not as severely decreased as with HJV mutations.\textsuperscript{3} The severity of the hemochromatosis phenotype therefore appears to correlate with the degree of hepcidin deficiency, corresponding to the loss of hepcidin responsiveness to iron loading. Lower hepcidin levels result in greater intestinal absorption and eventually lead to iron deposition in organs and their dysfunction (Figure 3A).

It is not known how HFE and TfR2 are involved in hepcidin regulation by iron. Since HFE disruption causes a much less penetrant phenotype, HFE is likely a modulator of signaling from the iron sensor but is not essential for the function of this pathway. Disruption of TfR2 causes a somewhat more severe phenotype, making the protein a strong candidate for being an iron sensor because it is predominantly expressed in the liver and its levels are regulated by transferrin saturation.\textsuperscript{14}

The exact function of hemojuvelin is still unknown. It belongs to the family of repulsive guidance molecules (RGM) which are involved in neuronal differentiation, migration, and apoptosis. Unlike other RGM, HJV is predominantly expressed in skeletal muscle, the liver and the heart.\textsuperscript{15} HJV is a GPI-linked protein but is cleaved to produce a soluble form,\textsuperscript{15,16} and this step is inhibited by increasing iron
concentrations, suggesting that HJV could be a part of the iron-sensing complex. The two forms of hemojuvelin have opposite effects on hepcidin mRNA expression in vitro: the addition of soluble hemojuvelin suppresses hepcidin mRNA expression but the membrane-bound form increases it.16 Considering that other molecules of the RGM family function as receptor ligands, it is likely that the mechanism of hemojuvelin action involves competition of the membrane-bound and soluble forms for binding to a transmembrane receptor, where only the interaction of the GPI-linked form with receptor initiates the signaling cascade regulating hepcidin expression.

Regulation of hepcidin by anemia and hypoxia; implications for iron-loading anemias

Hepcidin production is suppressed by anemia and hypoxia.17 In mice, bleeding or PHZ-induced hemolysis caused a decrease in hepcidin mRNA levels, as did exposure to a hypoxic atmosphere in mice and rats. While the molecular mechanisms are unknown, anemia could be regulating hepcidin through tissue hypoxia (possibly through involvement of hypoxia-inducible factor, HIF) or indirectly by decreasing transferrin saturation through stimulation of erythropoiesis and increased demand for iron. It is also possible that the degree of anemia (and the iron need for erythropoiesis) is communicated to hepcidin-producing hepatocytes through a circulating factor from the bone marrow. Patients with chronic anemias with dyserythropoiesis, such as thalassemia syndromes, congenital dyserythropoietic anemias and sideroblastic anemias, also suffer from iron overload and associated toxicity. Measurements of urinary hepcidin in these patients indicated that hepcidin levels were severely decreased, despite systemic iron overload reflected by the patients’ elevated serum ferritin levels.18 The ratio of urinary hepcidin to serum ferritin can be used as an index of appropriateness of the hepcidin response to iron load, and while this ratio in normal subjects is close to 1, it was severely decreased in patients with iron-loading anemias. Even in frequently transfused thalassemia patients, whose urinary hepcidin was increased in comparison to that of untransfused patients,18 the levels were still inappropriately low given the patients’ iron load. These findings suggest that anemia, especially when associated with increased and ineffective erythropoiesis, has a strong and dominant effect over iron on hepcidin production. The consequent low levels of hepcidin in hereditary anemias may be responsible for hyperabsorption of iron, thus contributing to systemic iron overload and associated organ damage.

Regulation of hepcidin by inflammation; anemia of inflammation

The sequestration of iron during infection is an important host defense strategy to limit the growth of invading microbes. However, this response can also limit the iron availability for the production of nascent erythrocytes in the bone marrow. The imbal-
Intraperitoneal injection of a single dose of synthetic hepcidin develops hypoferremia within hours, resulting in rapid increase in urinary hepcidin. In addition, mice injected intraperitoneally with a single dose of synthetic hepcidin developed hypoferremia within hours, while chronically, mice with tumors engineered to overexpress hepcidin develop more severe anemia and hypoferremia despite having increased liver iron stores when compared to mice with control tumors.

Unlike hepcidin regulation by iron and hypoxia, the molecular pathways of hepcidin regulation by inflammation are better understood and primarily involve the inflammatory cytokine interleukin (IL)-6. Treatment of primary hepatocytes with IL-6 in vitro, injection of IL-6 in mice or infusion of IL-6 in humans increased hepcidin production within hours. In addition to IL-6, IL-1 and possibly other cytokines may also regulate hepcidin production directly.

The proposed cascade that produces anemia of inflammation thus leads from a cytokine-mediated increase in hepcidin, to hypoferremia, and then to anemia of inflammation. Increased plasma hepcidin during inflammation induces the internalization and degradation of ferroportin in macrophages, hepatocytes and duodenal enterocytes, trapping iron in these cells and preventing the efflux of iron into plasma (Figure 3B). As the developing erythrocytes in the bone marrow continue to use iron, the plasma iron compartment becomes depleted within hours, causing hypoferremia. If hypoferremia persists, as in the setting of chronic inflammation, erythropoiesis will become iron-restricted. As pointed out earlier, hepcidin is regulated by iron levels and anemia; the hypoferremia and anemia will therefore inhibit hepcidin production and eventually, a new balance may be reached at a lower serum iron and blood hemoglobin concentration.

Anemia of inflammation is also characterized by a blunted response to erythropoietin and shortened erythrocyte lifespan, but it remains unexplored to what extent, if any, hepcidin contributes to these phenomena.

**Clinical uses of hepcidin**

Increased hepcidin contributes to the pathogenesis of anemia of inflammation while hepcidin deficiency is a common characteristic of most forms of hereditary hemochromatosis. Measurements of hepcidin concentrations in plasma or urine could therefore be useful in the differential diagnosis of anemia of inflammation and iron deficiency anemia, or in the diagnosis of hemochromatosis. Currently, assays for plasma or urinary hepcidin are not generally available and further development of such assays and their clinical validation is highly desirable. Additionally, development of pharmacological hepcidin agonists and antagonists could improve current therapies for iron disorders. Agonists may be helpful in the management of hereditary hemochromatosis or of hereditary anemias in which hyperabsorption of iron contributes to the iron load. Hecpidin antagonists should be beneficial in the treatment of anemia of inflammation when the primary disease is refractory to therapy.

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**References**


Pathogenesis and management of iron-loading anemias

The term iron-loading anemias defines a group of chronic conditions, genetic or acquired, in which iron overload is not the result of a primary defect of the iron regulation system, but of erythropoiesis. This shapes the stimulus for increasing intestinal iron absorption, the degree of anemia and the need for transfusion.

Strictly speaking the term iron-loading anemias should be reserved to three conditions: thalassemia syndromes, sideroblastic anemia and congenital dyserythropoietic anemias.

The thalassemia syndromes are a large group of hereditary anemias due to mutations in globin genes clusters and the decreased synthesis of one or more hemoglobin polypeptide chains. The most important clinical varieties are thalassemia major (transfusion-dependent) and thalassemia intermedia (transfusion-independent).1

The sideroblastic anemias are a heterogeneous group of inherited or acquired disorders characterized by hypochromic anemia, ringed sideroblasts in the bone marrow, the effect of mitochondrial iron loading, and progressive accumulation of iron.2

The X-linked form (XLSA) is due to mutations in the \( ALAS2 \) gene, which prevent efficient formation of heme, whereas X-linked sideroblastic anemia with ataxia is caused by a mutation in the \( ABC7 \) gene, encoding a protein important for export of iron/sulfur clusters from mitochondria.

The congenital dyserythropoietic anemias (CDA) are a heterogeneous group of macrocytic anemias, characterized by ineffective erythropoiesis with typical nuclear abnormalities in erythroblasts and progressive iron overload.3

Pathogenesis of iron overload

Two mechanisms of iron loading may be present: increased gastrointestinal absorption and secondary iron load from transfusions. The former is the hallmark of iron loading anemias, whereas the latter may be absent or, depending on the severity of anemia, may become the most important with time.

Increased gastrointestinal absorption

The mechanism of the sharp increase in iron absorption in the iron-loading anemias is not fully understood. The severity of anemia and the degree of erythropoiesis are important but not crucial. In fact many other forms of anemia, such as hereditary spherocytosis, sickle cell anemia, autoimmune hemolytic anemia, may be similarly severe, but do not stimulate iron absorption. In these anemias the destruction of mature red cells is peripheral, whereas in iron loading anemias the destruction regards mainly the erythroid precursors within an hyperplastic bone marrow (ineffective erythropoiesis).

It has been postulated that an erythroid regulator, through a soluble signal, tunes intestinal iron absorption according to erythropoietic needs, independently of body iron stores.4

Another factor that may explain the pathogenesis of iron load in iron loading anemias is the down regulation of hepcidin. Hepcidin inhibits the export of iron from enterocytes, macrophages and hepatocytes by binding to the exporter protein ferroportin and its synthesis is depressed by anemia and hypoxemia. Thus, a low level of hepcidin results in increased iron export from the aforementioned cells into the blood stream and deposition in parenchymal cells.5

In patient with thalassemia major and intermedia, urinary hepcidin levels were similar to those in normal controls, but the hepcidin to ferritin ratio was significantly lower, showing that hepcidin expression was inappropriate for the degree of iron overload.6

Three major types and more than four minor subgroups have been defined; the linkage to a disease gene has been demonstrated only in a part of cases, such as \( CDAN1 \) for CDA type I and \( CDAN3 \) in CDA type 3.
load. In thalassemia major transfusion significantly enhanced hepcidin production. In another study urinary hepcidin was found to be low in patients with thalassemia syndromes and congenital dyserythropoietic anemia type 1.

**Iatrogenic iron overload**

Sometimes patients with refractory microcytic anemias are misdiagnosed as being iron deficient and prescribed oral or parenteral iron, which aggravates the iron load.

**Transfusional iron overload**

Milder forms of iron loading anemias do not require regular transfusion, whereas transfusion may be life-saving in the most severe forms.

Among thalassemias, hydrops fetalis and β thalassemia major require transfusion since intrauterine life and since the first months of life respectively.

Sideroblastic anemias, unless misdiagnosed or pyridoxine-unresponsive, do not require transfusion.

Only some patients with congenital dyserythropoietic anemias require repeated transfusion during the first months of life. In children and adults, these anemias are usually mild to moderate and transfusion is required occasionally in association with infections.

In all these conditions, suppressing ineffective erythropoiesis and erythropoietic hyperplasia by regular transfusions may minimize dietary iron uptake, although the rate of iron accumulation is finally aggravated. Each unit of blood contains 200 to 250 mg of iron. After 10-20 transfusions, iron is present not only within the reticulo-endothelial cells but also in the parenchymal ones, where it causes significant oxidative damage. Free iron promotes generation of free hydroxyl radicals, propagators of oxygen-related damage.

**Management of iron overload**

In the past, iron overload in iron-loading anemias received limited medical attention. The poor prognosis of the most severe forms overshadowed the long-term risk of iron-related complications. Nowadays two major factors allow a more comprehensive approach: (i) advances in the treatment of the underlying disease and in the assessment of iron load, and (ii) availability of new oral chelators.

**Evaluation of iron overload**

An accurate assessment of iron status is a prerequisite for the evaluation of its clinical relevance, the need for treatment, its timing and its monitoring.

**Clinical features**

The time that signs or symptoms appear depends on the rate of iron overload. In severe conditions, such as thalassemia major, these may appear during childhood, with skin hyperpigmentation, growth impairment, delayed puberty, arthrythms and the onset of frank diseases such as congestive heart failure or diabetes. Otherwise, in other iron-loading anemias, as well as in genetic hemochromatosis, signs and symptoms may be very late and generic: weakness, fatigue, loss of libido, arthralgia. If the iron burden progresses and is not treated, all the clinical features may become manifest, with overt heart disease, diabetes, hypothyroidism, hypoparathyroidism, hypogonadism and cirrhosis. A clear association with the risk of developing hepatocarcinoma has been established, at least for thalassemias.

**Serum markers**

Serum iron and transferrin, as well as serum transferrin receptor, do not have a specific value in assessing an iron overload, but are necessary for calculating transferrin saturation.

A raised serum iron concentration, or an increase in serum ferritin or transaminases, may often be an incidental finding at routine tests and trigger the diagnosis of iron overload.

Serum ferritin is reliable at low and normal levels, but loses accuracy as the iron load rises and the predictive value of serum ferritin for iron load may be poor. Many factors independent from iron stores, such as infection, inflammation, hepatitis, hemolysis, and vitamin C deficiency may significantly alter the serum ferritin concentration.

**Iron toxicity markers**

When the iron load continues to rise and is not balanced by treatment, plasma transferrin becomes fully saturated and a toxic fraction of plasma iron appears. This is called non-transferrin-bound iron or labile plasma iron, according to the method used to detect it.

Other plasma markers of iron toxicity are products of lipid peroxidation, such as malondialdehyde, and the levels of physiological antioxidants, such as ascorbic acid and tocopherol.

**Tissue iron concentration/distribution**

The liver contains most of the body’s iron stores (70-80%) and it is the main crossroad of iron trafficking (storage from intestinal absorption and from red-cell catabolism, chelation by iron-chelating drugs, excretion through the bile). Histological liver biopsy, assessment of the histology may provide a semi-quantitative evaluation of iron load, its distribution, the effects of iron damage and possible independent factors such as viral hepatitis, alcohol, and steatosis.

Liver iron concentration (LIC) is the reference parameter to quantify iron stores, as many studies
have confirmed a close relationship between total body iron stores and LIC. After lyophilization and suspension of an adequate tissue sample, the iron is measured by atomic absorption spectroscopy.

In thalassemia LIC has a prognostic value, with concentrations above a threshold of 15 mg/g dry weight being associated with increased risk of cardiac disease and early death. Evidence for the existence of a critical LIC in transfusional iron overload has recently been demonstrated in unchelated patients with secondary iron overload, in whom transaminases remain normal with LIC below a threshold of around 6 mg/g dry weight.

Magnetic susceptometry using a superconducting quantum interference device (SQUID) is the most accurate non-invasive method for quantitative estimation of LIC, but accessibility to this technique is poor since there are only four systems functioning in the whole world.

The peculiar paramagnetic response of iron in ferritin and hemosiderin in a constant magnetic field is detected by the very sensitive SQUID. The system has been validated with chemically measured LIC, demonstrating direct linearity up to 12 mg/g dry weight. Carrying out the iron assessments at room temperature using a new susceptometer may make evaluation of iron load less expensive and more widely accessible.

Magnetic resonance imaging (MRI) is gaining importance, given that the system, the sequences and the acquisition methods are optimized for iron assessment. The iron concentration is quantified indirectly by its effect on shortening proton relaxation times.

Two methods are in use: the signal intensity ratio (SIR) between the studied tissue and a non-iron-loading reference tissue (skeletal muscle or fat), or the calculation of relaxation time constants (T2, R2, T2*). All approaches have been validated with liver biopsies and give reasonable quantification, R2 being the most robust method for the liver.

Cardiac MRI

As the primary cause of death in severe iron overload is heart failure, even if cardiac iron concentration is ten times lower than hepatic iron concentration, an affordable cardiac assessment is extremely useful for clinical management. The uneven iron distribution in the heart, blood flow and motion artifacts constitute an important challenge to heart assessment.

A retrospective study of thalassemia patients being treated with deferoxamine found a significant correlation between myocardial T2* and left ventricular function. Many patients with a T2* below 20 ms showed important impairment of ventricular function. Cardiac T2* did not correlate with serum ferritin or LIC in patients on long term chelation.

Iron load with transfusions

High-quality monitoring of transfusion-dependent patients includes accurate recording of transfused blood and a calculation of the amount of iron administered.

Patients with transfusion-dependent conditions such as severe thalassemias have a blood consumption of 100-200 mL/kg/year of pure red blood cells, corresponding to 0.32-0.64 mg/kg/day. Differences among patients may be substantial (from 0.15 to 0.80 mg/kg/day), depending on the underlying condition, transfusional scheme, spleen status and the presence of red cell immunization.

Treatment of iron overload

Phlebotomy

Phlebotomy is the first choice treatment of iron overload. By definition phlebotomy should be unsuitable in chronic anemias, but in milder forms or after stem cell transplantation it has been applied with success, with full normalization of iron stores and reversal of iron-related complications, including cirrhosis and cardiac dysfunction.

The amount of blood removed may range between 2 and 7 mL/kg every 4-30 days. The individual scheme is personalized according to the degree of anemia, the presence of hypotension, cardiac or liver disease and the individual tolerability.

In X-linked sideroblastic anemia, reversal of the iron overload by phlebotomy may result in a significantly better response to pyridoxine supplementation.

Iron chelation therapy

The aims of iron chelation are to prevent iron-related complications, to maintain safe tissue iron levels and to reverse iron-related complications. A single excellent drug, deferoxamine has been available now for some decades. In thalassemia regular slow subcutaneous infusions of deferoxamine give an impressive improvement in life expectancy and progressive lowering in the prevalence and severity of iron-related clinical complications. With experience it is possible to limit side effects and optimize compliance in most patients. Alternative treatment modalities, such as subcutaneous injections or continuous intravenous infusions can be used for a wide range of conditions and special needs.

During the past few years the advances in iron chelation research have been impressive, leading to the development of new oral chelators.

Deferiprone or L1, an orally active chelator from the bidentate hydroxypyridinone family, was first
synthesized in 1982, but was not developed in a systematic way. Several aspects of its safety and efficacy have been matter of serious discussions and controversies. The results from large and controlled studies allowed the approval of deferiprone in Europe and many other countries, often as a second line treatment for iron overload. The safety profile requires close monitoring. The growing evidence that regular use of this drug has a cardio-protective effect counterbalances these limitations. There are no consistent data on the value of deferiprone treatment in congenital dyserythropoietic anemias or in sideroblastic anemia.

ICL670 or deferasirox, a tridentate compound of the triazol family, has been recently approved by the Food and Drug Administration for treating transfusional iron overload in patients over the age of two years old. The overall results indicate that ICL670 is a well-tolerated, effective oral chelator that, taken once a day at a dose of 20-30 mg/kg/day, is as effective as standard subcutaneous deferoxamine. Preliminary results suggest a beneficial effect on cardiac iron both in animals and humans. Most of the clinical results have been obtained in thalassemia, but a significant group of iron-loading anemias has been studied with similar results.

The availability of more than one drug stimulated the search for benefits from combination therapy. Some in vitro data suggested the potential of an additive and even synergistic effect. Such an effect has been confirmed in patients, even if published data must be considered carefully, because they are often uncontrolled and heterogeneous.

As regards the safety of iron chelation, most of the side effects are caused by the subtraction of iron from iron-dependent physiological pathways. Age, high doses of chelator and low levels of iron overload are the main risk factors, whereas some side effects are characteristic to each drug. A close monitoring schedule should be individually tailored in order to detect any iron chelation toxicity earlier and minimize its consequences. This monitoring may include: auxological assessment (weight, body fat, standing and sitting height, pubertal stages, radiological assessment of bone age and the main metaphyses), bone densitometry, liver function tests, ophthalmological examination, audiometry, plasma zinc assays, rheumatological assessment, and absolute neutrophil counts. Specific attention must be paid to early signs of infection in order to diagnose and treat iron-related complications such as Yersinia enterocolytica septicemia.

Initiation of chelation therapy

In the absence of prospective studies, the recommendations on this point are based on empirical considerations.

In thalassemia a serum ferritin threshold level of 1000 µg/L is often taken to indicate the need to start chelation. This seems useful in thalassemia major, where this limit is reached after 10-20 transfusions. In thalassemia intermedia this threshold may lead to an underestimation of iron overload. In CDA serum ferritin levels of 1500 and 1000 µg/L have been proposed. These levels may not be sufficiently low to prevent significant morbidity from iron toxicity.

In some patients with CDA type I prolonged interferon therapy may reduce the iron overload.

A better understanding of the relationship between serum ferritin levels, extent of iron overload and degree of tissue damage in CDA and sideroblastic anemia will improve the prevention of long-term iron-related complications.

Conclusions

Iron overload in iron-loading anemias is caused by increased intestinal iron absorption and, when administered, by blood transfusion. The degree of ineffective erythropoiesis is crucial in determining the rate of iron absorption. The mechanisms are not fully understood. The direct action of an erythroid regulator is likely, and the down-regulation of hepcidin gives an important contribution.

The management of iron-loading anemias requires close evaluation and monitoring of the iron overload, with the integrated use of several iron indices. Recent advances in diagnostics make iron quantification more accurate and enable it to be carried out earlier. Treatment of iron overload is based on phlebotomy, when feasible, and iron chelation therapy. The new oral chelators greatly enhance the potential of full prevention of iron-related complications.

References


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Anemia of chronic disease

The association between chronic infections, rheumatic disorders, other inflammatory states, and anemia was recognized over 150 years ago. The anemia has been designated simple chronic anemia, the anemia of chronic disease (ACD), or the anemia of inflammation. The term anemia of inflammation most accurately represents the pathogenesis of the disorder, anemia resulting from the concerted action of inflammatory cytokines, but the term ACD is ingrained in the literature and will be used here.

In 1945, G.E. Cartwright, M.M. Wintrobe, and their associates began a series of experiments in animals and humans that characterized many of the clinical and biochemical features of ACD (summarized in ref. 2). The anemia is usually normocytic and normochromic, but hypochromia is frequently noted and occasionally the anemia is microcytic and hypochromic. Alterations in iron metabolism are prominent. Hypoferrremia is a consistent finding and the serum transferrin concentration is reduced. The reduction in serum iron is more marked than the reduction in transferrin, resulting in a subnormal percent saturation of transferrin in most cases. Iron uptake by developing red cells is dependent upon the diferric transferrin – transferrin receptor-mediated endocytic system and the reduced concentration of diferric transferrin results in iron-limited erythropoiesis. This is reflected by an increase in the concentration of protoporphyrin in red cells, a finding characteristic of iron-deficient erythropoiesis. In contrast to iron-deficiency anemia, iron stores are not diminished in ACD, macrophages sequester iron, and iron uptake from the gut is diminished.

An increase in plasma copper is found in most patients with ACD. The increase in copper is primarily due to an increase in plasma ceruloplasmin. Ceruloplasmin is a multicopper ferroxidase and is required for cellular iron export. Retention of iron by macrophages suggested that the ferroxidase activity of ceruloplasmin might be inhibited in ACD, but this proved not to be the case. The ferroxidase activity of ceruloplasmin is not altered and administration of exogenous ceruloplasmin does not correct the defect in cellular iron export.

The early studies of Cartwright and Wintrobe also demonstrated a modest reduction of erythrocyte survival time in ACD. Reduced erythrocyte survival is not due to a defect intrinsic to the red cell, as the survival of red cells from patients with ACD is normal when the cells are infused into normal subjects. Conversely, the survival of red cells from normal subjects infused into patients with ACD is shortened by a modest degree. Several early studies suggested that the shortened erythrocyte survival was due to an enhanced ability of macrophages to ingest and destroy red cells. The slight increase in red cell destruction in patients with ACD is not, by itself, sufficient to explain the anemia, as a normal marrow is capable of increasing red cell production six- to eightfold. These findings suggest that the capacity of the marrow to increase production in patients with ACD is markedly impaired.

In 1969, Ward et al., reported a reduction in serum erythropoietin levels in patients with rheumatoid arthritis and ACD. It was later demonstrated that erythropoietin levels vary directly with the degree of anemia in patients with iron deficiency or primary hematopoietic disorders. In contrast, there is no correlation between erythropoietin levels and the degree of anemia in ACD and erythropoietin levels are lower for the same degree of anemia found in iron deficiency and other hematopoietic disorders. It was subsequently observed that in ACD there was a blunted response to the administration of erythropoietin. These findings strongly suggest that erythroid precursors in ACD display some degree of resistance to the proliferative effects of erythropoietin.
Disease mechanisms

The presence of ACD correlates roughly with the activity of the associated inflammatory disease. This observation has led to numerous studies of humoral mediators of inflammatory responses including tumor necrosis factor, interleukin-1, and the interferons. Concentrations of these inflammatory cytokines are increased in the chronic disorders associated with ACD and in animal models of ACD. Administration of these inflammatory cytokines results in an anemia with all of the characteristics of ACD. These inflammatory mediators and other cytokines such as IL-6 have now all been implicated in the pathogenesis of ACD.

Iron-restricted hematopoiesis and macrophage iron sequestration

Virtually all of the observed abnormalities in iron metabolism associated with ACD can now be explained by the action of hepcidin. Hepcidin is a 25 amino acid disulfide-rich peptide generated in the liver in response to the inflammatory cytokine interleukin-6 (IL-6). The synthesis of hepcidin is induced by an increase in hepatocellular iron stores and by IL-6, whereas hepcidin expression is suppressed by hypoxia. The induction of hepcidin expression is not mediated by other inflammatory cytokines such as interleukin-1 or tumor necrosis factor alpha. This conclusion is enforced by the observation that IL-6-deficient mice do not induce hepcidin in response to inflammatory stimuli. Experiments in humans have conclusively demonstrated that the infusion of IL-6 in healthy volunteers rapidly induces hepcidin, and this is quickly followed by hypoferremia. Approximately 20 mg of iron enters the plasma and is bound to transferrin each day. Approximately 80% of the iron entering the plasma is derived from recycling of senescent erythrocytes by macrophages and, to a smaller extent, from mobilization of hepatic iron stores. During inflammation, the release of iron from macrophages and from liver iron stores is markedly inhibited. Studies in mice either lacking hepcidin or overexpressing hepcidin have demonstrated that hepcidin is a negative regulator of iron release from macrophages and is also a negative regulator of intestinal iron uptake. During inflammation IL-6 induces hepcidin production which, in turn, inhibits iron release from macrophages and other sites, rapidly leading to hypoferremia.

Hepcidin inhibits cellular macrophage iron export by binding to ferroportin, the cellular iron export channel, and inducing ferroportin’s internalization and degradation. Intestinal iron absorption in chronic inflammation is also diminished and this is also mediated by the hepcidin-ferroportin mechanism. Eventually, diminished gastrointestinal iron absorption can lead to depletion of iron stores, but this is uncommon in ACD except in clinical syndromes in which IL-6 expression is dramatically elevated. This appears to be the case in children and young adults with juvenile rheumatoid arthritis. The evidence incriminating hepcidin as the mediator of abnormal iron metabolism associated with ACD has been summarized in a recent review.

Shortened erythrocyte survival

The mechanism underlying the shortened survival of erythrocytes that is associated with ACD has been the most difficult to clarify. An extracorpuscular mechanism has been incriminated (see above) and levels of inflammatory cytokines such as interleukin-1 and red cell survival seem to be correlated in ACD associated with rheumatoid arthritis. It has been suggested that selective hemolysis of newly formed erythrocytes is mediated by activated macrophages and that this phenomenon may explain the extracorpuscular mechanism of shortened erythrocyte survival.

Blunted erythropoietin response

Several inflammatory cytokines including interleukin-1, tumor necrosis factor alpha, and transforming growth factor beta inhibit production of erythropoi-
etin by the kidney. It has also been demonstrated that tumor necrosis factor inhibits red cell production in \textit{in vitro} culture systems, an effect mediated by interferon-β.\textsuperscript{19} A very recent study also suggests that hepcidin may directly inhibit erythroid colony formation \textit{in vitro} and that this effect is independent of the effect of hepcidin on iron metabolism.\textsuperscript{20} A summary of the molecular mechanisms involved in the pathogenesis of ACD is shown in figure 1.

\textbf{Treatment of ACD}

The recommended approach to ACD has been direct treatment of the underlying disorder. It is clearly established that reversal of the underlying inflammatory state results in correction of the anemia. In general, ACD is not severe enough to merit specific therapy but in some cases, treatment is indicated. The blunted response to erythropoietin has prompted a number of studies evaluating the role of recombinant erythropoietin in the treatment of ACD. In a multicenter study, it was demonstrated that recombinant erythropoietin corrected ACD in patients with rheumatoid arthritis and that pre-treatment levels of endogenous erythropoietin did not correlate with response to the recombinant preparation.\textsuperscript{21} It has also become apparent that responses to recombinant erythropoietin are more likely to occur with concomitant oral iron therapy.\textsuperscript{22} Iron supplementation clearly plays a role in responsiveness to recombinant erythropoietin. Recombinant erythropoietin has generally been administered at a fixed dose of 40,000 units injected subcutaneously each week or as darbepoietin in a dose of 200 µg injected subcutaneously every two weeks.

Parenteral administration of iron is occasionally required in patients refractory to the combination of oral iron therapy and recombinant erythropoietin. The intravenous administration of sodium ferric gluconate results in the rapid saturation of transferrin and in a prompt reticulocyte response, but the need for weekly injections has led some authors to recommend the more conventional intravenous iron dextran preparations in combination with erythropoietin.\textsuperscript{23}

\textbf{References}

Enzyme deficiencies of erythrocytes can lead to several different clinical phenotypes: 1) anemia; 2) methemoglobinemia; 3) erythrocytosis; or may lead to no phenotype at all. Clinicians confronted by patients with anemia, methemoglobinemia, or with erythrocytosis need to consider hereditary enzyme deficiency in their differential diagnosis, but the diagnosis of enzyme deficiencies is complex, costly, and often not generally available. It is the purpose of this presentation to provide guidance to the clinician regarding the appropriate diagnostic approach for patients in whom red cell enzyme defects are a diagnostic possibility.

Anemia

The first step: is the anemia hemolytic?

Anemias that result from enzyme deficiencies are hemolytic. The first step in the differential diagnosis is to establish that the anemia is, in fact, due to a decreased red cell lifespan. The best clinical surrogate for the measurement of the red cell lifespan is the reticulocyte count. While ancillary measurements such as serum bilirubin, haptoglobin, and lactic dehydrogenase may have some utility, an elevated reticulocyte count is strong presumptive evidence that hemolysis is present, except in patients with active bleeding or in those treated recently for a deficiency of iron, vitamin B12, or folate. An exception to the value of the reticulocyte count exists in patients with intercurrent infections, in whom the erythroid response may be inhibited.

The second step: the history

Although acquired red cell enzyme deficiencies may occur secondary to neoplasia, the vast majority of patients with hemolytic anemia due to enzyme defects have inherited enzyme deficiencies. Therefore, the enzyme defect has been present for the lifetime of the patient, and a history of long-standing anemia can often be elicited. In some cases there is a history of neonatal jaundice. However, the absence of a positive history by no means eliminates the possibility that an inherited deficiency is present. In glucose-6-phosphate dehydrogenase (G6PD) deficiency, in particular, there is often no prior history of anemia. This is because the red cell lifespan is usually normal until an oxidative stress is imposed on the red cells. Such a stress may be caused by the administration of drugs, infection, or ingestion of fava beans. The occurrence of such events in the history may provide an important clue regarding the cause of hemolysis. In other types of red cell enzyme deficiencies the presence of anemia may be inapparent because the hemolysis may be well compensated until an aplastic crisis occurs and erythropoiesis is inhibited, usually as a result of an infection by parvovirus or other infectious agent. As noted above, in such patients the reticulocytosis that is usually the hallmark of hemolytic anemia will be absent.

It is important to try to establish whether a family history of anemia is present. Since anemia due to a variety of causes is very common in the population one must try to distinguish the existence of hemolytic anemia in family members from other types of anemia, such as iron deficiency or even tiredness that is presumed to be due to anemia. The existence of gall bladder disease or splenectomy may provide a valuable clue in this regard. If siblings of the patient are affected but the parents are free of the disease, then transmission is autosomal recessive. This is the mode of transmission of most of the red cell enzyme defects, including deficiencies of pyruvate kinase, glucosephosphate isomerase, and pyrimidine 5’ nucleotidase, but there are two red cell enzyme deficiencies that are transmitted as X-linked disorders. These are G6PD deficiency and phosphoglycerate kinase deficiency. The only red cell enzyme abnormality that is inherited in an autosomal dominant fashion is increased adeno-
Table 1. Fluorescent screening tests for red cell enzyme deficiencies.

<table>
<thead>
<tr>
<th>Enzyme deficiency</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosephosphate isomerase (GPI)</td>
<td>(10, 11)</td>
</tr>
<tr>
<td>Triosephosphate isomerase (TPI)</td>
<td>(11, 12)</td>
</tr>
<tr>
<td>Pyruvate kinase (PK)</td>
<td>(11, 13)</td>
</tr>
<tr>
<td>Glucose-6-P dehydrogenase (G6PD)</td>
<td>(11, 14, 15)</td>
</tr>
<tr>
<td>Glutathione reductase (GSSG)</td>
<td>(13)</td>
</tr>
<tr>
<td>NADH diaphorase</td>
<td>(16)</td>
</tr>
</tbody>
</table>

sine deaminase activity, a disorder that is so rare that we know of the existence of only four kindreds. Dominant inheritance is characteristic of hemoglobinopathies that produce hemolytic anemia, mainly those characterized by unstable hemoglobins, and by membrane defects. Accordingly, little is gained by performing enzyme panels when a family history reveals autosomal dominant inheritance.

The third step: laboratory diagnosis

The blood film

Examination of the blood film is a time-honored practice in hematology, and while it yields information of value at low cost, it is important to recognize its limitations. The appearance of the film will often confirm that the patient has hemolytic anemia by revealing the presence of polychromasia and anisocytosis. It is particularly useful in establishing diagnoses other than an enzyme deficiency, thereby preventing wasted effort in tracking down a deficiency that does not exist. Hereditary spherocytosis is such a diagnosis, and while there have been rare isolated instances of co-existence of this common disorder with PK deficiency and G6PD deficiency, red cell enzyme defects do not produce spherocytosis. Indeed, the hemolytic anemias caused by red cell enzyme deficiencies are in the group of disorders that have been named non-spherocytic hereditary hemolytic anemias. The same is the case with elliptocytosis; a patient with hemolytic elliptocytosis is not a candidate for the study of red cell enzymes.

The only morphologic finding that can be considered to be of diagnostic value in the differential diagnosis of red cell enzyme deficiencies is the basophilic stippling that is characteristic of pyrimidine 5'-nucleotidase deficiency. However, considerable confusion has been engendered by descriptions of morphologic features mistakenly regarded to be characteristic of other red cell enzyme deficiencies. For example, many hematologists have the mistaken idea that extensive spiculation of red cells is characteristic of pyruvate kinase deficiency. This misconception is probably based on a 1964 publication in which such an association was documented. Although occasional contracted, dense, spiculated cells may sometimes be seen on the blood film of a pyruvate kinase deficient patient, this is a quite nonspecific finding and of little diagnostic value.

The autohemolysis test

Introduced by Selwyn and Dacie in 1954 before any meaningful studies of red cell metabolism had been performed in patients with hemolytic anemia, it is quite remarkable that the autohemolysis test is occasionally still performed. It has been clear for decades that it has no diagnostic value.

Screening tests

With the discovery of G6PD deficiency a plethora of screening tests were developed for this disorder. The G6PD reaction:

\[
\text{Glucose-6-P} + \text{NADP}^+ \rightarrow 6\text{-phosphogluconolactone} + \text{NADPH} + \text{H}^+ 
\]

reduces NADP+ to NADPH, and earlier generations of these tests depend upon linking the NADPH formed to a visible substance – a dye, or hemoglobin. However, because NADPH fluoresces in the visible spectrum when illuminated with long-wave ultraviolet light, its formation can be observed directly. The same is true for NADH, which is the product of other enzymatic reactions in the red cell. This principle has been used to devise a series of simple to perform screening tests that can be used to identify red cell enzyme defects without the need for any equipment other than an inexpensive long-wave ultraviolet lamp. This type of test is available for the detection of the enzyme deficiencies enumerated in Table 1.

No fluorescent screening test is available for the diagnosis of pyrimidine 5'-nucleotidase deficiency. However, the difference between the ultraviolet absorption spectrum of pyrimidine and purine nucleotides has made it possible to diagnose the disorder provisionally without needing to perform the relatively complex assay required for quantification of the enzyme.

Enzyme assays

In some instances a screening test is adequate to establish the diagnosis. For example, the fluorescent screening test for G6PD deficiency establishes very clearly whether the deficiency exists in males. However, the detection of heterozygotes for X-linked disorders is difficult because of X inactivation. The red cells represent a mosaic of cells, some of which are enzyme deficient while others are normal. The deficient cells, like the cells of male hemizygotes, are susceptible to hemolysis. In the case of G6PD deficiency even quantitative enzyme assays are not sufficiently sensitive to detect heterozygotes with a high degree of reliability. DNA analysis (see below) is the method of choice for detecting heterozy-
gotes with confidence.

Although most of the screening tests are very reliable, it is probably best to confirm these with a quantitative enzyme assay. Moreover, screening tests are usually inadequate for the detection of heterozygotes, and quantitative assays are more likely to be helpful in family studies designed to detect carriers. An integrated series of enzyme assays has been developed, and these methods are used almost universally for the performance of red cell enzyme assays.

DNA analysis

Mutation detection by DNA analysis is complementary to the performance of enzyme assays in the diagnosis of red cell enzyme deficiencies. For example, DNA analysis can establish a diagnosis even when a patient has been extensively transfused. There are some patients, usually young children, whose hemolytic anemia is so severe that they require frequent blood transfusions. Red cells that are sampled from such patients are so contaminated with blood bank erythrocytes, that it is difficult to draw any conclusions from enzyme assays. But when patients have been transfused the leukocytes do not circulate, and the DNA that is purified from circulating white cells represents the somatic DNA of the patient, not that of the blood donor. DNA analysis is much more reliable in the identification of heterozygotes than are enzyme assays, and they are more suitable for prenatal diagnosis than enzyme assays, since the normal levels of enzymes in fetal tissues are not well established. Table 2 summarizes the advantages and disadvantages of the enzymatic and DNA approach.

With the technological advances of the past few years, complete sequencing of the coding regions and of the promoter of individual genes is quite readily carried out. On the other hand, without prior knowledge of which gene is at fault gene sequencing is currently an impractical approach. When sequencing the DNA encoding a red cell enzyme it is prudent to start seeking common, known mutations. Thus, a European patient with possible pyruvate kinase deficiency is most likely to have the common c.1529 G>A mutation, while a patient with Gypsy ancestry is likely to have a deletion of exon 10. In the great majority of cases complete sequencing of the coding region and in the promoter will reveal the pathogenic mutation. However, when no mutation is found enzyme deficiency is not ruled out with absolute certainty. It is always possible that the gene is not transcribed because of the action of some distant DNA element, such as an enhancer. Moreover, sequencing the coding regions will often not reveal aberrant splicing. Isolation of mRNA from erythroid cells is difficult, since only small amounts are present, and only in reticulocytes. Finally, even when a mutation is found, one cannot always be certain that it does not represent a benign polymorphism or family mutation that has no functional effect. Only when the mutation found has been associated with disease in previously studied families can one feel reasonably secure about the relationship between the genotype and disease phenotype.

Methemoglobinemia

The first step: does the patient actually have methemoglobinemia?

The use of automated spectrophotometers that measure the absorbance of blood samples at several different wavelengths has given rise to frequent errors in the diagnosis of methemoglobinemia. Small amounts of sulfohemoglobin can result in erroneous readings with some instruments. It is well to confirm the diagnosis of methemoglobinemia by making certain that the addition of cyanide eliminates the absorption band at 620 nm that is characteristic of hemoglobin.

The second step: the history

Patients with hereditary methemoglobinemia have a life-long history of cyanosis. Cyanosis that appears in late childhood or adult life is very unlikely to be hereditary in origin. It is probably due to the ingestion of or exposure to a methemoglobin-forming chemical.

If methemoglobinemia has been present for a long period of time, then the family history provides the most important clue as to its origin. Methemoglobinemia due to inheritance of a hemoglobin M shows a dominant inheritance pattern; recessive inheritance is characteristic of hereditary methemoglobinemia due to a deficiency of methemoglobin reductase (NADH diaphorase; cytochrome b5 reductase).

The third step: laboratory diagnosis

The enzyme deficiency can be diagnosed by performing a screening test (Table 1), an enzyme assay,
or by sequencing the gene. The caveats regarding these approaches are essentially the same as those outlined for the diagnosis of hemolytic anemia.

**Erythrocytosis**

Erythrocytosis is a rare consequence of red cell enzymopathies. It occurs when the enzyme deficiency results in lowered levels of red cell 2,3-bisphosphoglycerate (2,3-BPG; 2,3-DPG). Red cells with lower levels of this sugar phosphate have a higher oxygen affinity because of the allosteric effect that 2,3-BPG has on hemoglobin. This leads to an inappropriate signal to produce more erythrocytes.

There are only two red cell enzyme deficiencies that cause erythrocytosis: phosphofructokinase deficiency and a deficiency of the diphosphoglycerate mutase/phosphatase enzyme. These disorders are best diagnosed by performing a quantitative assay of the enzymes and by demonstrating that red cell 2,3-BPG levels are, in fact, diminished.

**What should our expectations be?**

Even when patients are carefully selected before red cell enzyme assays are performed a diagnosis is not established in many cases. Physicians were often surprised when they referred a sample from a young patient with all of the hallmarks of hereditary nonspherocytic hemolytic anemia to our laboratory and a complete panel of red cell enzyme assays failed to disclose an enzyme deficiency. Figure 1 illustrates the experience of our laboratory in performing a panel comprised of 26 red cell enzymes on 691 samples submitted to our laboratory between 1980 and 1986, the last time that we made this analysis. As shown in this figure some 80% of patients went undiagnosed. Admittedly, these results might be somewhat biased in that not all samples submitted were from patients who were good candidates for hereditary red cell enzyme deficiencies, and samples from patients with some of the more common deficiencies such as those with G6PD and pyruvate kinase might not have been submitted, the diagnosis having already been established at another laboratory. The experience of other reference laboratories has been quite similar. Figure 2 shows the distribution of diagnoses in those patients in whom a defect was detected. It is clear that the most common causes were pyruvate kinase and G6PD deficiency. Pyrimidine 5’ nucleotidase deficiency and glucosephosphate isomerase deficiency were less common and were in third and fourth place with respect to incidence. Other enzyme deficiencies were very uncommon; a few cases with unstable hemoglobins were also detected, since these hemolytic anemias are clinically very similar to red cell enzyme deficiencies.

**Summary and conclusions**

The history, blood films, screening tests, quantitative enzyme assays, and DNA analysis all play a role in the diagnosis of red blood cell enzyme defects. Appropriate use of these modalities, beginning with the simple means by which the physician can be guided to the correct conclusion, can save precious health care resources.

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Pyruvate kinase deficiency: genotype to phenotype

Mature red blood cells are optimally adapted to bind and transport oxygen, and delivery it to all tissues. The membrane, hemoglobin, and proteins involved in metabolic pathways of the red blood cell interact to modulate oxygen transport, protect hemoglobin from oxidant-induced damage, and maintain the osmotic environment of the cell. The biconcave shape of the red blood cell provides a very large area for respiratory exchange. The latter requires passage through microcapillaries, which is achieved by a drastic modification of the cell’s biconcave shape, made possible only by the loss of the nucleus and cytoplasmic organelles and, as a result, the ability to synthesize proteins. Consequently, erythrocytes depend solely on the anaerobic conversion of glucose by the Embden-Meyerhof pathway, or glycolysis, for the generation and storage of the adenosine triphosphate (ATP) required to maintain a number of vital cell functions (Figure 1). This pathway is subjected to a complex interplay of inhibiting and stimulating factors. The overall velocity of red blood cell glycolysis is regulated by three rate-limiting enzymes, hexokinase (HK), phosphofructokinase, and pyruvate kinase (PK), and by the availability of nicotinamide adenine dinucleotide NADH and ATP. Moreover, erythrocytes possess a unique glycolytic bypass, the Rapoport-Luebering shunt, for the production of 2,3-biphosphoglycerate (2,3-BPG) which decreases hemoglobin’s affinity for oxygen (Figure 1).

A number of red blood cell enzyme disorders have been described in the Embden-Meyerhof pathway. The lack of characteristic changes in red blood cell morphology differentiates the glycolytic enzyme disease from erythrocyte membrane defects and most hemoglobinopathies. In general, red blood cell enzymopathies cause chronic non-spherocytic hemolytic anemia (CNSHA), albeit to a variable degree. The clinical picture ranges from neonatal death to a mild and fully compensated hemolytic anemia, diagnosed during adulthood. The continuous lack of sufficient energy and other metabolic impairments results in a shortened lifespan of the mature red blood cell by, as-yet, unknown mechanisms. To induce clinically significant hemolysis, red blood cell enzyme function must be significantly impaired under physiological conditions. This is mainly due to the role of the affected enzyme in glycolysis as well as the underlying molecular alteration responsible for defective enzymatic function. The latter constitutes the primary basis of the associated hemolytic disease. The ability to compensate for the enzyme deficiency by overexpressing isozymes or using alternative pathways contributes to the clinical picture of patients with red blood cell enzymopathies. Diagnosing red blood cell enzymopathies may be complicated, thereby hindering the counseling of patients. This is especially problematic in cases that prenatal diagnosis would be useful. Therefore, it is important to combine information on genetics, biochemistry and structural consequences of mutations in the genes and the respective enzymes they encode. In other words, it is important to identify the molecular mechanisms by which a DNA-encoded error (genotype) eventually exerts its effect on the protein level, causing disease (phenotype). A simplified scheme that exemplifies the route from DNA to protein is displayed in Figure 2. Eventually, this knowledge will contribute to a better understanding of the clinical phenotype of defective glycolysis in red blood cells.

Though rare, deficiency of the key regulatory enzyme of glycolysis, PK, represents the most frequent cause of hereditary CNSHA due to defective glycolysis. To illustrate the above mentioned features regarding the relationship between genotype and phenotype we will use PK deficiency as an example.
The structure and function of PK

Pyruvate kinase catalyzes the irreversible phospho-
rylgroup transfer from phosphoenolpyruvate (PEP) to
adenosine diphosphate (ADP), yielding pyruvate and
the second molecule of ATP (Figure 1). Pyruvate is
crucial for several metabolic pathways. The enzyme
is active as a tetramer, and four different isozymes are
expressed in mammals. R-type PK expression is con-
fined to red blood cells whereas L-type PK is predom-
inately expressed in the liver. The PK-R and PK-L sub-
units are both transcribed from a single gene (PKLR),
located on chromosome 1q21, by the use of alterna-
tive promoters. PKLR consists of 12 exons and spans
9.5 kb (Figure 3). Exon 1 is erythroid-specific whereas exon 2 is liver-specific. Hence, exons 3 to 12 are included in both liver- and red blood cell-specific mRNA, and encode a PK-R subunit of 574 amino acids whereas the PK-L subunit comprises 531 amino acids. PK isozymes PK-M1 and PK-M2 are produced from a single gene (PKM) by means of alternative splicing. PK-M1 is expressed in skeletal muscle, heart, and brain and it is the only isozyme that is not sub-
jected to allosteric regulation. The PK-M2 isozyme is
expressed in early fetal tissues, but also in most adult
tissues, including leukocytes and platelets.

In basophilic erythroblasts, both PK-R and PK-M2
are expressed. During further erythroid differentia-
tion and maturation, a switch in isozymes occurs
whereby progressively increased PK-R expression
gradually replaces PK-M2. Human red blood cell PK
consists of two distinct species, PK-R1 and PK-R2.
PK-R1 predominates in reticulocytes and young ery-
throcytes, whereas mature red blood cells mainly
possess PK-R2. PK-R1 is a homotetramer composed
of four PK-R, also called L subunits (L-4). Limited pro-
teolytic degradation of this 63 kDa PK-R subunit ren-
ders a 57-58 kDa PK-L subunit that is incorporated in
the heterotetramer PK-R2 (L2L-2). The enzymatic
activity of PK decreases with increasing age of the
erthrocyte.

PK is allosterically activated by phosphoenolpyru-
vate and fructose-1,6-diphosphate (FDP), and nega-
PK deficiency is the most common cause of non-spherocytic hemolytic anemia due to defective glycolysis. The disease is inherited in an autosomal recessive manner. The estimated prevalence is 51 cases (i.e. homozygous or compound heterozygous patients) per million in the white population. To date, more than 180 mutations in PKLR have been reported to be associated with pyruvate kinase deficiency. A schematic overview is presented in Figure 3. Most mutations (70%) are missense mutations affecting conserved residues in structurally and functionally important domains of PK. In the European and North-American population, the most frequently detected mutations are missense mutants c.1456C>T (Arg486Trp), c.1529G>A (Arg510Gln), c.994G>A (Gly332Ser), and a nonsense mutant c.721G>T (Glu241Stop). The expression, biochemical characterization, and crystallization of various recombinant mutants PK has greatly enhanced our understanding of the relationship between the nature and location of the replaced amino acid and the type of molecular perturbation.

Hemolysis

Two major metabolic abnormalities result from PK deficiency: ATP depletion and increased 2,3-BPG content. However, the precise mechanisms leading to a shortened lifespan of the mature PK-deficient erythrocyte are still unknown. The increased 2,3-BPG levels ameliorate the anemia by lowering the oxygen-affinity of hemoglobin. Phenotypically, the clinical picture varies from severe hemolysis causing neonatal death to a well compensated hemolytic anemia. Some PK-deficient patients present with hydrops fetalis. Patients are often dependent on blood transfusions and may suffer from (secondary) hemochromatosis. Reticulocytosis is almost always observed. Splenectomy often ameliorates the hemolysis, especially in severe cases, and increases reticulocyte counts even further.

As stated, the patient’s PKLR genotype forms the basis of defective PK function and, hence, the associated hemolytic disease. However, many aspects of gene expression may be involved (Figure 2). Through elucidation of the molecular mechanism of disease in two patients with severe PK deficiency we illustrate how aberrant PK expression results from the PKLR genotype and discuss these findings in relation to the observed phenotype.

PKLR genotypes and PK-deficient phenotypes

Patient 1 – genotype

The first patient is a 6-year old boy who suffered from severe hemolytic anemia since birth and has...
been dependent on blood transfusions ever since. PK-deficiency was diagnosed at the age of 1 year (Table 1). The boy’s PK activity was too low considering the elevated reticulocytes and, consequently, hexokinase activity. The presence of transfused red blood cells also made these values difficult to interpret. DNA sequence analysis of the coding region revealed heterozygosity for one (common) missense mutation on the patient’s paternal allele: c.1529G>A, encoding an arginine to glutamine change at residue 510 (Arg510Gln, Figure 5A) A second mutation was not, however, detected. Subsequent RNA analysis showed only the mutant c.1529A allele, suggesting silencing of expression of the c.1529G allele. The putative erythroid-specific proximal promoter of PKLR was a likely candidate to harbor a mutation responsible for abolished expression. Therefore, approximately 500 bp of this part of the gene was investigated and this analysis detected two point mutations in cis on the maternal allele: -83G>C and -323T>A. Using transient transfection with wild type and diverse mutant constructs it was concluded that only the -83G>C mutation was capable of down-regulating PKLR promoter activity. In addition, nt -83G was found to be part of a regulatory element in the erythroid-specific PKLR promoter. In turn this regulatory element constituted a binding domain for an as yet unidentified trans-acting factor that mediates the effects of factors necessary for regulation of PK gene expression during red cell differentiation and matura-tion. The -83G>C mutation thus silenced erythroid-

Figure 3. Schematic representation of the PKLR gene and its erythroid-specific promoter, and the distribution of PKLR mutations that are associated with PK deficiency. Exons, but not introns, are drawn to scale. Exons are numbered and depicted as gray rectangles with 5’ and 3’-non-coding sequences in black. The open rectangle represents the liver-specific exon 2. Nucleotides are numbered starting from the ATG in red blood cell-specific exon 1. The location of the more than 170 mutations associated with PK deficiency is indicated by vertical lines. Double-sized vertical lines represent multiple base changes at the same nucleotide position. The three (large) deletions known to date are indicated by horizontal lines.

Figure 4. Ribbon representation of the human erythrocyte pyruvate kinase tetramer. The individual domains of subunit 1 are colored violet (N domain), slate (A domain), orange (B domain), and lime (C domain). Substrate analog phosphoglycolate and the allosteric activator fructose-1,6-diphosphate are shown in a stick representation and colored green and yellow, respectively. Metal ions in the active site are shown as blue (manganese) and purple (potassium) spheres. The figure was generated from the atomic coordinates of protein data bank entry 1I1U using the program PyMOL (DeLano, W. L. The PyMOL Molecular Graphics System (2002) on WWW http://www.pymol.org).
specific PKLR transcription completely and this explained the previously noted mono-allelic gene expression pattern. In conclusion, this patient, though compound heterozygosity at the DNA level, displayed de facto pseudo homozygosity for the Arg510Gln mutant. Therefore, the primary basis of hemolysis in this patient can be attributed solely to the aberrant kinetic properties of the PK variant.

**Patient 2 – genotype**

The second case concerns a female patient, also diagnosed at birth with severe hemolytic anemia due to PK deficiency. She underwent splenectomy at 4 years of age and further investigations were carried out at the age of 31 years. At this point she was anemic but only occasionally required blood transfusions. She showed massive reticulocytosis which was accompanied by a proportionately increased hexokinase activity (Table 1) but, in contrast, severely decreased PK activity. DNA sequence analysis displayed compound heterozygosity for two mutations that both concerned nucleotides involved in pre-mRNA processing. They were both located at the 5'-splice site at, respectively, nt +1 (IVS5+1G>A) and nt -1 (c.1436G>A). In addition, the latter mutation encoded an arginine to histidine substitution at residue 479 (Figure 5B). RNA analysis showed that normal splicing was completely abolished by the IVS5+1 mutation. Instead, two aberrant transcripts were produced that lacked either exon 5 or exons 5 and 6 together. Polysome profile analysis displayed that the first transcript was unstable. The latter nonsense transcript was only partially translated and, if stable, would encode a severely truncated PK monomer, not likely to be functional. RNA analysis of the second mutation (c.1436G>A) showed that correct splicing was largely maintained but, instead, mRNA levels were greatly reduced. Thus, ultimately, this mutation resulted in the production of low levels of an Arg479His PK monomer. This could be confirmed at the protein level, whereas no truncated protein could be detected that might have resulted from the other allele could be detected. In conclusion, like patient 1, this patient also displayed pseudo-homozygosity, in this case for the
Arg479His mutant. Though discrepancies have been noted with regard to the enzymatic behavior of this variant, the severe hemolysis in this patient can be attributed to the characteristics and, perhaps more importantly to the low levels of this mutant PK.

In summary, two patients suffering from severe hemolytic anemia due to PK deficiency were compound heterozygotes for a mutation in PKLR. Due to the effect of mutations that completely abolished transcription of PKLR (patient 1) and correct processing of PKLR pre-mRNA (patient 2), this compound heterozygosity at the DNA level resulted in pseudo-homozygosity at the protein level. In turn, this led to absent transcription of PKLR and correct processing of PKLR, this compound heterozygotes for a mutation in PKLR (patient 1) and PK-R 479His variants (patient 2) produced from the other allele in these patients. Clarifying the mechanism by which these mutations exert their effect on aberrant gene expression establishes the basis for a genotype-phenotype comparison.

Patient 1 – phenotype
Arginine 510 is located at the interface between the A/C domains in the PK monomer. Substitution of this residue by glutamine is likely to disrupt the local network of hydrogen bonds (Figure 5A). As stated, subunit and domain interactions are crucial for the allosteric transition from the inactive T-state to the active R-state and it seems likely that enzymatic function would be severely affected by the Arg510Gln mutation. Surprisingly, enzymatic characterization of the recombinant PK Arg510Gln mutant revealed a kinetic behaviour with respect to PEP and ADP that is very similar to that of the wild-type recombinant enzyme whereas the mutant is more susceptible to inhibition by ATP. The most striking feature of this mutant PK, however, is its dramatically lowered thermal stability and, consequently, accelerated intracellular proteolytic degradation. The PK Arg510Gln mutant thus results in a decreased level of enzyme in the cell, which accounts for the observed PK deficiency. In our patient, enzyme levels are likely to be decreased even further because the cells are only capable of about 50% of normal enzyme synthesis, thus aggravating a clinical manifestations.

In this case there is a clear and direct correlation between the patient’s genotype and phenotype. However, it is known that patients with identical genotypes may be affected differently. This is particularly clear in the case of the PK Arg510Gln mutant. The relative high frequency of this mutation enabled a direct comparison of 12 patients who were homozygous for this mutation. In theory these patients should have similar anemia. In practice, however, the severity of the disease and the well-being of the patients differed dramatically with clinical manifestations ranging from moderate to severe. Considering the decreased stability of this particular mutant, the differences in clinical expression in the homozygous patients may be (partly) attributed to, genetically determined, individual differences in intracellular proteolytic activity.

Patient 2 – phenotype
Arg479 is located in the C domain of the PK monomer. The arginine side chain is in the neighborhood of FDP but there is no direct interaction with this activator (Figure 5B). Furthermore, the crystal structure of the recombinant protein is identical to that of the wild-type recombinant protein and kinetic parameters are essentially unaffected by the amino acid substitution. Therefore, it is merely the severely reduced synthesis of mutant PK Arg479His that decreases the cellular PK content and leads to PK deficiency. There is one remarkable feature though which concerns the protein’s strongly reduced thermal stability as observed in patient 2. This was in agreement with the patient’s sensitivity to heat but contrasts with the nearly unaffected thermal stability of the recombinant mutant protein. This highlights the differences in fate and function of mutant enzymes in vitro and in vivo.

Enzyme levels in patient 2, like those in patient 1, are probably even more decreased because, again, only 50% of normal synthesis occurs in the cells. When comparing the clinical picture of patient 2 with that of two true PK 479His homozygotes, all three patients showed similar phenotypes. The true homozygotes were both severely affected during neonatal and infant life as was patient 2. After splenectomy and with increasing age the clinical picture improved, as frequently occurs among patients with PK deficiency. However, this did not apply to the sister of patient 2. This sister is 4 years older and has an identical PKLR genotype. She still requires blood transfusions on a more regular basis and, in addition, has more pronounced jaundice. Part of the explanation for the latter may involve the sister’s heterozygosity for a dinucleotide (TA) insertion polymorphism in the promoter of the gene that codes for UDP glucuronosyltransferase 1 (UGT1). This enzyme catalyzes bilirubin conjugation by the liver and the presence of the insertion is known to be actively involved in the pathogenesis of G6PD deficiency-associated neonatal hyperbilirubinemia. Patient 2 did not carry this polymorphism.

In summary, the PK-deficient phenotypes in both patients could be attributed to the enzymatic properties of the PK-R 510Gln (patient 1) and PK-R 479His (patient 2) mutants. These properties, however, appeared to be nearly unaffected in both cases.
Instead, the PK-R 510Gln is unstable whereas the PK-R 479His variant is produced in low amounts. This results in severely reduced intracellular levels of the respective mutant proteins in the red blood cells of both patients. Accordingly, this explains the PK-deficient phenotype although such a genotype to phenotype correlation is less evident in other patients with comparable genotypes.

**Other phenotypic modifiers**

The patients presented here are compound heterozygotes at the DNA level but can be regarded as pseudo-homozygotes at the protein level. Most PK-deficient patients, however, are compound heterozygotes for two missense mutations and will, therefore, also be compound heterozygotes at the protein level. Intracellularly, this results in the presence of two different mutant PK monomers, each with different enzymatic properties and different abilities to participate in tetramer formation. Consequently, if stable, five different PK-R tetramers may be assembled from two different PK monomers.

The two described patients clearly show that a patient’s phenotype is not solely dependent on the molecular properties of mutant proteins but rather reflects a complex interplay between physiological, environmental, and other (genetic) factors. Other putative phenotypic modifiers include differences in genetic background, concomitant functional polymorphisms of other glycolytic enzymes (many enzymes are regulated by their product or other metabolites), post-translational modification, epigenetic modification, ineffective erythropoiesis, and different splenic function.

The ability to compensate for the enzyme deficiency by overexpressing isozymes or using alternative pathways may also contribute to the PK-deficient phenotype. Persistent expression of the PK-M2 isozyme has been reported in the red blood cells of patients (and animals) with severe PK deficiency.20,21 It has been proposed that this compensatory increase in PK activity enables the survival of these patients. It has, however, also been reported that other patients survive in the absence of persistent expression of PK-M2.20 Interestingly, Basenji dogs that lack PK-R enzymatic activity as a result of a frameshift mutation express only the PK-M2 isozyme in their red blood cells.26 In addition, homozygous PK-deficient mice show delayed switching from PK-M2 to PK-R, resulting in delayed onset of the hemolytic anemia.27

It is important to consider that PK, and a number of other red blood cell enzymes are also expressed in other tissues. In general, the effects of deficiency of these enzymes are more pronounced in red blood cells, than in other cells, because of the long life span of the mature erythrocyte after the loss of protein synthesis. However, aberrant enzymatic function in non-erythroid tissues has been described in a case of PK deficiency associated with moderate cholestasis and increased aminotransferases.28

Future research aimed at determining the relationship between genotype and phenotype in PK deficiency will have to take these phenotypic modifiers into account.

### References

Glucose 6-phosphate dehydrogenase deficiency: from genotype to phenotype

Glucose 6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme critical in the redox metabolism of all aerobic cells (see Figure 1). G6PD deficiency has been a prototype of haemolytic anemias due to enzymopathy, i.e. to a primary abnormality of a red cell enzyme. G6PD deficiency is also a prime example of a hemolytic anemia due to an interaction between an intracorpuscular cause and an extracorpuscular cause, because in the majority of cases hemolysis is triggered by an exogenous agent.

Epidemiology

A distinction must be made between (i) the prevalence of G6PD deficiency as a genetic abnormality and (ii) the incidence of hemolytic anemia associated with G6PD deficiency. The genetic abnormality is distributed worldwide (see Figure 2): a conservative estimate is that at least 400 million people carry a G6PD deficiency gene. Areas of high prevalence are Africa, Southern Europe, the Middle East, South-East Asia and Oceania. In the Americas and in parts of Northern Europe G6PD deficiency is also quite prevalent as a result of migrations in relatively recent historical times. Although accurate quantitative data are lacking, fava beans are probably today the commonest trigger of hemolysis in G6PD-deficient subjects: therefore the incidence of this clinical manifestation can be identified with the epidemiology of favism. Fava beans are grown world-wide; they are a significant component of the diet particularly in the Middle East, in Iran and in Southern Europe.

Clinical manifestations

In view of the large number of people who have G6PD deficiency, it is important to note first of all that the vast majority remain clinically asymptomatic throughout their lifetime.

Neonatal jaundice

Although this is not always recognized, for reasons that are incompletely understood, G6PD deficiency is more likely to manifest during the neonatal period: indeed, the risk of developing neonatal jaundice is much greater in G6PD-deficient neonates than in G6PD-normal ones. The strength of the association between G6PD deficiency and neonatal jaundice appears to vary in different populations. The clinical picture of neonatal jaundice related to G6PD deficiency differs from the classical. Rhesus-related neonatal jaundice in two main respects: (i) it is very rarely present at birth, and the peak incidence of clinical onset is between day 2 and day 3; (ii) there is more jaundice than anemia, and the anemia is very rarely severe: in fact, it overlaps with physiological jaundice. Nevertheless, at the other end of the spectrum, neonatal jaundice can be very severe in G6PD-deficient babies, especially in association with prematurity, infection, and/or environmental factors (such as naphthalene-camphor balls, used in babies’ bedding and clothing), and it can cause kernicterus. Unfortunately inadequately managed neonatal jaundice associated with G6PD deficiency can produce permanent neurological damage.

Acute hemolytic anemia (AHA)

G6PD-deficient subjects are at risk of developing AHA in response to three types of triggers: (i) fava beans, (ii) infections, and (iii) drugs (see Table 1). Typically, a hemolytic attack starts with malaise, weakness, and abdominal or lumbar pain. After an interval of several hours to 2-3 days the patient develops jaundice and dark urine, due to hemoglobinuria. The onset can be extremely abrupt, especially with favism in children. The anemia is from moderate to extremely severe, it is usually normocytic and normochromic, and it is due largely to intravascular hemolysis: hence it is associated with hemoglobinemia, hemoglobin-
Table 1. Agents that can trigger hemolysis in subjects with G6PD deficiency.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Define association</th>
<th>possible association</th>
<th>Doubtful association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimalarials</td>
<td>Primaquine</td>
<td>Chloroquine</td>
<td>Quinacrine</td>
</tr>
<tr>
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<td>Sulfanilamide</td>
<td>Sulfanilamide</td>
<td>Sulfonamide</td>
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<tr>
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<td>Sulfacetamide</td>
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<td>Sulfasalazine</td>
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<tr>
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<td>Sulfapyridine</td>
<td>Sulfadoxine</td>
<td>Sulfadoxine</td>
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<tr>
<td>Sulfonamides</td>
<td>Sulfamethoxazole</td>
<td></td>
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<tr>
<td>Nitrofurans</td>
<td>Dapsone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antipyretic/analgesic</td>
<td>Nitrofurantain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antipyretic/analgesic</td>
<td>Acetanilid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other drugs</td>
<td>Nalidixic acid</td>
<td></td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>Other drugs</td>
<td>Nitrazole</td>
<td></td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Other drugs</td>
<td>Methilene blue</td>
<td></td>
<td>Vitamin K analogue</td>
</tr>
<tr>
<td>Other drugs</td>
<td>Phenaizopyridine</td>
<td></td>
<td>Ascorbic Acid</td>
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<tr>
<td>Other drugs</td>
<td>Septin</td>
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<td>p-Aminosalicylic acid</td>
</tr>
<tr>
<td>Other chemicals</td>
<td>Napthalene</td>
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<td>PAS</td>
</tr>
<tr>
<td>Other chemicals</td>
<td>Trinitrotoluene</td>
<td></td>
<td>Doxorubicin</td>
</tr>
</tbody>
</table>

Figure 1. The role of G6PD in intermediary metabolism. One of the products of the G6PD reaction, 6-phosphogluconolactone, is a precursor of pentose phosphate; whereas the other product, NADPH, is a coenzyme of many biosynthetic reactions, as well as an electron donor in the defense against oxidative stress. Several lines of evidence indicate that the latter is the most important role of G6PD in red cells.

Figure 2. World distribution of polymorphic G6PD-deficient mutants. The different shadings indicate the frequency of the G6PD deficient phenotype in the respective population. Modified from Vulliamy T, Luzzatto L. (2003).

uria and low or absent plasma haptoglobin. The blood film shows anisocytosis, polychromasia, spherocytes (Figure 3). The most typical feature is the presence of bizarre poikilocytes, with red cells that appear to have unevenly distributed hemoglobin (hemighosts), and red cells that appear to have had parts of them bitten away (bite cells or blister cells). A classical test, now rarely carried out, is supravital staining with methyl violet which, if done promptly, reveals the presence of Heinz bodies, consisting of precipitates of denatured hemoglobin, and regarded as a signature of oxidative damage to red cells (except for the rare occurrence of an unstable hemoglobin). The concentration of lactate dehydrogenase (LDH) is high as is that of unconjugated bilirubin, indicating that there is also extravascular hemolysis. The most serious threat from AHA in adults is the development of acute renal failure (this is exceedingly rare in children). Once the threat of acute anemia has passed, and in the absence of co-morbidity, full recovery form AHA associated with G6PD deficiency is the rule.
Chronic non-spherocytic hemolytic anemia (CNSHA)

A very small minority of subjects with G6PD deficiency has chronic anemia of variable severity. The patient is always a male, almost invariably develops neonatal jaundice, and in general he is investigated because of that or because of unexplained jaundice or because of gallstones later in life. Usually the spleen is moderately enlarged in small children, and subsequently it may increase in size sufficiently to cause mechanical discomfort, or hypersplenism, or both. The severity of anemia ranges in different patients from borderline to transfusion dependent. The anemia is usually normochromic but somewhat macrocytic, largely on account of reticulocytosis (up to 20 per cent or more). The red-cell morphology is not characteristic (hence the designation non-spherocytic). Bilirubin and LDH are increased. The bone marrow is normoblastic, unless there is superimposed folate deficiency. In CNSHA caused by G6PD deficiency, unlike in the AHA described above, hemolysis is mainly extravascular. However, the red cells of these patients are naturally also vulnerable to acute oxidative damage, and therefore the same agents that can cause acute hemolytic anemia in people with the ordinary type of G6PD deficiency will cause severe exacerbations in people with the severe form of G6PD deficiency.

Genetics

The gene encoding G6PD maps to the telomeric region of the long arm of the X-chromosome (band Xq28), physically very close to the genes for hemophilia A, dyskeratosis congenita and color blindness. The G6PD gene consists of 13 exons and spans some 18.5 kb. Structural and functional studies have revealed features of a housekeeping gene; this is in accord with the fact that G6PD is found in all cells. The X-linkage of the G6PD gene has important implications. First, as males have only one G6PD gene (i.e., they are hemizygous for this gene), they must be either normal or G6PD-deficient. By contrast, females, who have two G6PD genes, can be either normal or deficient (homozygous), or intermediate (heterozygous). Moreover, as a result of the phenomenon of X-chromosome inactivation, heterozygous females are genetic mosaics, and this in turn has clinical implications. Indeed, in most other (autosomal) enzyme deficiencies, heterozygotes are asymptomatic because cells with an enzyme level close to 50

Figure 3. Acute hemolytic anemia in G6PD deficiency. The blood film at the left is from a 22-year old Jamaican man who had received septrin; the blood film on the right is from a 4-year old Sardinian boy who had eaten a large dish of fava beans. Note the marked anisocytosis, numerous bizarre poikilocytes; in addition S, spherocytes; B, bite cells; hg, hemoghost; and nc, nucleated red cells.

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per cent of normal are biochemically normal. However, in the case of G6PD, as a result of X-inactivation, the abnormal cells of a woman heterozygous for G6PD deficiency are just as deficient as those of a hemizygous deficient man, and therefore just as susceptible to pathology. Thus, although G6PD deficiency is still often referred to as an X-linked recessive trait, this is a misnomer because a recessive trait is, by definition, not expressed in a heterozygote: instead, G6PD deficiency is expressed both biochemically and clinically in heterozygotes. Although on average heterozygotes have less severe clinical manifestations, individual heterozygotes may develop severe AHA.

**Biochemistry and pathophysiology**

G6PD catalyses the conversion of glucose 6-phosphate (G6P) to 6-phosphogluconolactone, with concomitant reduction of NADP to NADPH. NADPH in turn, via glutathione reductase, produces glutathione (GSH), required for the operation of GSH peroxidase; and NADPH also stabilizes catalase: these two enzymes are able to detoxify hydrogen peroxide, which is produced from oxygen radicals (such as superoxide) whenever cells are subjected to oxidative stress. Red cells are highly exposed to such stress for two reasons. First, oxygen radicals are generated continuously from within the red cells as hemoglobin cycles from its deoxygenated to its oxygenated form. Second, red cells are often directly exposed to a variety of exogenous oxidizing agents: for instance, phagocytosing granulocytes or certain glycosides present in fava beans.

The enzymatically active form of G6PD is either a dimer or a tetramer of a single protein subunit of 514 amino acids with a molecular mass of 59 096 Da. Some regions of the molecule critical for its functions have been identified because they are highly conserved in evolution. The G6P-binding site and the active center of the enzyme are located near lysine 205. Recently the three dimensional structure of G6PD has been solved (see Figure 4). In the dimer structure the two subunits are symmetrically located across a complex interface of β-sheets. The NADP binding site is near the N-terminus, and bound NADP is important for the stability of G6PD.

Since red cells have no protein synthesis, the activity of G6PD, like that of all other red cell enzymes,
decreases gradually during red-cell aging. For instance, in normal blood, reticulocytes have about five times more activity than that of the oldest 10% of red cells. As a result, during a hemolytic attack the oldest cells, which have less residual G6PD, will be selectively destroyed. With certain G6PD variants this phenomenon can be so marked that patients tested in the post-hemolytic period may be misclassified as G6PD normal: at this time, they may prove relatively resistant to further challenge.

Although there is a decrease in G6PD activity in most tissues in G6PD-deficient individuals, this decreased activity is less marked than in red cells, and it does not seem to influence the clinical expression. Only in some cases of CNSHA is the deficiency of G6PD so severe also in granulocytes that it becomes rate-limiting for their oxidative burst, with consequent increased susceptibility to some bacterial infections.

**Molecular basis of G6PD deficiency**

G6PD-deficient subjects have invariably been found to have mutations in the coding region of the G6PD gene (Figure 5). The current database of some 140 mutants consists, with few exceptions, of single missense point mutations, entailing single amino acid replacements in the G6PD protein. The exceptions are small deletions (of one to eight amino acids), and a few instances in which two point mutations rather than one are present (for instance, in G6PD A-, the variant most commonly encountered in Africa). In most cases these mutations cause G6PD deficiency by decreasing the in vivo stability of the protein: thus, the physiological decrease in G6PD activity that takes place with red cell aging is greatly accelerated. In some cases an amino acid replacement can also affect the catalytic function of the enzyme.

The mutations underlying CNSHA form a discrete subset. This much more severe clinical phenotype can be ascribed in some cases to adverse qualitative changes (for instance, a decreased affinity for the substrate, glucose 6-phosphate); or simply to the fact that the enzyme deficit is more extreme, because of a more severe instability of the enzyme. For instance, a cluster of mutations map at or near the dimer interface, and clearly they severely compromise the formation of the dimer. In such cases the steady-state level of G6PD is so low that, even in the absence of any oxidant challenge, it becomes limiting for the survival of red cells, which may have a life-span of between 10 and 50 days: this explains the CNSHA phenotype.

**Malaria selection**

G6PD is one of the best characterized enzyme protein polymorphisms in the human species. It would be quite extraordinary for a trait that causes significant pathology to spread widely and reach high frequencies in many populations without conferring some biological advantage. Indeed, clinical field studies and in vitro experiments strongly support the view that G6PD deficiency has been selected by *Plasmodium falciparum* malaria, by virtue of the fact that it confers a relative resistance to heterozygotes, and perhaps to hemizygotes as well, against this highly lethal infection. Different G6PD variants underlie G6PD deficiency in different parts of the world. Some of the more widespread variants are G6PD Mediterranean on the shores of this sea, in the Middle East and in India; G6PD A- in Africa and in Southern Europe; G6PD Vianchan and G6PD Mahidol in South-East Asia; G6PD Canton in China.
and G6PD Union worldwide. The heterogeneity of polymorphic G6PD variants is proof of their independent origin, and it supports the notion that they have been selected by a common environmental agent, in keeping with the concept of convergent evolution (see Figure 2).

**Laboratory diagnosis**
When clinical and hematological findings raise the suspicion of G6PD deficiency, this must be confirmed by measuring the red cell enzyme activity. A number of screening tests (the most popular being currently the fluorescence spot test) are available for diagnostic purposes in patients who are in the steady state. However, these semi-quantitative tests are not adequate for patients in the acute hemolytic or post-hemolytic period, or for those with other complications; nor can they be expected to identify all heterozygotes. Ideally, every patient found to be G6PD-deficient by screening should then be re-tested for confirmation by a quantitative assay. In normal red cells the range of G6PD activity, measured at 30°C, is 7 to 10 IU/g Hb. In G6PD-deficient males (or homozygous females) the level of G6PD in the steady state is, by definition, less than 50 per cent of normal; but with most variants it is less than 20 per cent and with some it is practically undetectable. In heterozygous females the level is intermediate and extremely variable; in some cases the diagnosis may, therefore, be difficult without family studies or DNA analysis. However, for practical purposes it is most unlikely that a woman will have clinical manifestations if her G6PD level is more than 70 per cent of normal.

**Management**

**Prevention**
The acute hemolytic anemia of G6PD deficiency in previously screened subjects is largely preventable by avoiding exposure to triggering factors. Of course, the practicality and cost-effectiveness of screening depends on the prevalence of G6PD deficiency in each individual community. Favism is entirely preventable by not eating fava beans. Prevention of drug-induced hemolysis is possible in most cases by choosing alternative drugs.

**Management of neonatal jaundice**
The management of this form of jaundice does not differ from that of neonatal jaundice due to causes other than G6PD deficiency. In most cases, prompt phototherapy is highly effective and sufficient; however, when bilirubin levels are above 300 µmol/L (or even less in babies who are premature, or who have acidosis or infection), exchange blood transfusion is imperative to prevent neurological damage.

A patient with AHA may be a diagnostic problem that, once solved, does not require any specific treatment at all; on the other hand, AHA may in some cases be a medical emergency requiring immediate action. In such cases immediate blood transfusion is imperative and may be life-saving. Hemodialysis may be necessary if there is acute renal failure.

**Management of CNSHA**
In general terms, CNSHA due to G6PD deficiency does not differ from that due to other causes (e.g. pyruvate kinase deficiency). If the anemia is not severe, regular folic acid supplements and regular hematological surveillance will suffice. It will be important to avoid exposure to potentially hemolytic drugs, and blood transfusion may be indicated when exacerbations occur, mostly in concomitance with intercurrent infection. In rare patients the anemia is so severe that they must be regarded as transfusion-dependent. Since, unlike in thalassaemia, there is no ineffective erythropoesis in the bone marrow of this type of CNSHA, a hyper-transfusion regimen aiming to suppress the bone marrow is not indicated. On the other hand, appropriate iron chelation should be instituted in patients requiring regular transfusions. Unlike in hereditary spherocytosis, there is no evidence of selective red-cell destruction in the spleen: however, in practice splenectomy has proven beneficial in severe cases. When a diagnosis of CNSHA is made, genetic counseling should be offered to the family. An important step is to establish whether the mother is a heterozygote; if she is, there is a 1 in 2 chance of disease in every subsequent male pregnancy. Prenatal diagnosis can be made by DNA analysis if the mutation is first identified in an affected relative.

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Molecular pathogenesis of the myelodysplastic syndromes, including the 5q- syndrome

This review summarizes the present state of our knowledge on the molecular basis of the myelodysplastic syndromes (MDS). In particular, the gene mutations and chromosomal abnormalities found in MDS are described. In addition, the early results of gene expression profiling by microarray analysis are discussed. It is probable that as our knowledge of the molecular basis of MDS grows, there will be a concomitant development of novel therapies targeting these abnormalities.

Point mutations in MDS.

Somatic mutations in several onco-genes, tumour suppressor genes or genes involved in the regulation of normal hematopoiesis have been described in MDS and implicated in the molecular pathogenesis of this disorder (Table 1). Most of the molecular abnormalities described have been associated with disease progression and there is little evidence to suggest that they represent initiating events in the development of MDS. None is specific for MDS; most occur in acute myeloid leukemia (AML) and usually at a higher frequency. The identification of the initiating events in MDS should be an important goal of future studies.

Mutations of the RAS gene family

Point mutations involving codons 12, 13, or 61 of members of the RAS gene family (primarily N-RAS) represent one of the most frequent molecular abnormalities found in MDS. These mutations act to prevent the hydrolysis of Ras-GTP and thus Ras is constitutively activated. The precise incidence of RAS mutations in MDS is controversial, but the incidence at diagnosis is probably in the range of 10% to 15% with some other patients acquiring these mutations during the course of their disease. RAS mutations have been associated with poor response to treatment and poor prognosis in most, but not all studies of MDS.1 Interestingly, hyper-activation of the RAS signaling pathway has been recently associated with MDS/AML with AML1/RUNX1 point mutations.2 The RAS/MAPK pathway is also frequently deregulated in the childhood MDS, juvenile myelomonocytic leukemia (JMML).

P53 mutations

The p53 tumor suppressor gene is involved in cell cycle regulation, apoptosis and the maintenance of genomic stability. Inactivating p53 gene mutations are found in approximately 5-10% of MDS cases, are generally demonstrable at the time of diagnosis and may have independent prognostic value. p53 gene mutations occur predominantly in the poor-risk FAB subtypes and are usually associated with deletions of the non-mutated allele (through a chromosome 17p deletion).3 The MDS cases harboring p53 mutations generally have complex karyotypic abnormalities, making it difficult to assess the contribution of the p53 mutations to the pathogenesis of MDS. These mutations are associated with the pseudo-Pelger-Huet anomaly and vacuoles in neutrophils giving rise to the 17p-syndrome, one of the rare phenotype-genotype associations reported in MDS (the principal one being the 5q-syndrome; see below).

FLT3 duplication in MDS

Internal tandem duplications (ITD) and other constitutively activating mutations of the receptor tyrosine kinase FLT3 represent the most frequent molecular abnormality found in AML. ITD of FLT3 occur less frequently in MDS and have been reported in approximately 5% of patients. Mutation of FLT3 is associated with a high risk of progression to AML.3

AML1/RUNX1 mutations

It has been argued that the most common point mutations detected in MDS to
Table 1. Point mutations in MDS.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Approximate frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>5-10%</td>
</tr>
<tr>
<td>NRAS</td>
<td>10-15%</td>
</tr>
<tr>
<td>FLT3</td>
<td>5%</td>
</tr>
<tr>
<td>PTPN11</td>
<td>30% juvenile myelo-monocytic leukaemia Rare in adult MDS</td>
</tr>
<tr>
<td>RUNX1/AML</td>
<td>23% advanced MDS</td>
</tr>
<tr>
<td>ATRX1</td>
<td>Mutated in acquired α thalassaemia MDS (ATMDS)</td>
</tr>
<tr>
<td>PIGA</td>
<td>Insufficient data</td>
</tr>
<tr>
<td>JAK2</td>
<td>5%</td>
</tr>
</tbody>
</table>

Recent studies have reported a high incidence of these mutations, particularly in the more advanced forms of MDS. Harada showed that 26/110 (23%) of patients with refractory anemia with excess blasts (RAEB), RAEB in transformation (RAEBt) and MDS/AML had RUNX1/AML mutations. Steensma described RUNX1/AML mutations in approximately 10% of patients with MDS, with 4/7 (57%) patients with RAEB-2 harboring these mutations. AML1/RUNX1 mutations have been associated with the presence of abnormalities involving chromosome 7 and with a high risk of progression to overt leukemia. However, there is no evidence to suggest that this mutation occurs even rarely in early MDS. Most AML1/RUNX1 mutants lose trans-activation potential, which leads to a loss of AML1/RUNX1 function. These data indicate that AML1 mutations are important in the molecular pathogenesis of MDS. Dr. Harada has recently suggested that MDS/AML with AML1 mutation represents a distinct clinicopathologic-genetic entity. It is interesting to note that AML1/RUNX1-deficient mice, in addition to splenomegaly and lymphomas, display features of an MDS.

JAK2 mutations in MDS

A somatic mutation in the JH2 autoinhibitory domain of JAK2 was recently described in polycythemia vera. The mutation leads to constitutive tyrosine phosphorylation activity that promotes cytokine hypersensitivity and induces erythrocytosis in a mouse model. Interestingly, JAK2 mutations have recently been described in 5% of patients with MDS, but there were no distinguishing clinical features among these patients. JAK2 mutations have also been described in approximately 8% of patients with CMML/aCML.

ATRX mutations in acquired alpha-thalassemia MDS

Specific gene mutations may be associated with unique MDS phenotypes. Rarely MDS is found in association with acquired alpha-thalassemia (ATMDS). This disorder has the features of MDS in addition to those of alpha-thalassemia, such as microcytic red cells and HbH on Hb electrophoresis. We demonstrated the marked down-regulation of the chromatin remodeling factor ATRX in the neutrophils of some patients with ATMDS using cDNA microarray analysis. This finding was the major clue that led to the identification of acquired somatic mutations in the ATRX gene in patients with ATMDS, now recognized as the common genetic basis of this disorder. Rarely ATMDS is associated with an acquired deletion of the alpha-globin gene cluster limited to the neoplastic clone.

Genetic predisposition to MDS- inherited gene mutations

Evidence in support of an inherited predisposition to MDS and related AML has been provided by studies of inherited constitutional genetic defects including the defective DNA repair of Fanconi anemia, deregulation of the RAS signal transduction pathway in NF1 and most recently mutation of SBDS in Shwachman-Diamond syndrome.

Important studies by Shannon and colleagues concerning familial MDS with chromosome 7q abnormalities led to speculation that genetic loci might exist that could predispose to chromosome instability, secondary loss of specific chromosomal regions (for example 5q, 7q, and 20q) and to the onset of MDS and AML in children and adults. Support for this hypothesis was provided by Song et al. in a study that determined the genetic basis of familial platelet disorder with leukaemia (FPD/AML). Affected individuals in the seven pedigrees studied all have a marked propensity to develop MDS and AML, often with abnormalities of chromosomes 5q and 7q. Inactivating mutations the AML1 gene were detected in these different pedigrees. The loss of function of a single allele of the AML1 gene is the genetic basis of FPD/AML and also appears to confer a susceptibility to the acquisition of secondary changes, including the loss of the chromosomal regions frequently associated with MDS and AML. This ground breaking discovery has led to a model for MDS/AML in which AML1 mutations predispose to chromosome instability resulting in the eventual loss of 5q and 7q chromosomal regions. It is interesting to note that some
Interestingly, associations between recurrent cytoge

tic abnormalities and provide us with important clues as to the loca

abnormalities found in MDS have prognostic value in therapy-re

hypothesis the two hit gene. In the absence of supporting data for Knudson’s mutations have yet been described in any candidate genes have been identified, no inactivating and normal hematopoiesis. Whilst several promising candidate genes have been identified, no inactivating mutations have yet been described in any candidate gene. In the absence of supporting data for Knudson’s two hit hypothesis the one hit mechanism also known as haploinsufficiency (a dosage effect resulting from the loss of a single allele of a gene) now appears most likely to be the mechanism of action.9

Table 2. Karyotype in MDS.

<table>
<thead>
<tr>
<th>Karyotype</th>
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<tbody>
<tr>
<td>Normal, del (20q), isolated del(5q), Y normal</td>
<td>IPSS good-risk markers</td>
</tr>
<tr>
<td>Complex karyotype, abnormalities of chromosome 7</td>
<td>IPSS poor-risk markers</td>
</tr>
<tr>
<td>Abnormalities of 11q23</td>
<td>Amplification or translocation of MLL gene</td>
</tr>
<tr>
<td>Abnormalities of 3q21, t(3:3) (q21;q26), inv (3) (q21;26)</td>
<td>Often associated with activation of EVI gene</td>
</tr>
<tr>
<td>t(5,12)(q33;p13)</td>
<td>PDGFR-B constitutive activation</td>
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</table>

Chromosomal deletions and monosomies are common in MDS and the del(5q) and -7/del(7q) are amongst the most frequently reported karyotypic abnormalities in MDS. Other recurrent deletions reported in MDS include del(20q), del(11q) and del(17p). It is unknown whether these chromosomal abnormalities are initiating events leading to the development of MDS or are secondary events. Notwithstanding the huge amount of research effort undertaken, the pathogenetic mechanism underlying most of the common deletions found in MDS remains undetermined. The chromosomal deletions commonly found in MDS are widely believed to harbour tumor suppressor genes, the loss of which may affect important processes such as growth control and normal hematopoiesis. Whilst several promising candidate genes have been identified, no inactivating mutations have yet been described in any candidate gene. In the absence of supporting data for Knudson’s two hit hypothesis the one hit mechanism also known as haploinsufficiency (a dosage effect resulting from the loss of a single allele of a gene) now appears most likely to be the mechanism of action.9

The 5q- syndrome

The 5q- syndrome is the most distinct of the MDS. It is important to recognize that the good prognosis for patients with the 5q- syndrome only applies to patients stringently defined: that is, they have <5% bone marrow myeloblasts and no additional chromosomal abnormalities.10 Importantly, there is a clear genotype-phenotype association in the 5q- syndrome, whereas, for most other chromosomal deletions in MDS and AML there is no such association. Using fluorescent in situ hybridization (FISH) and molecular mapping techniques our group identified the commonly deleted region (CDR) of the 5q- syndrome as the approximately 1.5 Mb interval at 5q32 flanked by D5S413 and the GLRA1 gene.11 We have been involved in the complete genomic annotation of this region. The CDR is gene rich and contains 48 genes, including the putative tumor suppressor genes MEGF1 and SPARC and several microRNA genes.11

The majority of the genes mapping within the CDR are expressed in CD34+ cells. Mutation and expression analysis of all 48 genes mapping to the CDR in patients with the 5q- syndrome is now essentially complete. No mutations have been identified. The majority of genes mapping within the CDR show a reduction in expression levels consistent with the loss of one allele in the CD34+ cells of patients with the 5q- syndrome. These data offer support for the proposal that haploinsufficiency (a gene dosage effect) for one or more of the genes mapping to the CDR is the pathogenetic basis of the 5q- syndrome. However, two genes mapping within the CDR showed expression ratios in the range consistent with loss of more than one allele (down-regulated by more than 50%) in the majority of patients with the 5q- syndrome and this observation requires further investigation.

The identification of the molecular targets of drug treatments in hematological malignancies can shed light on the molecular basis of the disease. Lenalidomide (Revlimid) is a new thalidomide analog that is particularly effective in patients with the 5q- syndrome.12 We, and others, are performing experiments to investigate the mode of action of lenalidomide on the bone marrow cells of patients with the 5q- syndrome. It is possible that such studies may shed some light on the pathogenetic basis of the 5q- syndrome. Interestingly, preliminary data from our collaborative studies with Eva Hellstrom-Lindberg showed expression of the SPARC gene, a counter adhesive protein mapping within the 5q- syndrome CDR.13

A recent study by Crescenzi et al. reported the deletion of several tumor suppressor genes in patients with sporadic MDS/AML have similar AML1 mutations.
with the del(5q) and additional karyotypic changes, but no additional deletions in patients with the 5q-syndrome. Similarly, we screened a group of patients with MDS and the del(5q) for mutations of FLT3, NRAS and p53; none of the patients with the 5q-syndrome was found to harbor mutations in these genes. These data suggest that the stability of the 5q-syndrome may be due to the absence of other additional abnormalities.

**Other MDS with the del(5q)**

The del(5q) is the most commonly reported deletion in *de novo* MDS and is found in 10-15% of all patients. The relationship between the 5q-syndrome and the other myeloid malignancies with the del(5q) is a complex issue. When the del(5q) occurs as the sole karyotypic abnormality in refractory anemia (5q-syndrome) it carries a good prognosis, otherwise it is among the worst prognostic indicators. Moreover, the del(5q) in AML, particularly secondary AML, invariably occurs together with other karyotypic abnormalities and frequently as part of a complex karyotype. Interestingly, mutations with loss of function of p53 are significantly associated with the del(5q) in t-MDS and t-AML after previous treatment with alkylating agents and are associated with genetic instability. The CDR at 5q31 identified in AML and the more aggressive forms of MDS by Le Beau et al. is 1-1.5Mb, and is flanked by D5S479 and D5S500. This region contains 18 genes including the candidate genes EGR1 and CTNNAL1. No inactivating mutations have been described in any candidate genes mapping to this interval. Low or absent expression of CTNNAL1 has been recently reported in a proportion of MDS/AML patients with a del(5q) by Liu et al. However, in our recent gene expression profiling study of MDS CD34+ cells none of the 20 patients with del(5q) investigated showed absent expression of CTNNAL1, most patients had expression levels consistent with the loss of one allele. The identification of more than one CDR of the del(5q) in malignant myeloid disorders suggests the existence of more than one pathogenetically relevant gene. This might be expected given the very different clinical features and prognoses observed in patients with the 5q-syndrome and patients with the more aggressive forms of MDS or AML and a del(5q).

**Del(5q) CDR animal modeling experiments**

Data from our laboratory and from others suggests that the deletions involving 5q may contribute to myeloid malignancy by haploinsufficiency. Using Cre-loxP based genome engineering we are currently producing chromosomal deletions in mice that mimic those seen in the 5q-syndrome in order both to define the effect of gene deletion on the development of MDS and to produce viable models of this disease (A. McKenzie, unpublished data). Similarly, mutant zebrafish harbouring deletions affecting the 5q-syndrome and MDS/AML 5q- CDRs syntenic regions are being generated in the laboratory of Dr T. Look.

**Other common deletions/monosomies in MDS -7/del(7q) and del(20q)**

The -7/del(7q) is found in 5-10% of patients with *de novo* MDS and in approximately 50% of all therapy-related cases. The del(7q) is invariably a bad prognostic marker in MDS/AML. It is now recognized that rearrangements and deletions involving chromosome 7 may be very complex and that multiple, distinct regions may contribute to the disease phenotype or progression. Whilst there is general agreement that 7q22 is involved in the majority of cases at least three other CDR have been identified mapping to 7q31, 7q32-33 and 7q35-36. Interestingly, methylation of p15 has been recently associated with the del(7q) in t-MDS, suggesting that inactivation of p15 and deletion of genes on 7q possibly co-operate in leukemogenesis. The del(20q) is found in 3-4% of patients with MDS. The AML/MDS CDR of the del(20q) has been narrowed to a 2.6Mb interval mapping to 20q12 and containing the candidate gene L3MBTL by Bench et al. The comprehensive mutational/expression analysis of all candidate genes assigned to the respective CDR on 7q and 20q in MDS is nearing completion in several laboratories. To date no inactivating mutations have yet been described in any candidate genes and it now seems probable that haploinsufficiency is the mechanism of action.

**Translocations**

In marked contrast to AML, balanced translocations are rare in *de novo* MDS. Balanced and unbalanced translocations are more frequent in therapy-related MDS. Most of the cytogenetic abnormalities found in AML are also found in MDS, however, certain balanced translocations found in AML, including the t(15;17), inv(16) and t(8;21) are never found in MDS. The cloning of translocation fusion genes in MDS has resulted in the identification of several novel fusion genes of importance to the pathogenesis in some patients. The leukemia-associated fusion genes identified include: AML1/MDS1-EVI1 in the t(3;21), TEL-PDGFBR in the t(5;12), PDGFBR-HIP1 in the t(5;7), NPM-MLF1 in the t(3;5), MEL1 in the t(1;3) and MLL-CBP in the t(11;16). The most frequent recurrent translocations in MDS are the t(5;8) (q21;q26) and the inv(3)(q21q26), which lead to the inappropriate activation of the EVI1 gene located at 5q26. Interestingly, EVI1 has recently been shown to
induce a MDS in a mouse model. Identification of MDS patients with translocations involving the PDGFR gene has immediate therapeutic implications since such patients are selectively responsive to PDGFR kinase inhibitors such as imatinib.

Cryptic translocations or insertions have also been reported in MDS, and it is likely that the incidence of such hidden abnormalities is underestimated. Recently, a case of adult MDS with a cryptic insertion producing a NUP98-NSD1 fusion was described by La Starza et al., this being a leukemia-associated fusion gene that we first identified in childhood AML.

**Complex karyotypes**

Complex chromosomal aberrations can be detected in a substantial proportion of patients with AML and MDS, de novo as well as therapy-related, and are associated with an adverse prognosis. The molecular basis of this genomic instability is unknown, but may be related to marked telomere shortening. The application of multiplex-FISH (M-FISH) has proven of value in the characterization of complex karyotypes and, in particular, has demonstrated the frequent copy gain or amplification of the MLL gene at 11q23. The del(5q) is commonly found in patients with MDS/AML and a complex karyotype and an interesting association between MLL amplification and the del(5q) has been noted in this setting.

**Gene expression profiling in MDS**

Gene expression profiling has the potential to provide novel insights into the molecular pathogenesis of MDS, to identify those genes/new molecular pathways important in disease evolution, and to derive a set of molecular markers that will have clinical utility in diagnosis, classification and prognosis. In a study by Hofmann et al. class membership prediction was used to identify a subset of 11 predictor genes expressed in CD34+ cells that could differentiate between patients with low-risk MDS, high-risk MDS and healthy controls. Preferential expression of the DLK1 gene in MDS was also reported. In an illuminating study, Chen et al. analyzed the gene expression profiles of CD34+ cells from MDS patients and reported distinct gene expression profiles, implicating specific pathogenetic pathways, for monosomy 7 or trisomy 8 MDS.

Most recently we have determined the gene expression profiles of the CD34+ cells of 55 MDS patients using a comprehensive array platform containing most human genes. These profiles showed many similarities to reported interferon-γ induced gene expression in normal CD34+ cells; indeed the two most up-regulated genes, IFIT1 and IFITM1, are interferon-stimulated genes (ISG). We suggest that alterations in the expression of ISG may play a role in the hematological features of MDS, such as peripheral blood cytopenias. The up-regulation of IFIT1 is a potential diagnostic marker for MDS. We determined whether distinct gene expression profiles were associated with specific FAB and cytogenetic groups. CD34+ cells from patients with refractory anemia with ringed sideroblasts (RARS) showed a particular gene expression profile characterised by up-regulation of mitochondrial-related genes, and in particular of those of heme synthesis (e.g. ALAS2). CD34+ cells from patients with the del(5q) had a distinct gene expression profile, characterized by downregulation of genes assigned to 5q, up-regulation of the histone HIST1 gene cluster at chromosome 6p21, and by the up-regulation of genes related to the actin cytoskeleton. Moreover, several markers were identified in this study that may prove useful for both diagnostic and prognostic purposes. This study provides important and new insights into the pathophysiology of MDS.

The relationship between the etiology of the 5q-syndrome and the other subtypes of refractory anemia (RA) remains unknown at a molecular level. Using gene expression profiling analyses of MDS CD34+ cells we recently identified a subset of genes able to discriminate patients with 5q- syndrome from the patients with RA and a normal karyotype. Seven patients with 5q- syndrome could be distinguished from seven patients with RA and a normal karyotype using 120 genes. Sixty-two of the 120 genes map to 5q of which five map to the CDR (RBM22, CSNK1A1, TCOF1, KIAA0194, TNIP1) (unpublished data).

**Methylation in MDS**

There is a common pattern of altered DNA methylation in malignancy. The bulk of the genome is less methylated than normal whereas some CpG islands become abnormally methylated. The CpG islands that become methylated in malignancy are frequently those associated with the promoters of genes involved in maintaining normal proliferation (e.g. p16, p15). There has been much interest in p15 methylation and MDS. A recent study examined four genes implicated in the development of AML for promoter CpG island hypermethylation in cells from patients with different stages of MDS. The highest rate of methylation was found for p15 (51%), followed by HIC1 (32%), CDH1 (27%), and ER (19%). Hypermethylation of p15 was associated with leukemic transformation of early MDS. Such findings of aberrant methylation in MDS have stimulated the use of DNA methyltransferase inhibitors such as 5-azacytidine and 5-aza-2'- deoxycytidine (decitabine).
Conclusions
Much of the enormous advance in our knowledge of leukemia stems from the study of specific translocations. Those of us studying MDS do not have this starting point with common translocations. Nevertheless it is our opinion that to move the field forward, a new classification of MDS based on genetics should be achieved. The acute leukemias and the lymphomas are clear evidence of the huge benefit to be gained from this approach. The central problem with MDS is its heterogeneity. Fortunately we now have the technology to achieve a better understanding of the genetic and biochemical basis for this heterogeneity.

References
Epidemiology and clinical outcome

Aging of the European population

It is a commonplace knowledge that myelodysplastic syndromes (MDS) affect mainly elderly patients, the median age being 70 years. However, most physicians are not completely aware of the repercussions that this fact will have on daily hematology practice in the future. The latest EUROSTAT projections on the aging of the European population are a matter of concern to all hematologists treating elderly people. The good news is that life expectancy has doubled in Western Europe over the last 200 years. However, the 2005 EUROSTAT projection indicates that the share of the population of working age (14-65) is expected to decrease strongly from 67.2% in 2004 to 56.7% in 2050. The share of the population aged between 0-14 will also be reduced, from 16.4% in 2004 to 13.4% in 2050, while the proportion of elderly people (aged >65) is expected to almost double in this period from 16.4% to 29.9% in 2050, or from 75.3 million to 134.5 million. The largest shares of elderly people in 2050 are expected in Spain (35.6%), Italy (35.3%), and Greece (32.5%). These countries are referred to as having hyperaging populations. The lowest proportions of elderly people are expected in Luxembourg (22.1%), the Netherlands (23.5%), and Denmark (24.1%). The proportion of very old people (>80 years) is expected to almost triple from 4.0% in 2004 to 11.4% in 2050, with the highest proportions expected in Italy (14.1%), Germany (15.6%), and Spain (12.8%). Given these developments, the European countries will have to face important health care problems both from a medical as well as from an economic point of view. The treatment of myelodysplastic syndromes with their increasing incidence at higher ages will be one of them.

Epidemiology of myelodysplastic syndromes

Epidemiological studies in MDS have been impeded for several reasons. Until 1982, when the updated French-American-British (FAB) classification was proposed, MDS were described by a plethora of terms making it almost impossible to use official statistics for the calculation of epidemiological data. The integration of MDS into the International Classification of Diseases coding systems was not performed until the 10th edition, which was published in 1994. Furthermore, the development of the WHO classification has again added complexity to the task. Morphologically speaking, two main problems persist: the lack of minimal diagnostic criteria for MDS and the insidious beginning of these disorders make it particularly difficult to correctly establish the diagnosis of early phases of the disease. Moreover, the notorious variability in the determination of blast cell counts between different pathologists makes central morphological review mandatory if data are to be used for epidemiological analyses. Finally, the completeness and accuracy of case registration in MDS databases is dependent on a multitude of factors including regional patient referral patterns, number of practicing haematologists with a special interest in the disease, and the intensity of examination (e.g. bone marrow punctures) of patients in the age groups with the highest incidences of MDS, i.e. over the age of 70 years. A number of centers with a specific interest in MDS have published robust data on the three main epidemiological aspects, i.e. age and sex distribution of the MDS population, incidence rates, and temporal trends of MDS occurrence.

Age and sex distribution

The median age of patients with MDS in European countries is about 70 years. A recent review reported median ages of 65 to 74 years by different European investi-
The largest database to date is the Düsseldorf Bone Marrow Registry that comprises over 28 is 71 years with no significant difference between the FAB or WHO subgroups. However, patients with secondary MDS are significantly younger, with a median age difference of 10 years, than patients with primary MDS. This reflects the fact that these patients have had treatment with chemotherapy or radiotherapy at younger age or had occupational exposure to organic solvents, herbicides, insecticides, or other environmental pollutants.

MDS occurs more commonly in men than in women. In most series, the male/female ratio varies between 1.1 and 2. This unbalanced ratio may indicate the importance of occupational factors in the development of some cases of MDS. For some entities the sex ratio is even more pronounced. The 5q- syndrome has a male to female ratio between 1:1.5 and 1:2, while chronic myelomonocytic leukemia is more common in men, with male/female ratio of 1.5 to 3.

The incidence of MDS

A small number of epidemiological studies have been conducted in Europe and have established figures on the crude and age-specific incidence rates of myelodysplastic syndromes. According to data in the Düsseldorf Bone Marrow Registry, the overall incidence of MDS between 1986 and 1990 was 4.1 per 100,000 inhabitants. As expected, incidence figures rose constantly with the increasing age of the population, amounting to 4.9 per 100,000 for people aged 50 to 70 years and 22.8 for people older than 70 years. Interestingly, a difference in incidence between men and women only became apparent in the population ≥70 years old, suggesting that long-term effects from previous occupational exposure to toxic substances may play a role in the development of MDS. Radlund and co-workers examined the incidence in the county of Jönköping in Sweden and found very similar results. During the period from 1988 to 1992 the age-specific incidence of MDS rates were 0.7 for people < 50 years, 1.6 for people aged 50 to 69 years, and 15 for people ≥70 years. Maynadié et al. examined the incidence of MDS in the area around Dijon, France. The crude incidence was 3.2 cases per 100,000 inhabitants per year, and again, incidence rates rose sharply in elderly people reaching a top value of 45 cases per 100,000 in men aged 80 and older. The highest incidence rates were reported by British investigators in the East Dorset Health District in England. By actively contacting 2,926 patients over the age of 55 years of a busy general practice and performing repeated blood samples (and bone marrow biopsies, if appropriate), they established that the incidence of MDS was 12.6 cases per 100,000 inhabitants per year. Age-specific incidence rates were 5.3 for patients aged 50 to 59 years, 15 for patients aged 60 to 69, 49 for patients aged 70 to 79 years, and 89 for patients aged 80 years and older. It is unclear why the incidence in the latter investigation is about three times higher than that in other studies. One factor may be the more active approach of the authors by inviting elderly patients to undergo blood examinations even if these patients were not complaining of any relevant symptom.

Apparent increase of myelodysplastic syndromes

The Düsseldorf Bone Marrow Registry experienced a steady rise in new cases during the first years of its existence. Since 1986, however, the annual new incidence in the town district of Düsseldorf has stabilized. The initial apparent rise in MDS incidence is likely to be due to several factors: Before 1982, when the FAB classification was not established, a number of cases might not have been diagnosed as MDS. Because of the special interest of the institution in MDS, there was an increase in the number of elderly patients being referred, and a greater likelihood of bone marrow examination in this elderly patient population. The proportion of patients older than 60 years among new entries to the register increased from 42% in 1975 to 54% in 1990. A rising number of cases of secondary MDS due to more intensive chemotherapy at younger ages may also partly account for an apparent increase of MDS cases, although this effect cannot be very important. The Düsseldorf Registry included only 5% of patients with therapy-related MDS at the time of publication of their epidemiological data, which precludes a relevant effect of secondary MDS cases on the overall incidence calculation.

Clinical outcome of myelodysplastic syndromes

The major clinical challenge in the care of MDS is their prognostic heterogeneity, and hence, the difficulty to assign the appropriate risk-adapted therapy to the individual patient. A number of research groups have developed scoring systems to refine the prognostic value of the FAB, which was based merely on morphological information. These systems have included, along with the bone marrow blast count, a number of easily obtainable parameters (e.g., age, neutrophils, hemoglobin, lactate dehydrogenase). Other scores based on the FAB classification included the karyotype of patients as an important prognostic marker. After the publication of the WHO classification for MDS, large databases reclassified their patient samples according to the WHO proposals. They were able to show a prognostic significance of this novel classification, although not uniformly. Today, the most widely accepted prog-
nostic score is the International Prognostic Scoring System (IPSS), devised by Greenberg et al. This scoring system reliably differentiates between four risk groups that show significant differences in both overall survival and in the transformation rate into AML (Figure 1). This system is based on three characteristics of the affected patients’ hematopoietic system: the bone marrow blast count, the number and extent of peripheral cytopenias, and the cytogenetic risk group.

**Prognostic impact of cytogenetic subgroups**

Cytogenetic abnormalities are detected in about 50% of patients with primary myelodysplastic syndromes. The IPSS encompasses three cytogenetic risk groups: the prognostically favorable group includes a normal karyotype, an isolated del(5q), isolated del(20q), and isolated -Y. The poor prognostic karyotypes are represented by chromosome 7 anomalies and complex karyotypic changes, defined as ≥3 karyotypic anomalies. A recent multicenter analysis from Germany and Austria on the prognostic impact of chromosomal aberrations on 2,124 patients may lead us to reconsider the dogma of the dismal prognosis of chromosome 7 anomalies. In fact, in this analysis, patients with isolated deletions of chromosome 7 and isolated del(7q) had an intermediate prognosis, defined as a median overall survival of 12 to 24 months. Moreover, patients with an isolated t(7q) ended up with a favourable prognosis with a median overall survival exceeding 24 months. Other patients who were shown to belong to the good prognostic subgroup were patients with isolated del(12p), isolated del(9p), and isolated t(5q).

Previously, Spanish investigators had also found del(12p) to be associated with a good prognosis and isolated del(7q) and single -7 anomalies to have an overall survival of >12 months. Although both the data of Solé and Haase are based to some extent on patients that had undergone chemotherapy (about 20% of patients had received some form of chemotherapy), these findings indicate that further analysis of these subgroups should be performed.

**What to do in the absence of cytogenetic data**

The gold standard for risk assessment in MDS still remains the IPSS, until additional investigations including long-term follow-up of evaluated patients corroborate the above-mentioned findings. Thus, to make a reliable assumption regarding the prognosis of a patient, it is mandatory to assess the bone marrow blast count correctly and to perform a bone marrow cytogenetic examination. However, although of crucial importance, the latter is not routinely being

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<th>Risk score</th>
<th>% pts</th>
<th>Median Overall Survival</th>
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<tr>
<td>Low</td>
<td>20%</td>
<td>82 months</td>
</tr>
<tr>
<td>Intermediate-1</td>
<td>30%</td>
<td>31 months</td>
</tr>
<tr>
<td>Intermediate-2</td>
<td>20%</td>
<td>19 months</td>
</tr>
<tr>
<td>High</td>
<td>30%</td>
<td>8 months</td>
</tr>
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done in a large proportion of patients. Two examples underline this statement. The Düsseldorf Bone Marrow Registry has compiled data on over 2,800 MDS patients, but only about 1,000 have been registered with complete cytogenetic data. The multicenter American trial that led to the approval of 5-azacytidine for all subtypes of MDS in the United States comprised 191 patients, but cytogenetic data were available for only 81 of them. Therefore, the clinical reality teaches us that there is still need for easy-to-use prognostic scoring systems for MDS patients for whom cytogenetic data are lacking. As mentioned above, a number of investigators have published such scoring systems, which enable the clinician to assess the patients’ fate reliably. The Düsseldorf Score comprising hemoglobin level, platelet count, bone marrow blast count and lactate dehydrogenase (LDH) level (Table 1) is especially powerful in determining both high and low risk patients. LDH levels are being used in this score as a surrogate for intramedullary cell turnover, i.e. as a marker of both apoptosis and proliferation. In multivariate analysis, the LDH level has proven to be an independent prognostic variable in MDS and has recently been added to the IPSS for refinement of its prognostic accuracy. The result was astonishing: by combining the IPSS with the patients’ LDH level, it was possible to identify both more benign and more aggressive risk groups than with the classic IPSS. The authors defined a very low risk category for patients with an IPSS low-risk score and a normal LDH and showed that their median overall survival reached 107 months, i.e. significantly longer than the median survival time of the original IPSS low risk category applied to this database (median survival 88 months). Moreover, the median survival of patients with a low risk IPSS who had an increased LDH was not better than that of patients with intermediate-1 risk with normal LDH levels (Table 2).

### Table 1. The Düsseldorf score

<table>
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<tbody>
<tr>
<td>Blast count</td>
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<tr>
<td>&lt;5%</td>
<td>0</td>
</tr>
<tr>
<td>≥5%</td>
<td>1</td>
</tr>
<tr>
<td>LDH</td>
<td></td>
</tr>
<tr>
<td>within normal range</td>
<td>0</td>
</tr>
<tr>
<td>elevated</td>
<td>1</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td></td>
</tr>
<tr>
<td>≥9 g/dL</td>
<td>0</td>
</tr>
<tr>
<td>&lt;9 g/dL</td>
<td>1</td>
</tr>
<tr>
<td>Platelet count</td>
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</tr>
<tr>
<td>≥100,000/µL</td>
<td>0</td>
</tr>
<tr>
<td>&lt;100,000/µL</td>
<td>1</td>
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Score A point count: 0: low-risk; Score B count 1-2: intermediate risk; Score C point count: 3-4: high risk.

### Table 2. Prognosis of risk groups according to IPSS+LDH for all FAB types

<table>
<thead>
<tr>
<th>IPSS</th>
<th>median survival (months)</th>
<th>LDH</th>
<th>median survival (months)</th>
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<tbody>
<tr>
<td>Low</td>
<td>263</td>
<td>normal</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td></td>
<td>elevated</td>
<td>52</td>
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<tr>
<td>Intermediate-1</td>
<td>295</td>
<td>normal</td>
<td>180</td>
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<td></td>
<td></td>
<td>elevated</td>
<td>108</td>
</tr>
<tr>
<td>Intermediate-2</td>
<td>153</td>
<td>normal</td>
<td>92</td>
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<td></td>
<td></td>
<td>elevated</td>
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<tr>
<td>High</td>
<td>181</td>
<td>normal</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>elevated</td>
<td>87</td>
</tr>
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</table>

All differences between subgroups in median overall survival were statistically significant (p<0.05)

### A WHO based scoring system

The IPSS is based on the FAB classification and includes refractory anemia with excess blasts in transformation (RAEB-t) (with 20 to 29% bone marrow blasts), as well as chronic myelomonocytic leukemia (CMML). However, with the WHO classification gaining increasing acceptance in the MDS community, there was need to establish a prognostic scoring system that includes the morphological subtypes defined by the WHO. Compared to the IPSS, this approach would have the additional advantage of including dysplasia as defined by the WHO into a prognostic scoring system. An Italian-German Cooperative Group has submitted a first proposal of such a scoring system, named the WHO-based Prognostic Scoring System (WPSS). The WPSS is based on the WHO morphology classification, the IPSS karyotype risk groups, and the transfusion requirements of the individual patients. The inclusion of transfusion requirements as a risk factor is based on data presented by Italian researchers showing a worse prognosis for patients who are dependent on regular transfusions. This scoring system allowed five risk groups to be differentiated by significantly different median overall survival times. The very low risk group comprises patients with unilineage MDS (erythroid dysplasia only with or without ring sideroblasts) with low risk cytogenetics according to the IPSS and no transfusion burden, or non-transfusion-dependent patients with the 5q-syndrome. Indeed, this subgroup has a median overall survival that is not different from the age-matched standard mortality ratios and therefore represents real low-risk MDS. On the other hand, although high-risk populations such as patients with RAEB-t and CMML, are excluded from the WHO MDS definition, the very high-risk population in the WPSS had median overall survival times that were comparable to those of patients with untreated AML.
Concluding remarks

Myelodysplastic syndromes are common diseases that affect about 4/100,000 patients per year. Given the rapid aging of the European population and the exponentially rising incidence of MDS in elderly patients, the prevalence of these syndromes is going to increase sharply in the near future. Detailed knowledge of risk factors and effective prognostic scoring systems are prerequisites for sensible therapeutic decisions in this heterogeneous disease. The presented data will enable us to use new tools that might affect the survival of patients by improving their risk stratification. A next step should be the development of dynamic prognostic factors and scoring systems that allow for prognostic assessment throughout the course of the disease, including during supportive care, immunomodulatory treatment, growth factor therapy, epigenetic or cytotoxic therapy.

References

Treatment of myelodysplastic syndromes represents a challenge for several reasons, which include the clinical heterogeneity of these conditions and the lack of therapeutic options that can be employed and are effective in most patients.1

The clinical heterogeneity of myelodysplastic syndromes is best illustrated by the observation that these disorders range from conditions with a near normal standardized mortality ratio to entities with a mortality rate close to that of acute myeloid leukemia (AML).1 The World Health Organization (WHO) classification of myeloid neoplasms (Table 1) provides clinicians with a very useful tool for defining the different subtypes. This classification is not only useful for diagnostic purposes but also has a relevant prognostic value, and can therefore be used to facilitate clinical decision-making in the individual patient.3

Several therapeutic tools have been proposed in the last decades but only a few have survived the evidence-based criteria of efficacy.1 In the last few years, Italian4 and British5 guidelines for the therapy of myelodysplastic syndromes have been published. This review article will examine both established and novel therapeutic options for myelodysplastic syndromes. Searches for ongoing clinical trials were performed at http://www.clinicaltrials.gov.

The need for an accurate prognostic assessment of the individual patient aimed at implementing a risk-adapted treatment

A proposal for standardized diagnostic and prognostic procedures for the myelodysplastic syndromes was prepared within the European LeukemiaNet (http://www.leukemia-net.org/ WP8_SOP.pdf).

Before taking any therapeutic decision it is recommended that the diagnostic process is carefully reviewed in order to be sure that the individual patient has a myelodysplastic syndrome. The current diagnostic approach includes assessment of peripheral blood and bone marrow morphology (to evaluate abnormalities of peripheral blood cells and hematopoietic precursors), bone marrow biopsy (to determine marrow cellularity and topography), and cytogenetics (to identify non-random chromosomal abnormalities). The combination of overt marrow dysplasia and clonal cytogenetic abnormality allows a conclusive diagnosis of myelodysplastic syndrome, but this is found in only a portion of patients.1

Although a bone marrow biopsy may be considered too invasive for elderly patients, it provides extremely useful diagnostic information (cellularity, fibrosis, malignant cells, topography). The British guidelines6 conclude that bone marrow histology complements the morphological information obtained from a marrow aspirate and hence a trephine biopsy should be performed in all patients with suspected myelodysplastic syndrome in whom bone marrow examination is indicated. Flow cytometry immunophenotyping may allow a quantitative evaluation of marrow dysplasia and is useful in the work-up of individual patients.6,7 In addition, novel molecular markers will likely make the diagnostic process more reliable.8

A risk-adapted treatment strategy is mandatory for disorders that range from indolent conditions lasting years to forms approaching acute myeloid leukemia. A patient’s individual risk is generally defined by using a prognostic scoring system.9-12 So far the International Prognostic Scoring System (IPSS)9— based on the percentage of marrow blasts, cytogenetic pattern and number and degree of cytopenias – has been commonly used for predicting survival and leukemic risk. Since cytogenetics analysis is not always technically successful, the WHO classification can be used for defining risk groups (Table 1). Moreover, a novel prognostic scoring system based on the WHO classification, cytogenetic, information and transfusion requirements (the so-called WPSS) may
further improve the capacity of the WHO classification to stratify patients with myelodysplastic syndrome and may be more useful than IPSS in clinical decision-making.13

Not all patients need to be treated: watchful-waiting strategy

The approach to a patient with myelodysplastic syndrome should begin with a period of observation, with sequential peripheral blood counts - and sometimes bone marrow examinations - to assess the rate of progression, if any.

Not all patients must be treated. If the IPSS risk is low and anemia is mild (Hb > 10 g/dL) patients do not need any treatment and can be just followed. This watchful-waiting strategy might change in the future if safe treatments capable of modifying the natural history of the disease are developed.

Supportive care, transfusion therapy and iron chelation therapy: critical issues for patients who may have a nearly normal life expectancy

According to evidence-based practice guidelines,4 the vast majority of patients should receive supportive therapy at present.1 Once anemia is symptomatic, regular red cell transfusions and iron chelation are the mainstay of therapy for many individuals with myelodysplastic syndrome. Pre-transfusion hemoglobin levels may range between 7 and 9 g/dL according to the patient’s performance status and co-morbidities.

The life expectancy of patients with isolated erythroid lineage dysplasia aged 70 years or older is not significantly shorter than that of the general population.13 Avoiding disease complications is, therefore, very important in these individuals. Only a few studies in the past have examined the impact of transfusion iron overload on survival in myelodysplastic syndromes.14-16 We recently showed that the development of transfusion dependency may worsen the survival of patients with myelodysplastic syndrome.3,17 Although this poor prognosis partly reflects the severity of bone marrow failure, our observations also suggest that development of secondary iron overload per se can worsen the survival of transfusion-dependent patients.17

Despite the limited evidence, both the British5 and Italian6 guidelines recommend iron chelation in patients with myelodysplastic syndrome and regular need for transfusion. Iron chelation should be considered once a patient has received between 20 and 50 units of red cells, but only in patients for whom long-term transfusion therapy is likely (see median survivals in Table 1), or when an allogeneic stem cell

Table 1. WHO classification and criteria for the myelodysplastic syndromes: information is from Vardiman et al.2 Survival data are from the ad hoc database of the Department of Hematology, IRCCS Policlinico San Matteo, Pavia, Italy.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Blood findings</th>
<th>Bone marrow findings</th>
<th>Median survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refractory anemia (RA)</td>
<td>Anemia, no or rare blasts.</td>
<td>Erythroid dysplasia only, &lt; 5% blasts, &lt; 15% ringed sideroblasts.</td>
<td>109* months</td>
</tr>
<tr>
<td>Refractory anemia with ringed sideroblasts (RARS)</td>
<td>Anemia, no blasts.</td>
<td>Erythroid dysplasia only, &lt; 5% blasts, ≥ 15% ringed sideroblasts.</td>
<td>103* months</td>
</tr>
<tr>
<td>Refractory cytopenia with multilineage dysplasia (RCMD)</td>
<td>Cytopenia (bicytopenia or pancytopenia), no or rare blasts, no Auer rods, &lt;1 x 10^9/L monocytes.</td>
<td>Dysplasia in ≥ 10% of cells in 2 or more myeloid cell lines, &lt; 5% blasts, no Auer rods, &lt;15% ringed sideroblasts.</td>
<td>40* months</td>
</tr>
<tr>
<td>Refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS)</td>
<td>Cytopenia (bicytopenia or pancytopenia), no or rare blasts, no Auer rods, &lt;1 x 10^9/L monocytes.</td>
<td>Dysplasia in ≥ 10% of cells in 2 or more myeloid cell lines, &lt; 5% blasts, no Auer rods, ≥ 15% ringed sideroblasts.</td>
<td>51* months</td>
</tr>
<tr>
<td>Refractory anemia with excess blasts-1 (RAEB-1)</td>
<td>Cytopenia, &lt;5% blasts, no Auer rods, &lt;1 x 10^9/L monocytes.</td>
<td>Unilineage or multilineage dysplasia, 5% to 9% blasts, no Auer rods.</td>
<td>24 months</td>
</tr>
<tr>
<td>Refractory anemia with excess blasts-2 (RAEB-2)</td>
<td>Cytopenia, 5-19% blasts, occasional Auer rods, &lt;1 x 10^9/L monocytes.</td>
<td>Unilineage or multilineage dysplasia, 10% to 19% blasts, occasional Auer rods.</td>
<td>10 months</td>
</tr>
<tr>
<td>Myelodysplastic syndrome, unclassified (MDS-U)</td>
<td>Cytopenia, no or rare blasts, no Auer rods.</td>
<td>Normal to increased megakaryocytes with hypolobated nuclei, &lt;5% blasts, no Auer rods, isolated del(5q)</td>
<td>37 months</td>
</tr>
<tr>
<td>MDS associated with isolated del(5q)</td>
<td>Anemia, &lt;5% blasts, platelet count normal to increased.</td>
<td>Unilineage dysplasia in granulocytes or megakaryocytes, &lt;5% blasts, no Auer rods.</td>
<td>104* months</td>
</tr>
</tbody>
</table>

*Survival estimates of RA, RARS and MDS associated with isolated del(5q) are not significantly different. **Survival estimates of RCMD and RCMD-RS are not significantly different.
transplantation is planned. Desferrioxamine 20-40 mg/kg per day should be administered by 12 h subcutaneous infusion or by two daily subcutaneous bolus administrations\(^\text{18}\) for 5 days per week. The target ferritin concentration is <1000 ng/mL.

Deferasirox (ICL670) has proven to be an effective oral iron chelator in patients with thalassemia major\(^\text{19,20}\) and in those with myelodysplastic syndrome.\(^\text{21}\) In November 2005, the US Food and Drug Administration (FDA) approved the use of deferasirox for treatment of chronic iron overload due to multiple blood transfusions in patients 2 years of age or older. Deferasirox can now be employed for treatment of patients with myelodysplastic syndrome in the US and Switzerland. A European Medicines Agency (EMEA) decision is awaited in 2006. Two phase II studies evaluating the safety and tolerability of oral deferasirox in adult transfusion-dependent myelodysplastic syndrome are currently ongoing (http://www.clinicaltrials.gov).

**Treatment of anemia with hematopoietic growth factors in low risk patients who are not candidates for allogeneic stem cell transplantation: who can actually benefit from these treatments**

This topic has been covered comprehensively by Hellström-Lindberg, and the reader is referred to her review article for details.\(^\text{22}\)

Erythropoietin therapy is largely employed but only a small proportion of patients really benefit from this treatment. Responsive patients are mainly those with early disease, inadequate endogenous erythropoietin productions and no regular need for blood transfusion.\(^\text{1}\) As a practical recommendation patients with moderate to severe anemia (Hb < 10 g/dL) and refractory anemia or refractory anemia with ring sideroblasts, (RARS), should have their serum erythropoietin level assayed. Those with serum erythropoietin levels lower than 200 mU/mL should be considered for erythropoietin therapy.

The British Expert Panel\(^\text{1}\) concluded that patients with RARS are more likely to respond to the combination of erythropoietin plus granulocyte colony-stimulating factor (G-CSF). The response to treatment with erythropoietin + G-CSF can be predicted by combining information about pretreatment transfusion need (≤2 units per month) and serum Epo (≤500 mU/mL) using the decision model developed by Hellstrom-Lindberg.\(^\text{21}\) Any treatment with erythropoietin + G-CSF should be performed within a clinical trial or a registry study.


**Allogeneic stem cell transplantation: who should be considered as a candidate for this potentially curative treatment?**

At present, the only treatment that can definitely prolong survival is allogeneic stem cell transplantation (SCT). In a BMTR study it was concluded that transplantation from an HLA-identical sibling offers the possibility of long-term, disease-free survival to patients with myelodysplastic syndromes, and that the best candidates are younger patients with a low percentage of blasts and preserved platelet counts.\(^\text{21}\) It can be estimated that approximately one third of patients receiving an allogeneic transplantation are cured with this treatment, but only about 8-10% of all patients are eligible and have a suitable donor.\(^\text{1}\)

The Italian Expert Panel\(^\text{1}\) agreed on the following recommendations concerning stem cell transplantation:

a) the decision to perform allogeneic SCT should be shared with the patient, whose risk aversion and performance status should be taken into account (Recommendation level D);

b) considering patients with a moderate risk aversion, allogeneic SCT from an identical sibling should be recommended to patients younger than 55 years with an IPSS risk class intermediate-1, intermediate-2 or high (Recommendation level B);

c) patients under 40 years old with a low IPSS risk score are also candidates for allogeneic SCT if they have moderate or severe anemia (Hb<10 g/dL) (Recommendation level D);

d) allogeneic SCT from an unrelated donor should be recommended to patients younger than 40 years who do not have a related donor but are in IPSS risk class intermediate-1, intermediate-2 or high. Patients younger than 40 years with a low IPSS risk score are also candidates for allogeneic SCT from an unrelated donor when they have a good performance status (ECOG 1-2), unfavorable cytogenetics or severe neutropenia (Recommendation level D).

The optimal timing of stem cell transplantation from HLA-identical siblings is a critical point when making a clinical decision for an individual patient. Considerable evidence exists that the earlier the transplantation is performed the better the outcome. However, many patients with low-risk myelodysplastic syndrome experience a long survival without sign of disease progression. For these patients, the risks of immediate morbidity and mortality associated with transplantation are often felt to be unacceptable high. A decision analysis from the International Bone Marrow Transplant Registry\(^\text{24}\) demonstrated that life expectancy of patients with low-risk IPSS scores (low- and intermediate-1-risk groups) who have HLA-identical siblings was higher when transplantation is delayed by some period but performed prior to the development of AML. When quality of
life utilities were incorporated into the decision model, a delayed approach to transplantation remained the dominant strategy. For high-risk (IPSS intermediate-2 and high) patients, transplantation soon after the diagnosis confers the best prognosis. Although potential sources of bias could have affected this analysis, it provides physicians with a base of evidence to plan the optimal timing of transplantation for their patients.

Another issue to be taken into account when implementing transplantation strategies tailored on the individual risk is the choice of the preparative regimen to be used. In the late 1990s, reduced-intensity and non-myeloablative conditioning regimens have been introduced into clinical practice. These schedules have been shown to reduce transplant-related toxicity and mortality, significantly, as well as to extend the eligibility to transplantation to patients in whom standard transplantation is contraindicated due to age or poor performance status. According to the Italian guidelines, no more than 10% of all patients are eligible for a myeloablative allogeneic stem cell transplantation. This proportion increases to about 20% when the British criteria, which consider the use of non-ablative allogeneic stem cell transplantation in 50-65-year-old patients, are implemented in a scenario analysis including a large cohort of MDS patients diagnosed at the Division of Hematology of the University of Pavia Medical School & IRCCS Policlinico San Matteo, Pavia, Italy (unpublished data).

In the estimation of the individual risk/benefit balance for allogeneic stem cell transplantation, besides defining disease-related and demographic variables, a critical task is identifying and assessing comorbidities that have a substantial impact on non-relapse mortality. Recently, a Hematopoietic Cell Transplantation-specific Comorbidity Index (HCT-CI) for predicting risk of non-relapse mortality has been developed and validated. Based on data from over 1000 MDS patients, the most significant comorbidities were identified and scored according to coefficients from Cox proportional hazards regression. Despite some limitations, this study provided a reliable base of evidence for assessing the risks of patients who are candidates for allogeneic stem cell transplantation.

Intensive AML-like chemotherapy: a therapeutic option for a subgroup of relatively young high-risk patients with favorable cytogenetics

Intensive chemotherapy can be employed in patients with an increase in marrow blasts, but complete remissions are mainly obtained in relatively young individuals with favorable cytogenetics. In a recent study, younger age, good performance status and favorable cytogenetics were found to be independent prognostic factors associated with survival.

In particular, among 82 patients younger than 65 years with a normal karyotype, the complete remission rate was 67% and 5-year survival rate 27%.

The Italian Expert Panel agreed that:

- AML-like chemotherapy is highly recommended for patients not candidates for stem cell transplantation, who are younger than 55 years and have an intermediate-2 or high IPSS risk score (Recommendation level A);
- AML-like chemotherapy is also recommended for patients aged between 55 years and 65 years with an intermediate-2 or high IPSS risk score and a good performance status (ECOG 0-1) (Recommendation level D);
- standard or high-dose ARA-C combined with anthracyclines or fludarabine are the recommended drug associations (Recommendation level B).

Since the median remission duration is usually short due to a high incidence of early relapses, transplantation with autologous stem cells after remission induction and consolidation chemotherapy has been employed. However, stem cell mobilization may be problematic in these patients, and the available evidence does not allow firm conclusions on any additional effect of autologous stem cell transplantation in patients with myelodysplastic syndrome who have achieved complete remission with intensive chemotherapy.

5-azacytidine and 5-aza-2-deoxycytidine (decitabine)

As discussed in a comprehensive review by Hellström-Lindberg, DNA hypermethylation may be responsible for the pathogenesis of myeloid neoplasms. The DNA hypomethylating pyrimidine analogs 5-azacytidine and 5-aza-2-deoxycytidine (decitabine) might reduce hypermethylation and induce re-expression of key tumor suppressor genes. However, the mechanism of action of azacytidine and decitabine in myelodysplastic syndromes remains uncertain, and these drugs might be, at least in part, cytotoxic.

A prospective randomized trial has shown that treatment of patients with myelodysplastic syndrome with 5-azacytidine resulted in significantly higher response rates, improved quality of life, reduced risk of leukemic transformation, and improved survival compared with supportive care. On May 19, 2004 the US FDA approved azacitidine as an injectable suspension for treatment of patients with the following myelodysplastic syndrome subtypes: refractory anemia or refractory anemia with ringed sideroblasts (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia. At
the time of writing this article, a decision from the European authorities (EMEA) is pending. A survival study in patients with high-risk myelodysplastic syndrome comparing azacitidine versus conventional care is currently recruiting patients world-wide (http://www.clinicaltrials.gov/ct/show/NCT00071799).

A multicenter phase II study on the effect of decitabine in elderly patients was reported in the year 2000."\(^\text{32}\) A phase III randomized North American trial of decitabine in advanced myelodysplastic syndromes has been reported in abstract form."\(^\text{31}\) The median time to AML or death in patients receiving decitabine did not differ significantly from that of patients receiving the best supportive care. A randomized, open-label, multicenter phase III trial comparing the effectiveness of low-dose decitabine with that of standard supportive care in individuals aged \(\geq 60\) years is currently recruiting patients in Europe (http://www.clinicaltrials.gov/ct/gui/show/NCT00043134).

**Immunosuppressive therapy: a therapeutic option for a subgroup of relatively young patients**

Findings of several studies suggest that the pathophysiology of bone marrow failure in myelodysplastic syndromes is mediated at least in part by the immune system, and immunosuppressive therapy have been employed for these patients."\(^\text{32}\)

Only uncontrolled observations are available so far on the use of antithymocyte globulin or cyclosporine A (or both combined) for the treatment of myelodysplastic syndromes. In two case series (training and validation cohorts, \(n=82\) and \(n=23\), respectively),"\(^\text{33}\) three pretreatment variables were found to be associated with a response to antithymocyte globulin: younger age, shorter duration of red cell transfusion, and positivity for HLA-DR 15. This indicates that antithymocyte globulin may abolish transfusion requirements in younger patients and does so mainly in those with transfusion dependence of short duration. With respect to marrow cellularity, in a previous study of 61 patients"\(^\text{34}\) it was concluded that hypocellularity was an almost independent factor predicting response to antithymocyte globulin; this was not confirmed subsequently."\(^\text{35}\) In a British study, transfusion independence was achieved in half of low risk patients."\(^\text{35}\)

Antithymocyte globulin administration involves not-negligible toxicity, especially in individuals over 60 years of age. Based on limited evidence and balancing potential beneficial and adverse effects, antithymocyte globulin may be reasonably considered in patients under 60 years of age with hypoplastic bone marrow and/or HLA-DR15 who are not candidates for allogeneic stem cell transplantation.

**Lenalidomide: a potentially useful agent for patients with the 5q deletion**

Thalidomide has been employed in MDS patients with the aim of utilizing its anti-cytokine and anti-angiogenic effects for improving the efficiency of hematopoiesis."\(^\text{22}\) Some transfusion-dependent patients can become transfusion independent with thalidomide, but achievement of response requires several weeks and treatment is significantly limited by neurological toxicity. Lenalidomide which lacks this adverse effect, appears more suitable for long-term treatment, and recent reports of its clinical use are interesting for several reasons.

List and co-workers"\(^\text{36}\) treated 43 patients with transfusion-dependent or symptomatic anemia with lenalidomide at doses of 25 10 mg per day or of 10 mg per day for 21 days of every 28-day cycle. Twenty-four patients had a response and 20 achieved sustained independence from transfusion. The response rate was highest among patients with a clonal interstitial deletion involving chromosome 5q13.1 and patients with lower prognostic risk. The most common adverse events were neutropenia and thrombocytopenia.

The effect of lenalidomide on myelodysplastic hematopoiesis appears dual, at least initially, resulting in increased red cell and decreased neutrophil and platelet production, as previously discussed."\(^\text{37}\) This observation cannot be explained only by a direct effect on clonal stem cells, which should result in parallel changes in peripheral blood cells. The observed effects may be consistent with an anti-cytokine activity of lenalidomide, although a direct effect of the drug on myelodysplastic clones with the 5q deletion cannot be excluded.

On December 28, 2005, the FDA approved the use of lenalidomide for the treatment of patients with myelodysplastic syndrome and the 5q deletion.

A multicenter, randomized, double-blind, placebo-controlled, three-arm study is currently evaluating, in Europe, the efficacy and safety of two doses of lenalidomide versus placebo in transfusion-dependent subjects with low- or intermediate-1-risk myelodysplastic syndromes associated with \(5q\) deletion (http://www.clinicaltrials.gov/ct/show/NCT00179624). In European countries lenalidomide should be employed only within clinical trials at present. Neutropenia needs to be monitored closely, particularly in the first weeks of treatment; patients who have severe neutropenia (grade 3 or more, absolute neutrophil count \(< 1 \times 10^9/L\)) at baseline or develop it during lenalidomide treatment should be given G-CSF.

**European guidelines for the treatment of myelodysplastic syndromes**

Within the MDS Working Package, European

References

The introduction of imatinib represented a major milestone in the treatment of chronic myeloid leukemia (CML) and heralded the coming of other magic bullets in cancer therapy. While the efficacy of imatinib is without doubt, the persistence of minimal residual disease and, more worryingly, the development of resistance to single drug therapy, have dampened the initial enthusiasm. The mechanisms of resistance to imatinib have been extensively studied and the search is now on for other more efficacious agents to improve response further and prevent or overcome drug resistance.

Resistance can be defined on the basis of its time of onset. Primary resistance is a failure to achieve a significant cytogenetic response, whereas secondary or acquired resistance is the progressive reappearance of the leukemic clone after an initial response to the drug. Resistance is also defined on the basis of clinical and laboratory criteria used for the detection of leukemia, and includes hematological, cytogenetic and molecular resistance. Hematological resistance is a lack of normalization of peripheral blood counts and spleen size; cytogenetic resistance is a failure to achieve a major cytogenetic response, i.e. less than 35% Philadelphia (Ph) chromosome positivity; and molecular resistance represents the failure to achieve or the loss of complete or major molecular response (MMR). A MMR can be defined as a three or more log-reduction of BCR-ABL/control gene ratio from a laboratory standardized baseline or an international scale converted BCR-ABL/control gene ratio of < 0.1%.1

Three major mechanisms of resistance have been identified. The two most common affect the BCR-ABL gene itself, namely mutations in its tyrosine kinase domain and overexpression of the BCR-ABL protein due to amplification of the BCR-ABL gene.2,7 The latter has been reported in a relatively small proportion of patients, with an overall percentage of 18%,3,3 but this may be an underestimate if its detection is only based on the cytogenetic findings of Ph chromosome duplication. Overexpression of Bcr-Abl leads to resistance by increasing the amount of target protein needed to be inhibited by the therapeutic dose of the drug. The third mechanism is not so well characterized and understood, and is represented by phenomena which lead to resistance independently of Bcr-Abl. Among these, upregulation of the multi-drug resistance P-glycoprotein (Pgp),4,10 binding of the α1-acid glycoprotein (AGP),11 overexpression of Lyn, a Src kinase,12 and other Bcr-Abl-independent mechanisms have been variably reported.13

**Mutations in the Abl kinase domain**

The development of point mutations in the Abl kinase domain is the most frequent mechanism of acquired resistance, but is very rare in patients who fail to show any response to the drug. It is important to emphasize that mutations are not induced by the drug, but rather, just like antibiotic-resistant in bacteria, arise through a process whereby the drug itself selects for rare pre-existing mutant clones, which gradually outgrow drug-sensitive cells.

Mutations can be broadly categorized into four groups: (i) those which directly impair imatinib binding; (ii) those within the ATP binding site; (iii) those within the activation loop, preventing the kinase from achieving the conformation required for imatinib binding; and (iv) those within the catalytic domain.

The substitution of the amino acid threonine with isoleucine at position 315 of the Abl protein was the first mutation to be detected in resistant patients.3 Based on the crystal structure of the catalytic domain of Abl complexed to a variant of imatinib,14 this substitution was predicted to reduce the affinity for the drug in two ways. Firstly, the oxygen atom provided
by the side chain of threonine 315 is not present, and this prevents the formation of a hydrogen bond with the secondary amino group of imatinib. Secondly, isoleucine contains an extra hydrocarbon group on its side chain and this sterically inhibits the binding of the inhibitor. Another amino acid that makes contact with imatinib is phenylalanine 317, and its mutation to leucine also leads to resistance.

Mutations can also cluster within the ATP-binding loop (phosphate or P-loop). This domain is a highly conserved glycine-rich sequence that spans amino acids 248-256 and interacts with imatinib through hydrogen and van der Waals bonds. These mutations modify the flexibility of the P-loop and destabilize the conformation required for imatinib binding. Apart from imatinib insensitivity, a feature of clinical relevance is that imatinib-treated patients who harbor P-loop mutations have been suggested to have a worse prognosis than those with non-P-loop mutations. However, this has not yet been confirmed in larger series.

The activation loop of the Abl kinase begins at amino acid 381 with a highly conserved motif of three amino acid residues (aspartate-phenylalanine-glycine). This region of the kinase can adopt a closed (inactive) conformation or an open (active) conformation. Imatinib forces Abl into the inactive conformation and is incapable of binding to the active configuration. Mutations in the activation loop may disturb the energetic balance required to stabilize the closed conformation of the loop and thus favor the open, active conformation.

Finally, some amino acid substitutions cluster in the catalytic domain, a region that has a close topologic relation to the base of the activation loop. Therefore mutations in this region can also influence the binding of imatinib. Thus far, 73 different point mutations leading to a substitution of 50 amino acids in the Abl kinase domain have been isolated from CML patients resistant to imatinib, and this number is likely to increase with more sensitive methods of detection (Figure 1).

**Bcr-Abl overexpression**

Another common mechanism of resistance is overexpression of the Bcr-Abl protein due to amplification of the *BCR-ABL* gene. This phenomenon was first observed in vitro when resistant CML cell lines were generated by exposure to gradually increasing doses of imatinib. Overexpression and gene amplification also occurs in patients. In one study, three out of 11 CML patients in blast crisis who relapsed after initially responding to imatinib were shown to have multiple copies of the *BCR-ABL* gene by fluorescence in situ hybridization (FISH). In another study, seven out of 55 patients showed a more than 10-fold increase in *BCR-ABL* transcript levels and two out of the 32 patients evaluated were found to have genomic amplification of *BCR-ABL* by FISH. In the latter two patients, resistance was primary and not acquired.

**Drug efflux and influx transporters**

Multidrug resistance (MDR) is a well-known phenomenon of cross-resistance of mammalian cells to a...
number of anticancer agents following exposure to one such drug. In many cases, this is mediated by an increased expression at the cell surface of the MDR1 gene product, Pgp, an energy dependent efflux pump, which primarily reduces intracellular drug concentrations, leading to the failure of effective levels of the drug reaching its target. Several studies have suggested that MDR may play an important role in imatinib-resistance. Imatinib and other tyrosine kinase inhibitors have been demonstrated to be substrates of Pgp, and the intracellular levels of imatinib were shown to be significantly lower in Pgp-expressing cells. An imatinib-resistant CML cell line generated by gradual exposure to increasing doses of the drug was shown to exhibit Pgp overexpression, and MDR1 overexpression in CML cell lines also confers resistance to imatinib. Although Pgp overexpression has not been reported in patients who are resistant to imatinib, the addition of a Pgp pump inhibitor, PSC833, to cultures of imatinib-treated cells from drug-resistant CML patients produced a significant decrease in colony formation, thus providing indirect evidence of a possible role of MDR1 overexpression in imatinib resistance.

Recently, two other drug transporters, breast cancer resistance protein (BCRP)/ABCG2 and human organic cation transporter 1 (hOCT1), have been implicated as possible mechanisms for promoting imatinib resistance. Imatinib is a substrate for the BCRP/ABCG2 drug efflux pump which is overexpressed in many human tumors and also found to be functionally expressed in CML stem cells. The drug transporter, hOCT1 mediates the active transport of imatinib into cells and inhibition of hOCT1 decreases the intracellular concentration of imatinib. The hOCT1 gene was also found to be expressed in significantly higher levels in patients who achieved a complete cytogenetic response to imatinib than in those who were more than 65% Ph chromosome positive after 10 months of treatment. This would suggest that patients with low baseline expression of hOCT1 may not achieve a complete cytogenetic response because of insufficient intracellular levels of imatinib.

Bcr-Abl independent mechanisms

The Src family kinases, Lyn and Hck, are activated in BCR-ABL-expressing cell lines. Lyn has been shown to be overexpressed and activated in an imatinib-resistant CML cell line generated by incubation of the parental line in increasing concentrations of imatinib, as well as in samples from CML patients who were resistant to imatinib. Lyn suppression by a Src kinase inhibitor resulted in reduced proliferation and survival of the imatinib-resistant but not the -sensitive cell line. Using microarray analysis, transcripts with anti-apoptotic or malignant transformation properties and transcripts with involvement in signal transduction/transcriptional regulation were found to be overexpressed in CML cells innately resistant to imatinib, suggesting that pathways downstream of Bcr-Abl and independent of its kinase activity may be important factors which confer resistance to imatinib.

Strategies to overcome imatinib resistance

There is a pressing need for the discovery and development of new compounds or novel combinations capable of circumventing imatinib resistance. Several other Abl tyrosine kinase inhibitors have been recently identified to have potent in vitro and in vivo activity in wild-type Bcr-Abl cell lines, as well as in cell lines harboring Abl kinase domain mutations (Figure 2). Two of these compounds also have favorable pharmacokinetic profiles and are now in early phase clinical trials. There is also an increasing body of pre-clinical evidence showing that combination therapy has a role to play in preventing or combatting imatinib-resistance.

Dual Src/Abl kinase inhibitors

Src was the first proto-oncogene to be discovered, and disruption of its function has been associated with the pathogenesis of human cancers. A number of synthetic small molecule inhibitors of Src-family kinases have been developed (Figure 2). These Src kinase inhibitors, eg, PD180970, AP23464, SKI606, CGP76030, BMS-354825 also inhibit Bcr-Abl, Kit and PDGFRα receptors, and have in vitro antiproliferative activity in imatinib-sensitive and -resistant CML cells. Several other Abl tyrosine kinase inhibitors have been recently identified to have potent in vitro and in vivo activity at picomolar concentrations. One of these dual Src/Abl kinase inhibitors, dasatinib (BMS-354825, Bristol Myers Squibbs) was found to be more potent than imatinib, and was capable of inhibiting the proliferation and kinase activity of wild type Bcr-Abl cell lines at picomolar concentrations. Another crucial advantage that dasatinib has over imatinib is that, similar to previously developed Src/Abl kinase inhibitors, it effectively targets the active, imatinib-resistant conformation of the kinase. This was validated in in vitro assays which revealed that dasatinib inhibited the kinase activity and proliferation of 14 out of 15 clinically relevant Bcr-Abl mutant cell lines. Only the T315I mutant remained resistant, even at micromolar concentrations of the drug. In vivo studies in a mouse model confirmed the activity of dasatinib in inhibiting the growth of leukemic cells and prolonging the survival of mice harboring the wild type Bcr-Abl and the M351T, but not the T315I mutant.

Clinical trials of dasatinib in imatinib-resistant and -intolerant CML and Ph chromosome-positive acute
lymphoid leukemia (Ph+ ALL) are currently underway, and the hematological and cytogenetic responses are summarized in Table 1.33-37 Dasatinib is generally well tolerated, although grade 3-4 myelosuppression is common, especially in the advanced phases. Non-hematological side effects include diarrhea, nausea, headache, peripheral edema and pleural effusion.

Second generation Abl kinase inhibitors

The N-methylpiperazine moiety was originally incorporated into imatinib to improve its solubility and oral bioavailability. It was hypothesized that a more potent compound could be created by the substitution of this amide moiety with alternative binding groups, while maintaining H-bond interactions to Glu286 and Asp381. Based on this approach, nilotinib (AMN107, Novartis) was discovered (Figure 2). Nilotinib also inhibits the activity of Arg, Kit, and PDGFα and β receptors, but not Src kinase. In cellular assays, it is 10 to 50 times more potent than imatinib in inhibiting the proliferation and autophosphorylation of wild-type Bcr-Abl cell lines. Similar to dasatinib, it inhibits the proliferation of most of the clinically relevant Bcr-Abl mutants at submicromolar concentrations, except the T315I mutant. Nilotinib was also shown to be superior to imatinib in reducing the leukemic burden and prolonging the survival of mice transplanted with marrow transduced with wild-type Bcr-Abl, the M351T and E255V mutants. 38 Results from phase I clinical trials with nilotinib are summarized in Table 2.39

Substrate-competitive inhibitors

Adaphostin is a tyrphostin which alters the binding of peptide substrates rather than the ATP-binding site. Imatinib-resistant cell lines were shown to remain sensitive to the inhibitory effects of adaphostin.40 Although adaphostin is not Bcr-Abl-specific, it inhibited colony formation from primary CML cells but not from normal cells.40

The resistance of the T315I mutant to the Src/Abl kinase inhibitors and nilotinib poses a therapeutic challenge, and it is likely that this mutant will remain insensitive to other ATP-competitive inhibitors. Recently, a substrate-competitive inhibitor of Bcr-Abl, ON012380, was reported to have potent in vitro inhibitory activity in cell lines expressing wild-type Bcr-Abl and all the Bcr-Abl mutants, including the T315I mutant. The activity against the T315I mutant was confirmed in vivo in mice expressing this form of Bcr-Abl protein in which treatment with ON012380 caused a decrease in leukemic cells.41

Allosteric inhibitors

A recent class of Bcr-Abl inhibitor compounds was uncovered by a differential cytotoxicity screen in a 384-well format of approximately 50,000 combinatorially derived kinase-directed heterocycles.42 This is a class of compounds that exert their activity through a newly described allosteric, non-ATP competitive mechanism, potentially involving binding to the myristate pocket in the C-loop of the Bcr-Abl kinase domain.

Other compounds with activity against imatinib-resistance

A multitude of compounds have been described to have in vitro activity against imatinib-resistant cells. These include the inhibitors of the Bcr-Abl chaperone heat shock protein 90, geldanamycin and 17-allylaminkaldanamycin (17-AAG);43 the MEK kinase inhibitor, PD184352; 44 the cyclin-dependent kinase inhibitor, flavopiridol;45 the histone deacetylase inhibitors, suberoylanilide hydroxamic acid (SAHA) and sodium butyrate;46 and the proteasome inhibitor, bortezomib.47 Other small molecule compounds have also been identified to have in vitro anti-proliferative activity against the T315I mutant and these include a phosphoinositide-dependent kinase-1 inhibitor, OSU-03012;48 an Aurora kinase inhibitor, VX-680;49 a p38 inhibitor, BIRB-796;49 and an Abl kinase inhibitor, SGX-70430.50

Combination therapy with inhibitors of effectors in Bcr-Abl downstream pathways

Another strategy for overcoming and possibly preventing resistance is to combine imatinib with inhibitors of pathways downstream of Bcr-Abl. Farnesyl transferase inhibitors (FTI) inhibit protein farnesylation and thereby antagonise the onco-
genecity of Ras, a protein that plays a central role in leukaemogenic transformation by Bcr-Abl. Lonafarnib (previously SCH66336, Schering-Plough) is an FTI which is equally effective in inhibiting the proliferation of wild type Bcr-Abl, Bcr-Abl overexpressing and T315I mutant cell lines. Although the drug itself did not induce apoptosis, it enhanced imatinib-induced apoptosis in the first two cell lines but not the T315I mutant.51 Lonafarnib was also shown recently to enhance imatinib-induced cytotoxicity in primitive quiescent CML cells, a population of cells known to persist in vitro in imatinib-treated primary CML cells.52 Nitrogen-containing bisphosphonates, via their inhibition of Ras prenylation, have also been effective in inducing apoptosis and inhibiting proliferation of imatinib-sensitive and -resistant CML cells.53,54

Effectors along the phosphatidylinositol-3 (PI-3) kinase/Akt pathway are also attractive candidates for targeted molecular therapy. Inhibitors to the mammalian target of rapamycin (mTOR), a serine-threonine kinase activated by the PI-3 kinase, have been shown to inhibit the proliferation of CML cells. The combination of rapamycin or its derivative, RAD001, with imatinib was effective in overcoming imatinib-resistance in cell lines which overexpressed Bcr-Abl or harbored mutants which retained a moderate sensitivity to imatinib. However this combination was not effective in mutants which were highly resistant to imatinib, for example, T315I and E255K.55,56 A novel phosphoinositol-dependent kinase-1 inhibitor, OSU-03012, acting via an Akt-dependent mechanism, was shown to synergize with imatinib in inducing apoptosis and inhibiting proliferation in both the T315I and E255K mutants.46

The Jak-STAT pathway is the third major pathway downstream of Bcr-Abl. Mycophenolic acid, an inosine monophosphate dehydrogenase inhibitor that depletes intracellular guanine nucleotides, reduced phosphorylation of STAT5 and S6 ribosomal protein, a substrate downstream of mTOR. When combined with imatinib, mycophenolic acid produced synergistic antiproliferative and pro-apoptotic effects in imatinib-sensitive CML cells. However the effect on imatinib-resistant cells was not determined.57

Conclusions

Although imatinib-resistance has reduced the initial enthusiasm concerning targeted therapy, new approaches to circumvent this problem are now possible and are being investigated pre-clinically and clinically. It is likely that the emergence of subclones with new Bcr-Abl mutations may develop in response to these new small-molecule inhibitors, leading again to resistance to these compounds.

However, it may be possible to delay or suppress the emergence of mutants by combining imatinib with the newer Abl kinase inhibitors or with inhibitors of Bcr-Abl downstream effectors.

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Update of first-line imatinib therapy in chronic phase chronic myeloid leukemia

Imatinib is a selective Bcr-Abl protein kinase inhibitor that suppresses Philadelphia positive cells. Chronic myelogenous leukemia (CML) represents an ideal disease target for therapy with imatinib, given that the Bcr-Abl kinase is widely believed to play a dominant role in the deregulated myeloid cell proliferation that is the hallmark of this disease. Imatinib had shown selectivity for the Abl protein-tyrosine kinase at in vitro, cellular and in vivo levels. Soon after the demonstration of its anti-leukemic effect imatinib showed encouraging activity in CML patients in late chronic phase who were resistant to therapy with interferon-α, as well as in patients who were intolerant of such therapy. Inhibition of the Bcr-Abl kinase is most likely to have anti-leukemic effects during the chronic phase of CML since additional chromosomal abnormalities may drive the malignant process during accelerated phase and blast crisis.

Based on the first phase I and II trials, imatinib was established as a safe and effective treatment for CML, which led regulatory authorities to grant approval for its use in patients in whom previous therapy had failed. However, it was reasonable to assume that the drug would also be effective in patients who are treated at an earlier stage of the disease. In order to demonstrate the benefit of imatinib over an interferon-α-based regimen, it was decided to set up a large international study. This study (also referred to as the IRIS study: International Randomized Study of IFNα + Ara C vs Imatinib) was carried out in newly diagnosed patients with chronic phase CML to compare the efficacy, safety and tolerability of imatinib administered as monotherapy with that of standard treatment with interferon-α plus Ara-C as the first-line treatment of the disease. In this trial, imatinib produced significantly higher rates of major (MCyR) and complete (CCyR) cytogenetic responses and improved progression-free survival. Imatinib was also better for compliance, toxicity and quality of life. In addition, the molecular response with imatinib was significantly better.

Although Imatinib is effective in treating CML, some patients ultimately relapse with resistant disease. Resistance may develop through several mechanisms, such as point mutations within the tyrosine kinase binding site, gene amplification, clonal evolution and decreased bioavailability of imatinib. Thus research is currently in progress to further elucidate the mechanisms of imatinib resistance and develop strategies that will expand the usefulness of this drug.

Update on the superiority of standard dose of imatinib as a single agent over an interferon-based regimen

The objective of the IRIS trial was to determine the progression-free survival in adult patients with newly diagnosed previously untreated Philadelphia-positive CML in chronic phase randomized to receive either imatinib as a single agent or a combination of interferon-α plus Ara-C. Between June 2000 and January 2001, the IRIS study enrolled 553 patients into each arm. The design of the trial allowed patients who had no response, had a loss of response, increasing white blood cell count, intolerance of treatment or failure to achieve MCyR at 12 months to cross over to the other treatment. Mainly because of intolerance and poor responses, a substantial number of patients crossed over from the interferon-α plus Ara-C arm to the imatinib arm. Thus overall survival analysis based on an intention-to-treat basis was not assessable. The subsequent analysis of the trial confirmed the initial impressive results. At 42-months of follow-up, 75% of the 553 patients randomized to imatinib remained on treatment. Of the 553 patients randomized to interferon-α plus Ara-C only 4% were still receiving this treatment.

At the last update, 358 patients (65%) had crossed over to imatinib, the reasons...
for crossing over being lack of response (18%), loss of response (14%), intolerance of treatment (28%) and reluctance to continue interferon-α (7%). With an average duration of 38 months of Imatinib therapy the best observed rates of complete hematologic response (CHR), MCyR and CCyR were 96%, 88% and 81%, respectively. Most of the patients achieved their MCyR by 6 months and their CCyR by 12 months (Figure 1). The overall estimated survival at 42 months was 91% (considering all deaths). The estimated survival was lowest in patients with high risk Sokal score (84%), better (91%) in the intermediate risk patients and (94%) in the low risk patients ($p<0.001$). Similarly, the best observed CCyR in the high, intermediate, and low risk groups were 69%, 80% and 88% respectively ($p=0.002$). In the subset of patients with CCyR the estimated survival at 42 months was 92%, 93% and 97% in the high to low risk groups ($p=0.50$), indicating that once patients achieve a CCyR, their survival is not significantly affected by Sokal risk groups. In this trial, for patients who achieved CCyR and a reduction in BCR-ABL transcripts of at least 3-log at 12 months, the probability of remaining progression-free at 42 months was greater than that of patients who had CCyR but a less than 3 log reduction (98% versus 90%), and significantly greater than that observed for patients who were not in CCyR at 12 months. Thus the achievement of at least a 3-log reduction after 12 months of treatment predicts subsequent progression-free survival. In addition, when the definition of progression is restricted to accelerated phase or blast crisis, for patients who had achieved at least 3-log reduction at 12 months, the probability of remaining free from progression to accelerated phase or blast crisis was 100% at 42 months compared to 95% for patients in CCyR but not in molecular response, and 91% for patients not in CCyR at 12 months ($p=0.0013$). The estimated progression-free rate at 42 months was 84%; additionally 94% were estimated free of progression to accelerated phase or blast crisis (97% of the patients with CCyR and 73% of the patients without CCyR during the study, $p<0.001$).

The risk of relapse remains low with no apparent increased risk over time. The yearly hazard for progression to accelerated phase or blast crisis was 1.5%, 2.8%, 1.6% and 0.9% at 1, 2, 3 and 4 years respectively. Overall survival rates were analyzed according to MCyR by the landmark method. Of the 509 patients who were still on treatment at 12 months and had achieved a MCyR by then ($n=436$), the proportion without progression to accelerated phase or blast crisis at 42 months was 97% whereas it was only 83% for the 78 patients who did not achieve a MCyR at 12 months ($p<0.001$). A subsequent analysis of the outcome of 101 patients with a sustained complete response who had blood collected for polymerase chain reaction (PCR) analysis at 1 and 4 years was performed. At 1 year the BCR-ABL transcript levels had fallen by at least 3 log in 47 (46%) of the 101 patients. At or after 44 months from the start of the study, 76 (75%) patients showed a reduction of at least 3 log. Of these 76 patients, 39 (51%) had already had a >3 log reduction at 1 year, whereas 37 (49%) had not. It was also concluded that for patients in sustained complete cytogenetic response with a moderate decline of their BCR-ABL transcript at 1 year, there is still a chance of achieving major molecular response at 4 years. The best alternative approach to estimate the survival benefit produced by imatinib would be a comparison between a group of patients receiving imatinib and an homogeneous group of patients included in a prospective trial assessing the combination of interferon-α plus Ara-C as first line therapy.

Thus, it was decided to perform a retrospective analysis comparing the outcome of patients first treated with imatinib in the IRIS trial, and patients assigned to interferon-α and Ara-C in the French CML91 trial. This historical comparison showed that for first line therapy for newly diagnosed chronic phase CML, imatinib was superior to prolonged therapy with interferon-α and Ara-C with regards to the rates of MCyR, CCyR, progression-free survival and overall survival.

Exploratory strategies of higher dose imatinib or combination therapies for improving molecular responses

High dose imatinib

Several observations suggested that higher dosages of imatinib may be more effective. The initial phase I trial showed a clear dose-response relationship. In accelerated phase CML, patients treated with 600 mg imatinib daily had higher response rates, longer responses and longer survival.

Thus a study investigated the efficacy and toxicity
of high dose imatinib (800 mg daily) in patients with newly diagnosed chronic phase CML. Among the patients given the 800 mg regimen, 90% achieved CCyR. The estimated 2-year survival rate was 94%. Using QPCR, it was found that BCR-ABL/ABL percentage ratios decreased to less than 0.05% in 71 (63%) of 112 patients and to undetectable levels in 31 (28%). The response rate for the historical control group of patients in that study who received 400 mg/day were 74% with CCyR and 56% with BCR-ABL/ABL ratios less than 0.05%. High dose imatinib was well tolerated but resulted in more frequent myelosuppression. The Australian group conducted a phase II trial (TIDEL in de novo CML patients who initially received imatinib 600 mg, with the dose increasing to 800 mg in the case of insufficient response, defined as no MCyR at 6 months, no CCyR at 9 months and ≥4 log reduction in BCR-ABL at 12 months. Of the 101 patients included in the trial, 81 were assessable for molecular response at 24 months. By 12 months 89%, 45% and 13% had achieved CCyR, ≥2 log and ≥4 log reductions, respectively. By 24 months 92%, 65% and 29% achieved these response levels. It was concluded that a more dose intense approach to the treatment of newly diagnosed CML patients produces a better rate of major molecular response than do lower doses, and that maintenance of dose intensity in the first 6 months of therapy is predictive of molecular response.

**Imatinib in combination**

In order to obtain a higher rate of cytogenetic response and to overcome resistances, new strategies using combinations of cytotoxic drugs have emerged. Drugs currently being tested are those that have been selected in the past for their high antileukemic activity. Interferon, in pegylated and non-pegylated forms, and cytarabine at various dosages are being actively tested. The conjugation of a 40kDa branched polyethylene glycol molecule to interferon-2α (Peg-IFN2α) results in the formation of a novel interferon with properties including sustained absorption and a prolonged half-life, allowing for a once-weekly dosing regimen. This new compound, might, therefore be better tolerated by CML patients. An open-label trial included 144 patients in order to compare subcutaneous Peg-IFN2α 450 µg once weekly with regular interferon-2α, 9 MU/day. After 12 months MCyR, CCyR complete cytogenetic response as well as hematologic response were significantly better with Peg-IFN2α as compared with regular interferon-2α, being 35% and 18% (p=0.0016), 15% and 7%, 66% and 41% (p=0.0008) respectively.

The French CML group performed a phase II study of imatinib at a daily fixed dose of 400 mg in combination with Ara-C at 20 mg/m² on days 14 to 28 days with cycles repeated every 28 days. Thirty previously untreated CML chronic phase patients were enrolled within six months of diagnosis. Adverse events were frequently observed with grade 3 or 4 hematologic toxicities and non-hematologic toxicities in 53% (n=16) and 27% (n=8) of patients, respectively. The cumulative incidence of CCyR at 12 months was 83% and at 6 months 100% of the patients had achieved aCHR.

An exploratory study was conducted in order to investigate the effects of a standard dose of 400 mg imatinib daily and a variable dose of pegylated interferon (PegIFN) (50 µg/wk, 100 µg/wk, and 150 µg/wk). The criteria for dose adjustment were designed in order to ensure the delivery of the imatinib dose and to protect quality of life. Seventy-six patients with previously untreated Philadelphia chromosome-positive CML were enrolled in the study. Three patients discontinued imatinib and 45 patients discontinued PegIFN. The severity of adverse events increased with increasing PegIFN dose. The imatinib dose could be administered to the patients who were assigned to receive 50 µg/wk or 100 µg/wk PegIFN but not to those who were assigned to receive 150 µg/wk. The median administered dose of PegIFN ranged between 32 µg/wk and 36 µg/wk. In this group of patients 70% achieved a CCyR and 83% a MCyR. The level of BCR/ABL transcripts decreased by at least 3 logs in 68% of the patients with a CCyR. These phase II trials were essential for the design of the current large phase III trials.

**Ongoing prospective randomized trials**

The first line therapy for patients with chronic phase CML is currently imatinib at a dose of 400 mg daily. Phase I and II trials have clearly shown a dose-response effect. In addition in vitro data and preliminary results using combination therapies are promising. Thus several national groups are currently conducting trials exploring various dosage of imatinib (400 mg, 600 mg and 800 mg) and combinations therapies with cytarabine or interferon-α. The UK and US groups are exploring a comparison between 400 and 800 mg. The GIMEMA (Italian) is conducting a phase II trial of imatinib 800 mg in patients with intermediate Sokal risk early chronic phase CML. Out the 44 patients who completed 6 months of treatment, the CHR rate was 100%, and the rates of MCyR and CCyR were 90% and 81%, respectively. The major molecular response rate at 6 months was 56%. Within the frame of LeukemiaNet, multinational working group (Italy, Nordic Countries, Turkey and Israel), is exploring 400 mg versus 800 mg in high risk patients. At present 80 patients have been enrolled. Two groups are conducting in parallel large
phase III trials exploring dosage of imatinib as well as combination therapies. In July 2002, the German COML study group activated a four-armed, randomized, controlled trial comparing imatinib 400 mg with imatinib plus interferon-α, imatinib plus cytarabine and imatinib after interferon-α failure. In this trial high risk patients are randomly assigned to primary imatinib-based therapies including a treatment arm with 800 mg daily. A recent evaluation was based on 416 patients with 12 months of follow-up. Of the 355 patients with cytogenetic evaluation, 63% achieved a MCyR and 53% a CCyR. The number of patients who progressed each year was very low and 27% of patients achieved a major molecular response. In September 2003, the French CML study group started a similar phase III trial. The experimental arms are imatinib 400 mg daily in combination with Peg-IFN-2α (90 µg weekly) or imatinib 400 mg daily in combination with Ara-C, (20 mg/m²/day, days 15-28 of 28-day cycles) or imatinib 600 mg daily. The reference arm is imatinib 400 mg daily. A first evaluation based on 515 patients with a median time of observation of 12 months demonstrated the feasibility of combination therapies with a CHR rate of 82% at 3 months. Cytogenetic data were available from 154 patients. At 6 months, 135 patients (87%) achieved a MCyR, being complete in 105 patients (68%). A substantial number of patients experienced grade 3/4 hematological as well as non-hematological toxicities.

**Imatinib in young patients**

CML is a very rare disease in children, accounting for 2% to 3% of leukemias in children and adolescents with an annual incidence of one case per million children. The characteristics of CML in this age range seem to differ from those in adults. Moreover, prognostic factors and prognostic scoring systems are not well defined in children with CML. There are currently two main treatment options for children with CML. The first option is hematopoietic stem cell transplantation (SCT), which is a potentially curative therapy in children with a suitable donor. The reported event-free survival in children with CML transplanted in chronic phase is about 70% when the donor is matched and related and from 40% to 60% with grafts from matched unrelated donors. The second option is treatment with imatinib. There are some data regarding imatinib therapy in children with CML. The Children’s Oncology Group conducted a phase 1 study testing imatinib mesylate in 31 children with Philadelphia chromosome-positive leukemia suggesting efficacy of this drug in this age-group. According to this phase 1 study, doses of 260 to 340 mg/m² administered to children provide drug exposures similar to those of the doses of 400 mg to 600 mg used in adult studies. The results obtained in children with CML receiving imatinib mesylate compare favorably with those reported in adults. However, there is no broad consensus on the use of imatinib in children with CML. In spite of significant cytogenetic and molecular responses, there is no evidence that imatinib is curative and long-term side effects of this drug remain to be determined in children. Moreover, the long-term tolerability and the durability of responses must be evaluated. Studies are under way to evaluate the activity of imatinib in children and adolescents with CML in first chronic phase.

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**References**


The role of allogeneic stem cell transplantation for chronic myeloid leukemia

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**ABSTRACT**

Hematopoietic stem cell transplantation (HSCT) was the standard of care for all younger patients with chronic myeloid leukemia (CML) and a compatible donor. The introduction of imatinib mesylate changed this attitude and numbers of HSCT have declined in recent years. In parallel, results of HSCT have improved, the main risk factors have been defined and treatment-related mortality has been reduced. Risk-adapted therapy has become the approach of choice. HSCT remains an early option for patients with high risk disease and low risk donors. It can be the preferred choice in economically disadvantaged situations. It should be considered in all patients with an inadequate response or loss of response to imatinib.

About thirty years ago, a change in the treatment of CML was initiated. The first reports of successful transplants with bone marrow from syngeneic twin donors in patients with CML marked the beginning of a new era in the treatment of this disease. Proof was provided that CML could be eradicated by intensive chemo-/radiotherapy and transplantation of healthy donor cells. This concept was followed by transplants from HLA-identical siblings and was confirmed in large series recorded by international transplant registries in Europe and the USA. Successful immuno-therapy of a hematological malignancy had become reality. Allogeneic HSCT became the standard of care for patients with CML. The introduction of reduced intensity conditioning transplants made HSCT feasible for older patients and those with associated co-morbidities. By 1999, CML was the most frequent indication for an allogeneic transplant.

The introduction of a new specific tyrosine kinase inhibitor imatinib mesylate (Glivec®) changed attitudes. Imatinib blocks BCR/ABL expression and can induce hematological and cytogenetic complete remissions far more frequently than previous therapies with interferon-α or other agents. Even molecular remissions, though not durable, can be achieved in some patients. These excellent short-term results are obtained with minimal side effects and with an only once daily oral medication. HSCT, in contrast, is associated with substantial early morbidity and mortality. Although the long-term results of imatinib are lacking, it is evident that the marked and obvious difference in early outcome has challenged the previous concept of early HSCT for all patients with CML. Numbers of HSCT have decreased markedly (Figure 1). It is of interest to note, that numbers of transplants for CML declined even before imatinib became available. It is also of interest to note that the numbers of HSCT for CML stabilized in 2004. Of the more than 20,000 patients treated with HSCT in Europe in 2004, about 800 were treated for CML; the vast majority of these underwent allogeneic HSCT. Two-thirds were in 1 first chronic phase and one third in an advanced phase of the disease. Numbers were stable in Western, countries but increase in Eastern European countries.

Current results of HSCT for CML

HSCT has considerable side effects, morbidity and mortality. Complications are due to toxicity of the conditioning regimen, to immunological complications such as rejection or graft-versus-host disease, to early and late infections or to relapse of the disease despite the transplant. All have their specific risk factors and different time frames of onset. Interpretation of outcome requires considerable care. Short and long term outcome can be influenced by diverse factors. For example, reduction of conditioning reduces early toxicity and mortality but increases late risk of relapse and vice versa. The documented advantage of HSCT lies...
in its potential to eradicate the disease and to achieve a consistent lasting BCR/ABL negative state on a regular basis. Data are now available for a substantial number of patients with follow-up of 20 years and more, as recently presented by the EBMT (Figure 2) in an analysis of 2,628 patients transplanted between 1980 and 1989. Of these, 1,492 were alive (57%) and 1,136 had died. The follow-up exceeded 15 years for 253 patients and 16 patients had been followed up for 20 years or more. The probability of survival at 20 years was 34% with a cumulative incidence of transplant-related mortality at 20 years of 47% and of relapse of 26%. Survival was better for patients those transplanted from an HLA-identical sibling, transplanted in 1 first chronic phase and those with a low EBMT risk score. In a subgroup analysis, survival at 20 years was 40% for all patients transplanted in first chronic phase and 50% for those transplanted with an EBMT risk score of 0-1.

There have been huge changes in transplant procedures since this technique was introduced 30 years ago. Age has increased, peripheral blood is now the preferred source of stem cells source instead of bone marrow, unrelated donors are used for nearly half of all allogeneic transplants, reduced intensity conditioning has cut down early toxicity and donor lymphocyte infusions have eased management of relapse. Early transplant-related mortality has been substantially reduced. This reduction in transplant-related mortality was most marked for low risk patients and for those receiving transplants from unrelated donors. It can therefore be predicted that results of patients transplanted today will be substantially superior to earlier results. In a comparison with a cohort transplanted between 2000 and 2003, the probability of survival at 2 years was 61% compared to the earlier value of 53% with a cumulative incidence of transplant-related mortality at 2 years of 30% (versus 41%) and of relapse of 22% (versus 14%). The probability of survival at 2 years for the subgroup of patients transplanted in 1 first chronic phase from an HLA-identical sibling was 74% (versus 61%) with a cumulative incidence of transplant related mortality at 2 years of 22% (versus 57%) and of relapse of 18% (versus 11%). Improvements were observed in all patient subgroups, for survival as well as for reduction in transplant-related mortality. Survival was most markedly improved for patients with an unrelated donor (from 29% to 55%), for patients transplanted in 1 first chronic phase (from 54% to 70%) and for patients with low EBMT risk score (from 65% to 80%). Transplant-related mortality in the same groups decreased from 41% to 30% for the whole group, from 65% to 37% for unrelated transplants, from 38% to 26% in patients transplanted in first chronic phase and from 31% to 17% for patients with a low EBMT risk score of 0-1.

Main factors influencing outcome

CML was the model disease for establishing the main risk factors influencing outcome in allogeneic HSCT: these factors are stage of the disease at time of transplant, donor type, the amount of time elapsed between diagnosis and transplant and the donor-recipient sex combination. A scale from 0 to 7 can be calculated according to the presence or absence of these risk factors, (Table 1). The results are clear: survival is better for patients transplanted in chronic...
phase than in other stages or blast crisis because of the increased transplant-related mortality and relapse among patients with more advanced disease. Survival is better for patients transplanted within one year from diagnosis because of their reduced transplant-related mortality and reduced relapse rate. Survival is worse for male patients given a transplant from a female donor compared to another family donor or an unrelated donor. These risk factors are cumulative, e.g., the higher the risk score, the higher the risk for transplant-related mortality. They were established more than 10 years ago but validated in several independent data sets. They remained valid in a series of patients transplanted with reduced intensity conditioning and they remained valid in most recent cohorts of patients. The EBMT risk score still provides a solid basis for decision-making today.11-13

**Table 1. EBMT risk score for allogeneic HSCT for CML.**

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<tr>
<td>Blast crisis</td>
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<tr>
<td>Unrelated (other)</td>
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<table>
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<table>
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<th>Donor recipient sex combination</th>
<th>Score</th>
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<tbody>
<tr>
<td>Other</td>
<td>0</td>
</tr>
<tr>
<td>Donor female, recipient male</td>
<td>1</td>
</tr>
</tbody>
</table>

Score 0 (min) to 7 (max).

Decision making

The general recommendation for early HSCT for all patients14 can clearly no longer be maintained. Current possibilities with imatinib need to be considered. Patients with high risk disease and low risk for HSCT need a different approach than patients with low risk disease and high risk donors. These changes in philosophy were reflected in recent debates12,14 and in consensus guidelines for the treatment of CML. Risk assessment and risk-adapted therapy forms the basis for current strategies. At diagnosis, the risk of the disease, evaluated by the Hasford or Sokal score, must be determined and the availability of a donor examined. Response to therapy and risk assessment remain ongoing tasks throughout therapy. Assessment of response, disease progression and risk profile can change at any time. For patients with high risk disease (Sokal/Hasford intermediate or high risk) and an EBMT risk score of 0 or 1, early HSCT remains the treatment of choice. The prospects of disease-free survival of 80% or greater and long-term eradication of the disease compare favorably with the results of tyrosine kinase inhibitors. For all other patients, an initial trial with imatinib might be favored and HSCT used if imatinib fails or disease progresses after an initial response. Imatinib failure was recently defined based on hematological, cytogenetic and molecular responses at defined time points as well as on progression and signs of warning (Table 2).

A novel finding is the observation, that cost considerations might favor HSCT as a once-in-a-lifetime procedure in countries with limited resources where lifelong drug treatment with an expensive drug represents too great of a burden. A recent report from South America and Mexico as well as findings of a higher transplant rate in Eastern, compared to Western, European countries are indicative of costs being a factor influencing decisions concerning HSCT.15

Technical aspects

Despite decades of investigations, there is little indication that more recent conditioning regimens or more recent methods for preventing or treating graft-versus-host-disease provide an advantage over older standard regimens. Cyclophosphamide and total body irradiation or cyclophosphamide and busulfan can still be considered as the conditioning of choice. The only exception being that intravenous busulfan given after cyclophosphamide might reduce risk of veno-occlusive disease. Cyclosporine and short-term methotrexate remain the method of choice for preventing graft-versus-host disease. Approaches using T-cell depletion have not yielded better long-term outcomes than those with T-cell replete transplants. Reduced intensity conditioning appears to provide a short term benefit in early toxicity, but at the expense of a higher relapse rate. It is considered appropriate in patients above age 55 to 60 years. It cannot yet be considered the best approach for patients below the age of 55 to 60 years old.11 Peripheral blood the preferred source of stem cells for patients with advanced stage CML. Results are open for patients with early disease and more time is required to have conclusive data from the prospective randomized trials.16 Busulfan and interferon were shown to increase the transplant-related mortality of subsequent HSCT compared to hydroxyurea pre-treatment in earlier cohorts. Only preliminary information is available concerning the impact of imatinib pretreatment on subsequent HSCT. So far, there is no indication of a detrimental effect.17 On theoretical grounds, some researchers recommend withdrawal of imatinib dur-
The finding that patients with no response to imatinib have a worse outcome is not surprising. These patients might represent a higher risk category and these findings are compatible with the observation from earlier CML studies that, in general, patients who responded to interferon had a better survival after HSCT than patients with no response to interferon.13

**Recommendations**

Guidelines were published several years ago by an ASH committee.4 An update of these guidelines is currently being prepared.14 They are based on the most recent data, which provide an estimate of long-term survival probability with HSCT. This long-term benefit has must balanced against the early mortality related to HSCT and the early ease of response but the unknown prospect of a 20-year treatment with tyrosine kinase inhibitors. The data also provide an estimate of the increased risk to be faced when transplants are deferred and disease stage changes. Consequences of these estimates were recently discussed in a debate. Clearly, the general recommendation that all patients with CML and a HLA-identical donor should be transplanted within the first 12 months after diagnosis can no longer be upheld. The approach to patients with high-risk disease, e.g. high Hasford or Sokal risk score and with a low-risk donor is no longer the same as that for a patient with low-risk disease and high-risk donor. Patients with high-risk disease and an EBMT risk score of 0 or 1 should be considered for early transplants. In contrast, in patients with low risk disease, e.g. low Hasford or Sokal risk score, a prior trial with imatinib might be favored. Assessment of response and progression then becomes mandatory.

An additional element must be taken into account. Various successful strategies are now available. A patient’s preference might not be the same as the physician’s preference. Patients might prefer a low-risk treatment despite its uncertain future or they might prefer an early risk with a late benefit. Costs need to be considered as well.12, 15 All these points must be discussed with patients at diagnosis and at defined timepoints. In addition, new developments in drug treatment, e.g. new tyrosine kinase inhibitors, vaccination strategies and safer transplant procedures, will increase long term perspectives for all patients.18,19

**Treatment of relapse after HSCT**

Relapse remains a risk despite HSCT. The risk of relapse is higher in patients treated during advanced disease, in those in whom the interval between diagnosis and transplant is longer and in patients not responding to pre-transplant therapy. The risk of relapse is inversely related to the risk of graft-versus-host disease: the higher the risk and the more intense grade of effective graft-versus-host disease, the lower the risk of relapse and vice versa. Optimal survival is seen in patients with just grade I acute and limited chronic graft-versus-host disease. The risk of relapse is also inversely correlated with the dose intensity of conditioning. The higher the conditioning intensity, the lower the risk of relapse and vice versa.

Several treatment modalities are available for patients who relapse. Donor lymphocyte infusions have become a firmly established option.20 Such infu-
sions are most powerful if given for early (molecular or cytogenetic relapse) relapse; they are of limited value for relapse in advanced phase, e.g. accelerated phase or blast crisis. Donor lymphocyte infusions should preferably be given in incremental repetitive doses and months might be needed to achieve a response. Imatinib is an option for patients with relapse. Complete molecular remissions with full return of donor type hematopoiesis have been observed. The best modality is still an open question and the goal of a current prospective trial of the EBMT (www.ebmt.org). For advanced phase relapse, which occurs more than 6 months after first HSCT, re-transplantation from the same or an alternative donor remains a valuable option. Despite marked progress in the management of relapse, relapse still remains a risk and the hazard of subsequent death is 4-fold higher in patients who relapse than in those with no relapse after HSCT.6

Concluding remarks
Modern treatment approaches have changed the previously grim outlook of patients with CML. These different strategies, targeted therapy, interferon-based therapy, transplantation and immunotherapy are not exclusive but synergistic, each with specific advantages and disadvantages. Risk-adapted therapy, tailored to the needs and profile of individual patients has become the strategy of choice.

Acknowledgments
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References
Idiopathic erythrocytosis (IE) is a clinical condition characterized by an absolute increase of red blood cell mass without a definite cause. The diagnosis is based on the exclusion of other causes of erythrocytosis, such as polycythemia vera (PV), secondary polycythemias and congenital erythrocytoses. The frequency of IE in the general population was estimated to be 1.1 every 1,000 subjects, definitely higher than that observed for PV. Clinically, IE is a stable disease with a low thrombotic risk and a low, if any, tendency to spontaneous progression to overt PV, myelofibrosis or acute leukemia. Phlebotomy is the treatment of choice in patients with elevated hematocrit values but the optimal target hematocrit is still uncertain. Myelo-suppressive drugs should be avoided since their use was associated with evolution into acute leukemia in about 10% of patients.

**Abstract**

Idiopathic erythrocytosis (IE) is a clinical condition characterized by an absolute increase of red blood cell mass without a definite cause. The diagnosis is based on the exclusion of other causes of erythrocytosis, such as polycythemia vera (PV), secondary polycythemias and congenital erythrocytoses. The frequency of IE in the general population was estimated to be 1.1 every 1,000 subjects, definitely higher than that observed for PV. Clinically, IE is a stable disease with a low thrombotic risk and a low, if any, tendency to spontaneous progression to overt PV, myelofibrosis or acute leukemia. Phlebotomy is the treatment of choice in patients with elevated hematocrit values but the optimal target hematocrit is still uncertain. Myelo-suppressive drugs should be avoided since their use was associated with evolution into acute leukemia in about 10% of patients.

**Frequency**

IE is a frequent disorder. The prevalence has recently been evaluated in a prospective cohort study of 10,000 healthy subjects carried out in Italy. The study was preceded by a pilot phase in which the hematocrit at presentation was measured in 100 consecutive patients with definite PV, diagnosed according to the Poly-cythemia Vera Study Group criteria which include increased red cell mass. The hematocrit in all male and female PV patients was greater than 0.51 or greater than 0.48, respectively. These hematocrit values were chosen as the upper limits of normal. Hematocrit was evaluated at presentation in all participants and at a second follow up in 88 persons with increased baseline values. Thirty-five patients with confirmed high hematocrit were extensively investigated for a diagnosis of PV or secondary polycythemias and all subjects with an increased hematocrit at enrollment were followed for at least 5 years. At the end of the study, 11 patients were diagnosed as having IE that was stable after 5 years. The estimated prevalence was 1.1 cases per 1,000 persons. In the same study, the prevalence of PV and secondary polycythemias was 0.3 and 2.2 per 1,000 persons, respectively. Thus, IE is about four times more frequent than PV in the general population.
Differential diagnosis

By definition, the diagnosis of IE requires the exclusion of other causes of primary or secondary polycythemias (Figure 1). This diagnostic work-up has become increasingly frequent because an elevated hematocrit is a relatively common finding since the introduction of automated blood cell counts.

By definition, the diagnosis of IE requires the exclusion of other causes of primary or secondary polycythemias (Figure 1). This diagnostic work-up has become increasingly frequent because an elevated hematocrit is a relatively common finding since the introduction of automated blood cell counts.

Differentiation of IE from PV

The first and clinically most important step in diagnosing IE is to rule out the presence of a clonal hematopoietic disorder, namely PV. The diagnostic criteria for PV have been recently reviewed. It is important to emphasize that an accurate differentiation of IE from early stages of PV can sometimes be difficult and may require the use of sensitive diagnostic techniques. These include measurement of serum erythropoietin levels, bone marrow histology, assays of in vitro endogenous erythroid formation, and screening for the constitutive JAK2 mutation V617F.

We recently found the JAK2 mutation in only 10% of our patients with IE but in 90% of those with PV. Other investigators confirmed that this mutation is rarely present in patients with IE. Adopting these tests, when indicated, in the initial evaluation of a patient with absolute erythrocytosis facilitates the differential diagnosis of PV and IE. The clinical relevance of the proper differentiation of IE from PV is underscored by prospective clinical studies of these patients (see below).

Exclusion of secondary polycythemias

The next step is to exclude the most frequent causes of secondary polycythemias. Routine screening procedures include measurement of arterial oxygen saturation and, especially in smokers, COHb levels, abdominal ultrasound scan and erythropoietin levels. The finding of an arterial oxygen saturation below 92% indicates a diagnosis of secondary hypoxemic erythrocytosis. However, some patients desaturate markedly during sleep, but have a normal awake oxygen saturation. Thus, it is important to explore the presence of both underlying lung disease as well as sleep apnea causing transient arterial oxygen desaturation. Cigarette smoking per se is an uncommon cause of erythrocytosis. Smoking, with the concomi-

Table 1. Pathophysiological classification of absolute erythrocytosis.

<table>
<thead>
<tr>
<th>Primary polycythemia</th>
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<tr>
<td>Congenital</td>
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<tr>
<td>Primary familial congenital polycythemia (including mutations of the EPO receptor)</td>
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<tr>
<td>Acquired</td>
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<td>Polycythemia vera</td>
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<tr>
<th>Secondary erythrocytosis</th>
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<tbody>
<tr>
<td>Congenital</td>
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<tr>
<td>Mutant high oxygen-affinity hemoglobin</td>
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<tr>
<td>Congenital low 2,3 BPG deficiency</td>
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<tr>
<td>Methemoglobinemia</td>
</tr>
<tr>
<td>Chuvash polycythemia (autonomous high erythropoietin production)</td>
</tr>
<tr>
<td>Acquired</td>
</tr>
<tr>
<td>Hypoxemia (chronic lung disease, high altitude, cyanotic congenital heart disease)</td>
</tr>
<tr>
<td>Renal disease (tumors, cysts, hydronephrosis, renal artery stenosis, renal transplantation)</td>
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<tr>
<td>Liver disease (hepatoma, cirrhosis)</td>
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<tr>
<td>Endocrin</td>
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<tr>
<td>(adrenal tumours)</td>
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<tr>
<td>Tumors</td>
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<tr>
<td>(uterine fibroids, cerebellar hemangioblastoma, bronchial carcinoma)</td>
</tr>
<tr>
<td>Drugs</td>
</tr>
<tr>
<td>(erythropoietin, androgens)</td>
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| Idiopathic erythrocytosis |

Abbreviation: 2,3 BPG, 2,3-biphosphoglycerate.

Figure 1. Diagnostic work-up of a patient with suspected idiopathic erythrocytosis. Epo= Erythropoietin, P50 = oxygen pressure at 50% hemoglobin-oxygen saturation.
tant rise in COHb, is more commonly additive to other factors, such as lung disease, sleep apnea and obesity. Erythrocytosis, may also be caused by various kidney pathologies including multiple cysts, renal artery stenosis, renal cell carcinoma, or after renal transplantation (post renal transplant erythrocytosis). In addition, illicit or excessive iatrogenic administration of androgen and erythropoietin should always be considered in the evaluation of patients with erythrocytosis.

Differentiation of IE from congenital polycythemias

The final diagnostic step is to consider rare causes of congenital polycythemias. In patients with apparent IE, it is important to take a careful family history, bearing in mind that the recessively inherited erythrocytoses/polycythemias are seen more commonly in consanguineous unions and typically affect only siblings. Although time-consuming, it is imperative to obtain blood counts from the distant past. Three groups of congenital polycythemia have been described: a) primary familial and congenital polycythemia (PFCP); b) secondary congenital polycythemia; and c) polycythemias with an autonomous cythemia (PFCP). Patients with either normal or elevated erythropoietin levels.12 With a majority, neither features of PV nor a hematocrit level for a given hematocrit. PFCP is characterized by low serum erythropoietin, normal P50 (oxygen pressure at 50% hemoglobin-oxygen saturation) and a typically autosomal dominant inheritance. However, it should be kept in mind that the family history may be misleading if PFCP derives from a new mutation in a studied individual, if information on paternity is incorrect, or if previous hematological data in these frequently asymptomatic patients and their relatives are not available. If needed, a diagnosis of PFCP can be confirmed by demonstrating the hyper-responsiveness of erythroid progenitors to erythropoietin in in vitro assays. Some, but not all, of these patients have a truncated erythropoietin receptor (gain of function mutation).13

Secondary congenital polycythemias, characterized by a low P50, may be caused by high oxygen-affinity hemoglobins, methemoglobinemia, and 2,3-bisphosphoglycerate mutase deficiency. Patients with any of these secondary polycythemias may have either normal or elevated erythropoietin levels.12

Polycythemias with autonomous high or inappropriately normal erythropoietin levels for a given hematocrit are the most difficult to differentiate from IE. However, in this group several separate entities with defined molecular defects have been identified. The 598G>T mutation of the von Hippel Lindau (VHL) gene constitutes Chuvash polycythemia that is endemic in the Chuvash Republic of Russia14 and the island of Ischia in Italy15 and presents sporadically in other racial and ethnic groups worldwide. Other germline VHL mutations, typically present in both VHL alleles in conjunction with the Chuvash VHL mutation or not, can also account for this polycythemic syndrome. The results of an in vitro assay of erythroid colonies are heterogeneous, demonstrating erythropoietin hypersensitivity (a feature of primary polycythemia) in Chuvash polycythemia, but may be normal or hypersensitive in patients with other VHL mutations.11 However, while VHL mutations are the commonest identified cause of congenital polycythemia, the molecular basis of congenital polycythemia and high or inappropriately normal erythropoietin levels remains obscure in the majority of patients.

Clinical presentation and course

Since diagnostic criteria to identify PV (and therefore exclude IE) changed significantly over the years we have distinguished recent from older clinical studies.

Early studies

Three studies specifically designed to evaluate the clinical course of IE were published between 1968 and 1981.2,3,14 An abstract updating the observations of one of these studies was reported later.15 The median age at presentation was between 54 and 65 years, like that for PV, but there was a greater male predominance, ranging from 2.2 to 5.5:1, than seen in PV. Patients were frequently diagnosed after vascular complications (63% of 50 patients in one series) or other symptoms, including headaches, gout and pruritus. Only 20% of patients were detected based on an abnormal routine blood count.

Evolution into PV during follow-up was reported in 10-40% of cases. Other patients were found to have a secondary erythrocytosis, notably hypoxemia. In the majority, however, neither features of PV nor a cause of secondary erythrocytosis emerged. The incidence of vascular occlusions was high in all three series of patients. In the first published series, the incidence of vascular occlusion was similar to that observed in PV.2 Examination of the cause of death in two studies showed that a cerebro-vascular accident or cerebral thrombosis was the cause in half of 21 patients. A reduction in cerebral blood flow was observed in IE, as in PV,16 and a limited study of treatment of IE demonstrated that the risk of vascular occlusion was six times greater in patients with hematocrit values above 0.50 than in those with values below this level.1 From these observations, it was proposed that reduction of hematocrit should be a therapeutic aim, but the heterogeneity and retrospective design of the series makes it difficult to formulate a specific management strategy for all IE patients.
Recent case series

In the current era, asymptomatic IE patients are often discovered as a result of routine blood counts, mandating the need to update the natural history of IE. In a prospective cohort study, we evaluated the clinical course of 74 patients (66 males, 8 females; median age 56 years, range 14-82) diagnosed as having IE in two Italian institutions.17 Twelve patients (16%) presented with a history of thrombosis (7 ischemic cardiac disease, 4 cerebral ischemic events, 1 deep vein thrombosis), whereas the great majority were investigated because of an incidentally found increased hematocrit. At presentation, the median hematocrit was 0.54 (range 0.48-0.68) and, by definition, red cell mass was increased by 25% or more above the mean normal predicted value. Normal leukocyte and platelet counts, spleen volume, erythropoietin levels, and bone marrow histology argued against PV. Normal arterial oxygen saturation and other routine screening procedures excluded a secondary polycythemia. In selected cases, congenital polycythemia was excluded by appropriate investigations. All patients were followed for a median of 3.5 years (range 1-23) and 23 of them (31%) were followed for more than 8 years. Treatment included phlebotomy to maintain the hematocrit below 45% and aspirin, 100 mg/day, in 24 patients (52%) with previous thrombosis, microvascular symptoms or cardiovascular risk factors. No cytotoxic drugs were given. During follow-up, no hematological transition to overt PV, myelofibrosis or acute leukemia was observed and no disease potentially associated with secondary erythrocytosis emerged. Two thrombotic events (one cerebral ischemia and one deep vein thrombosis) occurred, with an incidence of 0.8% patient-year, significantly lower than the 3.5% patient-year ($p<0.05$) incidence of major vascular complications observed in 205 patients with PV followed during the same period in one of the two Institutions (Bergamo).

Other case series have been reported recently. In a prospective study Kiladjian et al.18 evaluated 140 patients with PV (median age 62 years, M/F ratio 1.46) and 39 with IE (median age 57 years, M/F ratio 3.33) treated with pipobroman. The diagnosis of IE was based on elevated red cell mass, normal platelet and leukocyte counts, no splenomegaly, and no evidence of secondary erythrocytosis. After a median follow-up of 11.4 years (range 1-28), six IE patients (15.4%) developed leukemia; this proportion did not differ from that in the PV group (18.6%). Four patients with IE (10.2%) and 28 with PV (20%) presented with a major vascular event. Although this difference was not statistically significant, the rate of thrombosis in IE was about half that in PV; more interestingly, the calculated incidence of thrombosis per year in IE was approximately 0.9%, very close to the 0.8% observed in our study of untreated patients, and the development of leukemia in this group raises the question of a pipobroman-induced complication.

Peter Johansson in Sweden (personal communication) is currently following 13 patients with IE (median age 58 years, 9 males, 4 females) without apparent cause who are being treated with phlebotomy alone aiming for a target hematocrit < 50%. After a median follow-up of 6 years (range 2-22), one myocardial infarction has occurred, giving an estimated incidence of major thrombosis of 1.2% patients per year. None of the patients developed PV or any other hematological progression.

Treatment recommendations

Some patients with IE have been treated with myelosuppressive agents, with the assumption that this condition was a variant of PV.13,19-21 Similarly many patients with Chuvash polycythemia were also treated with chemotherapy.1 It should be noted that acute leukemia developed in 8-10% of patients treated with phosphorus 31 and in 15% of those given long-term pipobroman therapy.18 In contrast, leukemic transition was not observed in those patients in whom treatment was restricted to phlebotomy.1,14,17 Thus, we feel that potentially leuke-mogenic agents should not be used in IE.1

Phlebotomy has been the treatment most frequently used in these patients, although there is no controlled study to show that this therapy reduces the incidence of vascular occlusive events compared to the incidence in an untreated group. In addition, there is uncertainty regarding the threshold value of hematocrit to be used for starting phlebotomy and the target hematocrit to reach and to maintain. Translating evidence from PV management strategies,7 reduction of cerebral blood flow in patient with IE and raised hematocrit values has been interpreted as a predictor of cerebral thrombosis;14 thus, the recommendation is judicious phlebotomy to maintain the hematocrit below 0.45 if the basal hematocrit is above 0.54, or there is increased risk of thrombosis, i.e. evidence of ischemia, previous history of thrombosis, peripheral vascular disease, diabetes or hypertension.1

Low-dose aspirin (100 mg daily) has been recently found to reduce the incidence of major vascular complications in a randomized clinical trial in PV, leading to the recommendation of introducing this drug in the initial therapy of all PV patients.35 Whether this advice should also be applied to patients with IE has not been formally proven. For the time being, aspirin can be recommended to patients at increased risk of...
occlusion, such as those with evidence of ischemia, a previous history of thrombosis, peripheral vascular disease, diabetes mellitus or hypertension.

References

Management of pregnancy in polycythemia vera and essential thrombocytemia

Over the last few years, a major discovery has been made every year in myeloproliferative disorders (MPD) other than chronic myelogenous leukemia (results of the ECLAP study in 2003, results of the PT1 trial in 2004, the JAK2 mutation in 2005). Interest in these diseases has never been so great since the era of the Polycythemia Vera Study Group, and it is to be hoped that this will result in better evaluation and management of patients. Polycythemia vera (PV) and essential thrombocytemia (ET) are MPD characterized by clonal proliferation of hematopoietic stem cells leading to increased production of mature circulating cells. The main phenotypic difference between these two entities, with major pathophysiological consequences, is the expansion of the red cell compartment in PV. Otherwise, clinical complications (i.e. thrombotic, hemorrhagic and ischemic events) and hematological evolution to myelofibrosis, myelodysplastic syndromes or acute leukemia are observed in both diseases, although with a higher incidence in PV. The recent finding of a unique acquired mutation of JAK2 kinase in most cases of PV and about one half of ET allows better understanding of disease pathophysiology. ET patients with the V617F JAK2 mutation appear to display many features resembling PV, including higher hemoglobin levels, neutrophil counts or bone marrow myelopoiesis, but also a higher risk of developing venous thrombosis. Thus, cases of ET and PV with the JAK2 mutation may, perhaps, be considered as part of a continuum rather than clear-cut separate diseases. Future analysis of large prospective series will determine the impact of the JAK2 mutation but one can probably already assume that new risk stratifications of ET will also rely on JAK2 mutational status.

The mean age of patients at diagnosis of MPD is around 60 years. In ET (but not in PV), a second peak of incidence is observed in younger female patients. The proportion of patients diagnosed with PV in people younger than 40 years is clearly smaller (about 15%). But if one considers that the wide use of automated platelet counts played a part in the observed increasing annual incidence of ET, it is possible that screening for the highly specific JAK2 mutation could increase the rate of PV patients diagnosed earlier in coming years, once this test becomes easily accessible in clinical practice. Although younger patients with MPD usually belong to the clinically low risk categories, due to their lower age and lower incidence of associated risk factors (arterial hypertension, hypercholesterolemia...), this population may have an increased risk of atypical and major complications such as splanchnic vein thrombosis.

Thus, most clinical hematologists now follow female MPD patients of childbearing age, and pregnancy will become a non-exceptional challenge in clinical practice. Similar types of complications have been observed during pregnancy in both ET and PV, and (according to retrospective evaluations) the overall success rate of pregnancy is only about 50-60%.

Pregnancy outcome in ET and PV

Available data about pregnancies in ET and PV are mainly retrospective and, therefore, possibly biased; randomized or controlled studies do not exist in such patients. In the recently published guidelines for the management of ET by the Italian Society of Haematology and management of PV by the British Committee for Standards in Haematology, recommendations for pregnancy were mainly based on experts' opinions. However, a significant number of cases have been now reported, particularly in ET, making it possible to evaluate the impact of MPD on the outcome of pregnancy.

In reported cases, the success rate of pregnancies (live births) was around 50%. Unsuccessful outcomes were mainly due to first trimester miscarriage (50%...
to 40%, compared to 15-20% in the general population. Other complications included a higher prevalence of late pregnancy loss compared to that in the general population (5-10% versus 0-5%, respectively), stillbirth (3%), pre-term delivery (5-8%), intrauterine growth retardation (IUGR) (5%), and abruptio placentae (2-4%). Maternal thrombotic episodes seemed to be more frequent in ET patients than in the general population, occurring in about 5% of patients, and including atypical sites such as sagittal sinus thrombosis or Budd-Chiari syndrome. However, thromboses were usually minor, and occurred mainly in the postpartum period. In PV, maternal complications seemed to be more frequent and more severe, and included major thromboses and hemorrhages.

**Risk factors in pregnancies occurring in ET and PV patients**

Major risk factors used to stratify the vascular risk in ET patients are a history of major thrombosis, or of major hemorrhage, a platelet count higher than 1500×10^9/L, and older age. This last factor, in the context of women with childbearing potential, is clearly below the threshold for entering a high-risk group. These clinical characteristics, however, do not appear to be strong predictive factors for obstetric complications, due in part to the physiological changes in blood cell counts during pregnancy. Indeed, the platelet count progressively declines by 15 to 20% during pregnancy, this phenomenon usually being more pronounced in ET patients. The mechanism responsible for such decline is unclear. However, the 1500×10^9/L threshold that usually triggers platelet lowering therapy in ET is probably not suitable during pregnancy. An analysis of pooled outcome data from 461 pregnancies in ET showed that the average platelet count was 1010×10^9/L in patients with successful pregnancies, compared to 977×10^9/L in those with unsuccessful outcome, suggesting that platelet count *per se* is not a reliable criterion to predict pregnancy outcome. Similarly, in PV, the natural fall of hematocrit observed during pregnancy makes the threshold value of 42% (value below which female PV patients are considered to have a low risk of vascular complications) not adequate.

Neither ET-related symptoms prior to and during pregnancy nor a history of vascular events were clearly demonstrated to be of prognostic value for maternal or obstetric outcomes. Similarly, there is no significant evidence that the occurrence of a complication during the first pregnancy will affect the outcome of subsequent pregnancies.

Some biological markers have been demonstrated to be adverse factors for thrombosis in ET, including PRV-1 overexpression, monoclonal hematopoiesis as determined by X-chromosome inactivation patterns or the presence of the V617F JAK2 mutation. None of these has yet been studied in the particular context of pregnancy. In our own experience, pregnancy complications were observed both in patients with and without these factors.

**Pathophysiology**

Normal uterine blood flow and adequate placental development are crucial factors that may affect pregnancy outcome. Anomalies of this subtle system may result in severe pregnancy complications, such as placental abruption, IUGR, or pre-eclampsia. In ET, placental infarction due to thrombosis was consistently found in cases of IUGR, pre-term deliveries and late fetal losses. Thus, although the pathophysiology of obstetric complications in MPD is not completely understood, the acquired prothrombotic state probably participates in the higher complication rates observed in MPD patients. In the absence of prospective data, management of pregnancy in MPD may be improved using the large experience available in patients with other prothrombotic states such as anti-phospholipid syndrome and inherited thrombophilia.

**Cytoreductive drugs in the context of pregnancy**

As seen above, well-known validated risk factors used to stratify ET and PV patients are modified or controversial during pregnancy, making the decision to use cytoreductive therapy difficult and unfortunately not evidence-based. This assessment is of importance, since none of the cytoreductive agents currently efficient in the treatment of MPD has a product license for use in pregnancy.

Interferon-α is probably the drug of choice in this setting as, to date, it has not been shown to be teratogenic at standard doses. Furthermore, many successful pregnancies have now been reported in interferon-treated ET patients, showing that the use of this drug is possible. Interferon-α is probably the drug of choice in this setting as, to date, it has not been shown to be teratogenic at standard doses. Furthermore, many successful pregnancies have now been reported in interferon-treated ET patients, showing that the use of this drug is possible. Interferon-α is probably the drug of choice in this setting as, to date, it has not been shown to be teratogenic at standard doses. Furthermore, many successful pregnancies have now been reported in interferon-treated ET patients, showing that the use of this drug is possible. Interferon-α is probably the drug of choice in this setting as, to date, it has not been shown to be teratogenic at standard doses. Furthermore, many successful pregnancies have now been reported in interferon-treated ET patients, showing that the use of this drug is possible. Interferon-α is probably the drug of choice in this setting as, to date, it has not been shown to be teratogenic at standard doses. Furthermore, many successful pregnancies have now been reported in interferon-treated ET patients, showing that the use of this drug is possible. Interferon-α is probably the drug of choice in this setting as, to date, it has not been shown to be teratogenic at standard doses. Furthermore, many successful pregnancies have now been reported in interferon-treated ET patients, showing that the use of this drug is possible.
reduce the incidence of thrombosis in ET, its use is undoubtedly contraindicated during pregnancy due to its teratogenic potential, documented in many animal species.28-33 Harrison recently reviewed the outcome of 55 pregnancies with exposure to hydroxyurea.34 Eight pregnancies were therapeutically terminated, and one was terminated for unstated reasons. Forty-two live births were observed in the 46 remaining pregnancies, and only one newborn presented major abnormalities (the mother having also been exposed to interferon after the fourth month). The frequency of minor abnormalities did not seem different to that observed in the general population. Thus, hydroxyurea at therapeutic dosage could be less toxic than expected in humans. However, there were no follow-up data in those infants beyond one year, and long-term toxicity (including secondary malignancies) of in utero exposure to hydroxyurea could not be established.

Anagrelide, which was mainly used in younger patients given its lack of leukemogenicity,44 is also contraindicated during pregnancy because the drug is small enough to cross the placenta and the effects on the human fetus have not been studied.

**Aspirin and heparin**

The ECLAP study4 showed that low dose aspirin could reduce the risk of thrombotic complications in patients with PV. Such an effect has not yet been demonstrated in ET, but aspirin is clearly beneficial in platelet-mediated microvascular disturbances.4 It was also demonstrated that low dose aspirin is safe during pregnancy.46 Studies by Griesshammer et al. suggest that aspirin may indeed improve pregnancy outcome in ET.47 Although the beneficial effect of aspirin of reducing the incidence of complications during MPD pregnancies is debated,48 it does seem reasonable to propose low dose aspirin in all cases, in the absence of clear contraindications. Aspirin is clearly recommended in patients with microvascular symptoms.

Low molecular weight heparin (LMWH) has been extensively studied in pregnancy. LMWH is safe, and beneficial in pregnancies at high-risk of thrombosis, reduces fetal morbidity, and has fewer side effects than unfractionated heparin.49-51 Gris et al. recently showed that LMWH was superior to aspirin in terms of the rate of healthy live births rate in patients with inherited thrombophilic disorders.53 There are not sufficient data to firmly establish that LMWH may similarly improve obstetric outcomes in MPD in which the prothrombotic state involves blood rheology and cell-mediated mechanisms.50-51 Combinations of aspirin and unfractionated heparin or LMWH were reported to improve pregnancy outcome in small series of patients with ET,52,53 but these retrospective data (using different schedules of administration) need to be confirmed before such combinations can be definitely recommended. In case of previous thrombosis, prophylactic doses of LMWH are indicated.

**Practical management of pregnancy in ET and PV**

**Before pregnancy**

Cytoreductive therapies should be avoided at the time of conception and during the first trimester of pregnancy. In PV patients, hematocrit can be controlled by venesection. In ET and PV with marked thrombocytosis, the expected natural fall of the platelet count during pregnancy may often allow interferon cytoreductive treatment to be avoided. In situations in which cytoreduction is deemed necessary, interferon is the drug of choice as discussed above. In patients treated with hydroxyurea or anagrelide before pregnancy, the drug should be progressively withdrawn, preferably with a wash-out period of 3 to 6 months before conception.13,52 According to the ECLAP study results, all PV patients should receive low dose aspirin (unless contraindicated), and there is obviously no reason to stop aspirin before or during pregnancy in the absence of evidence of acquired von Willebrand’s disease. In ET, we also recommend that patients without clear contraindications should receive low dose aspirin before conception and throughout the pregnancy.

**During pregnancy: guidelines to start cytoreductive treatment**

Recently published comprehensive reviews and guidelines have delineated factors that should be used to assess the risk of complications in pregnancies with MPD.12,13 These factors include previous major thrombosis or major bleeding (whether pregnant or not), and a high platelet count. The platelet cut-off value is, however, debated and lies somewhere between 1000\*10^9/L (for authors who consider that the risk of acquired von Willebrand’s syndrome secondary to extreme thrombocytosis must be avoided) and 1,500\*10^9/L (for those who consider that the spontaneous decline of the platelet count during pregnancy would reduce the severity of such an acquired von Willebrand’s syndrome). The existence of complications during a previous pregnancy is also a controversial risk factor.27,28 For some authors, such a history places the subsequent pregnancies at higher risk.53 As discussed above, cytoreductive treatment in these situations should be interferon. The target platelet count is not clearly defined in pregnant women with thrombocytosis, some authors suggesting that platelet count should be simply reduced to a level that allows correction of symptoms in low-risk patients (in particular in the case of acquired von Willebrand’s disease).17 It
does, however, seems logical to try to normalize the platelet counts once cytotherapeutic treatment is decided. Plateletpheresis is effective for rapidly reducing the platelet count and has probably no adverse effect on the fetus, but the procedure is cumbersome, and effects are transient, lasting only a few days. Although it may be useful in urgent situations, this technique is usually not feasible for controlling the platelet count throughout the whole pregnancy.

Delivery and postpartum

The use of antithrombotic therapy during pregnancy implies discussion with the patient and anesthetist of its implications for a Cesarean section or epidural anesthesia. In particular, aspirin must be stopped 1 to 2 weeks before delivery to reduce the risk of epidural hematoma due to anesthesia or wound hematoma following a Cesarean section. Prophylactic doses of LMWH can then be used until delivery. For patients already being treated with LMWH, it is important to note that invasive procedures (epidural anesthesia, Cesarean section) are contraindicated for 12 and 24 hours after the last prophylactic and therapeutic dose, respectively.

The platelet count may rise rapidly after delivery. This rise is associated with a high risk of thrombosis in MPD patients. Thus, blood counts should be carefully monitored in order to start cytotherapeutic agent if needed (breast feeding is contraindicated once such treatment has been initiated). In addition, the prothrombotic changes of pregnancy do not revert completely to normal until 6 weeks after delivery. LMWH prophylaxis should be started within a few hours after delivery when there is no bleeding complication, and continued for at least 4 to 6 weeks.

Conclusion

With advances in knowledge regarding PV and ET, these conditions are being diagnosed earlier in younger patients, and the challenge of pregnancy will probably become less and less exceptional in clinical practice. Pregnancy in PV and ET is definitely possible, and the majority of cases result in successful delivery of a healthy baby. However, these pregnancies are clearly high-risk events like those in other prothrombotic states. Both the hematologist and the obstetrician in charge of pregnant MPD patients should be aware of the current data on beneficial as well as deleterious strategies, and manage each pregnancy in close collaboration. Information on pregnancy should also be systematically provided to MPD women of childbearing age, in order to avoid unanticipated pregnancies. A European registry of pregnancies in women with ET, created by M. Griesshammer on behalf of the LeukemiaNet, could be the first step of a large international collaboration that is required to address the numerous uncertainties concerning management of pregnancy in women with MPD.

References


Recent clinical trials in polycytemia vera and essential thrombocythemia

The last two years have seen major advances in our understanding of both the pathogenesis and the management of myeloproliferative disorders. Firstly, the two largest randomized clinical trials in myeloproliferative disorders have been published, one in polycytemia vera (PV) and the other in essential thrombocythemia (ET). Secondly, the discovery of the JAK2 V617F mutation, present in >90% of cases of PV and about half the patients with ET and idiopathic myelofibrosis (IMF) when sensitive detection methods are employed, promises to redefine the diagnostic work-up, classification and ultimately management of these disorders.

The classical myeloproliferative disorders (MPD) are three diseases, PV, ET and IMF, which share many clinicopathological features and yet retain several important phenotypic differences. The pathognomonic feature of PV is a raised red cell mass, and that of IMF collagen fibrosis of the bone marrow. These two characteristics have historically been used to distinguish the three disorders, and all randomized clinical trials to date have studied just one of the three diseases based on this historical division. However, even before the discovery of the JAK2 mutation, it was well recognized that there was considerable heterogeneity among patients within one disorder, and, additionally, considerable overlap in clinical and laboratory features of patients with different disorders. Thus, hemoglobin concentrations can sometimes be in the normal range in untreated patients with PV, due to iron deficiency, plasma volume expansion or splenomegaly, whereas patients with V617F-positive ET can have hemoglobin concentrations towards the upper limit of normal together with frankly suppressed erythropoietin levels. Similarly, bone marrow fibrosis can be present at diagnosis of ET and PV, can develop during the course of the disease, and may or may not be associated with other features of IMF such as splenomegaly and anemia (reviewed in ref. 10). Given that the identical JAK2 mutation is prevalent in all three disorders, it is now perhaps not surprising that there is considerable overlap among the three disorders in clinical and laboratory findings, although why there is also considerable inter-patient heterogeneity remains unexplained.

The interpretation of recent clinical trials in MPD needs to reflect these considerations. Firstly, shared molecular and clinical features suggest that results of trials in one disease can be generalized to some extent to the other. Secondly, as diagnosis and classification schemes evolve to incorporate JAK2 status and clarify the role of bone marrow histology, it will be important to recognize that treatment guidelines will be based on clinical trials using earlier inclusion criteria. Thirdly, the heterogeneity of disease outcome between patients highlights the importance of individualized risk assessment and treatment. Ultimately, though, the most important cause of morbidity and mortality in both PV and ET is thrombosis, and treatment should be directed towards managing this risk, while minimizing the chances of myelofibrotic and leukemic transformation in the longer term.

Polycytemia vera: the ECLAP trial

The ECLAP trial was a randomized comparison of aspirin with placebo in patients with newly or previously diagnosed PV, nested within a larger prospective observational cohort study that captured data on all-comers with PV. In addition to aspirin or placebo, all patients had cytoreduction or phlebotomy to control hematocrit and/or platelet count managed according to their local hematologists’ preferences. Patients who had a clear indication for aspirin (ie, high risk patients) were excluded from the randomization, and these represented a larger number of patients (742 patients) than those actually randomized (518), indicating that the
ECLAP trial represented the approximately 40% of PV patients at lowest risk of thrombosis. Thus, the mean age of randomized patients was about 61 years, compared to 65 years for the full cohort, and <5% had a previous arterial thrombosis in the aspirin trial compared to 28.7% in the overall study.²

The key finding of the randomized trial was a significantly reduced risk of the combined primary endpoint of non-fatal myocardial infarction, non-fatal stroke, pulmonary embolism, major venous thrombosis or death from a cardiovascular cause in patients randomized to receive aspirin.² Subgroup analyses showed that the benefits from aspirin were homogeneous across groups stratified by duration of disease, cytoreductive therapy or not, and platelet counts and hematocrit at trial entry. The benefits of aspirin came at no increased risk of major hemorrhage, and minimal if any increase in minor bleeding (relative risk 1.83; p=0.10).²

Overall, 3.2% of patients in the aspirin arm and 7.9% in the placebo arm suffered a primary endpoint event over a mean follow-up of nearly 5 years. This means that about 60 patients need to be treated for one year in order to prevent one primary endpoint event. This compares well with studies of aspirin prophylaxis in other clinical settings, in which, for example, 30 patients need to be treated following myocardial infarction, 110 in acute stroke and 45 for other high-risk vascular groups to prevent one vascular event in a year.¹² Given that the population studied in the ECLAP randomization represented the most prognostically favorable group, the number needing to be treated to prevent one event for higher risk patients with PV is likely to be substantially less than 60. Therefore, it seems clear that all patients with PV should be treated with aspirin, unless there is an absolute contraindication, and for those patients, consideration should be given to other anti-platelet therapy.

The larger observational cohort study of PV has also collected important information on the natural history of PV, and the predictors of vascular events and leukemic transformation. Overall survival was significantly poorer than that of the age- and sex-matched reference population, with rates of death from all causes being 1.2 times higher, death from cardiovascular causes 1.4 times higher and death from leukemia 36.1 times higher.¹¹ The two most important prognostic factors were age >65 years and a previous history of thrombosis. Treatment modality and disease duration were not independently associated with survival. For leukemic transformation, increasing age, and busulfan, pipobroman and radioactive phosphorus treatment at trial entry were predictive of increased risk.¹¹ Importantly, treatment with hydroxyurea alone was not associated with an increased risk of leukemic transformation (hazard ratio 0.86; 95% confidence interval 0.3-2.9). However, this finding needs to be interpreted with caution since the median follow-up was only 2.8 years, and also because the treatment was not randomized.

**Essential thrombocythemia: The PT-1 trial**

The Medical Research Council (MRC) primary thrombocythemia-1 (PT-1) trial 1 randomized high-risk patients (prior thrombosis, age >60 years or platelets >1000x10⁹/L) to receive either hydroxyurea plus aspirin or anagrelide plus aspirin. It has two ongoing sister trials: an intermediate-risk study, which is a randomized comparison between hydroxyurea plus aspirin and aspirin alone in patients between 40 and 60 years old with no high-risk features; and the low-risk study, an observational study of aspirin therapy alone in ET patients less than 40 years old. With over 500 patients randomized and with central clinical and histological review of end-points, the high-risk PT-1 trial is the largest and most comprehensive study of ET performed to date. The results demonstrate several major differences between the two arms. Compared to hydroxyurea plus aspirin, treatment with anagrelide plus aspirin was associated with increased rates of arterial thrombosis, major hemorrhage, myelofibrotic transformation and treatment withdrawal, but a decreased rate of venous thromboembolism.

It is informative to compare these results with the only other published randomized trial in this condition.¹⁴ The actuarial rate of first thrombosis at 2 years was 4%, 8% and 26% for patients receiving hydroxyurea ± aspirin (both studies), anagrelide plus aspirin (PT-1) or no cytoreductive therapy (Italian study), respectively. Notwithstanding the difficulties of such comparisons, these data suggest that anagrelide plus aspirin provides partial protection against arterial thrombosis.

In marked contrast to arterial thrombosis, the rate of venous thrombosis was significantly lower in the anagrelide plus aspirin arm. The incidence of venous thrombosis in untreated patients with high-risk ET is unknown and so it is not clear whether this rate is increased by hydroxyurea plus aspirin or decreased by anagrelide plus aspirin. The optimal management of a patient with prior venous thrombosis will depend on individual circumstances. Arterial thrombotic events are >3 fold more common than venous thrombotic events in ET, and are generally associated with greater morbidity and mortality, thus suggesting that hydroxyurea would in most cases remain the first-line treatment of choice in this situation.

The difference in rates of thrombosis in the two
arms of PT-1 is intriguing given the equivalent long-term control of the platelet count. These data imply that, in addition to lowering the platelet count, hydroxyurea or anagrelide may modulate thrombosis by other mechanisms (e.g., altered white cell count or function; altered endothelial function). The unexpected increase in major hemorrhage observed in patients receiving anagrelide plus aspirin may reflect an ability of anagrelide to interfere with platelet function in a way that synergizes with that of low-dose aspirin. Although most assays of platelet function are normal in patients with ET receiving anagrelide, some subtle effects on platelet function have been reported. The PT-1 results suggest that, if anagrelide is used, the decision whether to use concurrent aspirin should depend on the relative risk of arterial thrombosis and hemorrhage in each individual patient.

Patients receiving anagrelide plus aspirin experienced an increased rate of transformation to myelofibrosis compared to that in patients receiving hydroxyurea plus aspirin. It is important to emphasize that a diagnosis of myelofibrotic transformation required not only a trephine biopsy showing grade 3 fibrosis, but also the development of other clinical or laboratory evidence of transformation, which was absent at trial entry. The higher rate of myelofibrotic transformation was not an artifact of the precise definition used, since making the diagnostic criteria more or less stringent did not affect the statistical significance. The incidence of myelofibrosis in untreated ET is unknown, and so it is not clear whether the observed differences reflect a protective effect of hydroxyurea or an acceleration of myelofibrosis by anagrelide. Hydroxyurea has been reported to reduce reticulin fibrosis in a variety of myeloproliferative disorders, including ET. On the other hand, anagrelide blocks megakaryocyte differentiation, and the resultant relative increase in immature forms could conceivably result in altered production of profibrotic cytokines.

It is worth noting that the inclusion criteria for the PT-1 trial were based on the PVSG criteria for ET. Since the trial’s inception, the World Health Organization (WHO) published its controversial criteria on the diagnosis of MPD, in which the categories of prefibrotic and early manifest myelofibrosis were created. It is claimed that these categories represent an early stage of idiopathic myelofibrosis, with high probability of progression to end-stage myelofibrosis. However, it is clear from PT-1 that many patients were enrolled who would be labeled as having prefibrotic or early stage IMF under the WHO criteria, and yet only 2.6% (21/809) of the cohort underwent myelofibrotic transformation during a median follow-up of 39 months, suggesting a low rate of disease progression in the short to medium term.

Much of the debate about the results of the PT-1 study underscores the importance of rigorous methodology when running large, multicenter clinical trials. For example, it is possible that the increased myelofibrosis noted with anagrelide treatment reflects a chance excess of the prefibrotic form of myelofibrosis in the anagrelide arm. However, the randomization process should ensure an equal distribution of these cases in the two arms. Thus, the probability that the results can be explained by such a chance occurrence is 1 in 100 (p value = 0.01). The same considerations apply to other baseline characteristics, such as the five patients who had a hemoglobin level towards (but not above) the upper limit of normal. Similarly, it could be suggested that inclusion of complications without a gold-standard diagnostic test, such as transient ischemic attacks, in the composite primary and secondary end-points may be open to bias and misinterpretation. However, the components of the end-points and their definitions were all determined before the randomization code was broken and data analyzed. Furthermore, the clinical, diagnostic and histological material for all primary and secondary end-point events were reviewed centrally by a panel of hematologists who were blinded to treatment allocation, and each reported event arbitrated before the randomization code was broken and data analyzed. Thus, while discussion of the clinical significance of the results can and should continue, it is difficult to sustain arguments that the differences between anagrelide and hydroxyurea were the result of some bias of either the randomization or end-point validation. Exactly the same considerations apply to the ECLAP study, whose investigators used the same methodological rigor.

The JAK2 V617F mutation: relationship between V617F-positive ET and PV

DNA samples were stored at trial entry from 776 patients entered in the PT-1 trial and the on-going trials in low-risk and intermediate-risk ET, and have been analyzed for presence of the JAK2 V617F mutation. Just over half had the mutation and this divided patients into two biologically distinct subgroups. V617F-positive patients displayed multiple features resembling PV, with significantly higher hemoglobin levels, neutrophil counts, bone marrow erythropoiesis and granulocytopenia, more venous thromboses and a higher incidence of polycythemic transformation. In addition, mutation-positive patients had lower serum erythropoietin and ferritin levels than V617F-negative patients with ET. These results imply that V617F-positive thrombocythemia and polycythemia may be better viewed as a contin-
uum, and not as two distinct entities (Figure 1). V617F-negative individuals with ET, do nonetheless, exhibit features characteristic of an MPD, including cytogenetic abnormalities, hypercellular bone marrow with abnormal megakaryocyte morphology, PRV1 over-expression, growth of erythropoietin-independent erythroid colonies, and a risk of myelofibrotic or leukemic transformation. These facts suggest that ET should be subclassified as either V617F-positive or V617F-negative ET, with both being bona fide MPD, although future studies may prove V617F-negative ET to be biologically heterogeneous.

Interestingly, analysis of the response to therapy and complications in the PT-1 trial according to JAK2 mutation status revealed that V617F-positive patients were much more sensitive to hydroxyurea. Compared to V617F-negative patients, they required substantially lower doses of hydroxyurea and yet had greater reductions in platelet counts, white cell counts and hemoglobin levels. No such effect was seen in patients receiving anagrelide. Furthermore, the rate of arterial thrombosis appeared to be lower in V617F-positive patients receiving hydroxyurea compared to those receiving anagrelide, an effect that was not evident in V617F-negative patients.

These findings have several implications for the management of patients with ET. Firstly, in addition to risk stratification into high, intermediate and low risk, it is worth classifying patients as V617F-positive or negative, since hydroxyurea seems particularly important for the first-line management of high-risk JAK2-positive patients. Secondly, doses of hydroxyurea may need to be higher in V617F-negative patients to control the platelet count, but this may come at the expense of other cytopenias. Thirdly, if V617F-positive ET and PV do form a continuum, this raises the tantalizing possibility that V617F-positive patients with ET should have their hematocrit managed to a threshold of 0.45, and conversely that cytoreduction should be strongly considered for PV patients who develop thrombocytosis while being treated with phlebotomy, a previously controversial notion.

JAK2 may in the future provide a target for therapy in V617F-positive disease, although the relatively benign prognosis of PV and ET means that the initial phase II and III trials are likely to be performed on V617F-positive idiopathic myelofibrosis, known to have a poor prognosis.

Directions for future clinical trials in MPD

While the ECLAP and PT-1 studies have given clear guidance on the management of ET and PV, several questions remain. One of the most important unanswered questions in both PV and ET is the optimal target range for blood counts. Thus there are no randomized data to guide the choice of the threshold for target hematocrit in PV, target platelet count in ET, whether the platelet count should be reduced in PV and whether the hematocrit should be targeted in V617F-positive ET. Another undecided issue is whether cytoreduction should be undertaken in low- and intermediate-risk patients with ET, although the ongoing PT-1 trials should clarify this issue and are open to recruitment throughout Europe. The risk of leukemic and myelofibrotic transformation in the long-term with cytotoxic agents such as hydroxyurea, while partly addressed by PT-1 and ECLAP, will need further follow-up to be determined. Finally, there needs to be an on-going interplay between the long-term follow-up of patients with MPD and the results of diagnostic investigations such as JAK2 mutation testing and bone marrow biopsy in order to establish diagnostic frameworks that are clinically meaningful and reflect our greater understanding of the pathogenesis of these disorders.

References


Identification of mutant genes causally implicated in the pathogenesis of leukemia has been challenging, in part because leukemia is a rare disease, and the vast majority of cases are sporadic. Thus, strategies that are informative in heritable diseases, such as generalized linkage analysis, are not applicable for the elucidation of disease alleles in leukemia. There are several lines of evidence that acute myeloid leukemia (AML) is a clonal disorder that is the consequence of acquired somatic mutations occurring in a hematopoietic progenitor. These include recurring cytogenetic abnormalities in leukemia cells, which have been valuable clues to the genomic localization of leukemia-associated alleles, as detailed below. However, in recent years there has also been a marked expansion of the number of disease alleles in AML that are not evident by conventional cytogenetic analysis. In all, well over 100 disease alleles have been identified, far exceeding the number of subtypes of AML that we recognize by other diagnostic criteria. Thus, one would expect that many of these mutations would target similar transcriptional or signal transduction targets.1 Mutations and gene rearrangements will be presented in a functional context that emphasizes the targeting of shared pathways of transformation.

**Mutations that target signal transduction intermediates**

There were several clues that mutations that activate signal transduction pathways and confer proliferative and/or survival advantages to hematopoietic progenitors would be important in AML. First, rare cases of de novo AML are associated with t(9;22), resulting in expression of the BCR-ABL fusion, a constitutively activated tyrosine kinase. Similarly, cases of AML arising from chronic myeloid leukemia (in myeloid blast crisis) also harbor activating mutations in BCR-ABL. In addition, it had been demonstrated that activating mutations in RAS family members, primarily N-RAS and K-RAS, are present in AML, as well as in solid tumors. More recently, FLT3 activating mutations have been identified in AML at a frequency of ~30%, making FLT3 the most commonly mutated gene in AML. Collectively, mutations that constitutively activate signal transduction pathways have been identified in ~50% of AML, and it is plausible that appropriate screens will identify similar mutations in the remainder. These mutations appear to form a complementation group, in that they do not occur together in the same AML patient. For example, FLT3 and RAS mutations, though frequent in leukemia as individual mutations, do not occur together in the same patient except on very rare occasions. This observation suggests that these mutations subserve similar functions in AML in providing proliferation and survival signals to leukemic blasts. Because these mutations may occur with disease progression, it is possible that on those very rare occasions when both mutations are observed in a patient with AML, they have arisen independently in separate clones.

**Mutations that constitutively activate receptor tyrosine kinases**

Activating mutations have been identified in FLT3 (~30%) and c-KIT (< 5%) in AML.2,3 In the context of FLT3, about 20-25% of AML cases harbor tandem internal tandem duplications (ITD) of the juxtamembrane (JM) domain. These in-frame...
ITD are highly variable between AML patients and range in size from several to >50 amino acids. Recent structural data indicate that the FLT3 JM domain is an autoinhibitory domain whose function is disrupted by the ITD mutations, resulting in constitutive kinase activation. In an additional 5-10% of cases, there are so-called activating loop mutations that occur near position D835 in the tyrosine kinase, and also result in constitutive kinase activation. Analogous activating loop mutations at position D816 have also been reported in C-KIT in a fraction (< 5%) of cases of AML.

Expression of FLT3-ITD by retroviral transduction into bone marrow cells in a murine bone marrow transplant model results in a myeloproliferative disease (MPD) phenotype rather than AML. The MPD is similar to that observed with myeloid leukemia-associated tyrosine kinase fusion genes, such as BCR-ABL or TEL-PDGFβR in retroviral transduction models, and is characterized by neutrophilia, myeloid hyperplasia in the bone marrow, extramedullary hematopoiesis in liver and spleen comprising maturing myeloid elements, and lack of transplantability into secondary recipient mice. As discussed in more detail below, FLT3 mutations in humans may occur in conjunction with known gene rearrangements such as RUNX1/ETO, PML/RARA, CBFβ/MYH11, or MLL, and KIT mutations have been reported to occur at a higher frequency in association with the CBFβ/MYH11 fusion. These data suggest that cooperation between these alleles may be important for the AML phenotype.

Several large studies indicate that FLT3 mutations confer a poor prognosis, at least in patients under the age of 65, and occur at high frequency in both adult and pediatric populations. For these reasons, mutant FLT3 is an attractive candidate for molecularly targeted therapy with small molecule tyrosine kinase inhibitors (reviewed in ref. 2). Four small molecule inhibitors of FLT3 are currently in clinical trials: PKC412 (Novartis Pharma AG), MLN518 (Millennium), SU11248 (SuGen), and CEP-701 (Cephalon). Although these are still in the early phases of clinical development, there are several generalizations that can be made concerning the various inhibitors. Each appears to be well tolerated, with modest toxicity, and has activity in relapsed AML. Each of the inhibitors has been demonstrated to inhibit the FLT3-ITD target. The peripheral blood blast response has been striking in a number of cases, although complete remissions have been infrequent and in most cases the bone marrow blast response has been less impressive. Responses are short lived, and suggest that resistance to FLT3 inhibition develops rapidly.

In some cases, resistance to small molecule FLT3
inhibitors has been attributable to acquired point mutations in the context of FLT3, analogous to imatinib-resistant mutations in BCR-ABL. These include a FLT3-ITD N676K mutation that is resistant to PKC412, and had been predicted to be a resistance allele based on in vitro mutational analysis. With this in mind, it is reasonable to begin efforts to identify small molecule FLT3 inhibitors that will overcome resistance. The potential availability of several active FLT3 inhibitors with differing chemical structures may be of value in preventing or overcoming resistance. However, as for the T315I BCR-ABL, which is resistant not only to imatinib but also to the second generation BCR-ABL inhibitors dasatinib and AMN107, prospective mutational screens have identified a FLT3-ITD G697R mutation that appears to be resistant to current classes of FLT3 inhibitors in clinical trials and should be a focus of activity.

Phase I and phase II trials of FLT3 inhibitors as single agents in relapsed AML have shown activity as denoted above, but only rarely induce complete remission, demonstrate more impressive blast responses in peripheral blood than in bone marrow, and the responses they produce are short-lived. There are several potential explanations for these observations. It is possible that the bone marrow microenvironment provides survival signals to AML blasts even when FLT3-ITD signaling is inhibited, whereas such support is not present in the peripheral blood. In addition, there are clear data indicating that in some patients FLT3-ITD mutations occur as late events in disease progression, so killing all FLT3-ITD-expressing blasts might still leave a residual population of blasts that lack this mutation.

In any case, it appears that FLT3 inhibitors are not likely to be of significant benefit as single agents in relapsed disease. Current clinical approaches are focused on bringing FLT3 inhibitors up-front in combination with intensive induction chemotherapy, and for treatment in combination with conventional chemotherapy in relapsed disease. Trials are ongoing for the latter indication with the CEP-701 compound, and are planned for PKC412 and MLN518. The recent approval of SU11246 (Sutent) for other cancer indications may allow for its application in this context as well, although cardiac and other toxicities in treated AML patients led to the exclusion of such patients from the clinical trials that resulted in registration of the drug.

KIT mutations are also attractive targets for therapy, in that the native KIT kinase can be inhibited by imatinib (Gleevec). In addition, deletion mutations in the JM domain of KIT occur at a high frequency in gastrointestinal stromal cell tumors, and confer sensitivity to imatinib in that clinical context. Unfortunately, KIT D816X mutations confer resistance to imatinib, and thus it is unlikely that imatinib will be of value in treating this subset of patients. However, selected FLT3 inhibitors, such as PKC412, have activity against the KIT D816X mutants, and thus may be of therapeutic value.

**Oncogenic RAS mutations in AML**

Activating RAS mutations have been reported in both AML and MDS, typically at codons 12, 13 or 61 of N-RAS or K-RAS. The incidence varies widely between studies, ranging from 25-44% (reviewed in ref. 15). Mutant RAS alleles were assessed by direct sequencing in one study that reported an 18% incidence of N-RAS or K-RAS mutations, which conferred a poor prognosis.16 Because of the high frequency of oncogenic RAS mutations in AML and many other human tumors, considerable effort has been focused on developing RAS inhibitors. RAS activity in mediating signals from upstream effectors is dependent on RAS localization to the plasma membrane. Farnesylation of RAS is required for membrane localization, and thus one attractive strategy for inhibiting RAS activity has been to develop farnesyl transferase inhibitors (FTI).17,18 There is convincing evidence that FTI have activity in AML, though responses have not correlated with the presence of activating mutations in RAS.17,18 The basis for the response is not understood, but may be due to inhibition of other prenylated proteins in AML, off-target effects of FTI, or inhibition of native RAS that is activated by upstream effectors such as FLT3 or KIT.

Murine models of oncogenic K-Ras expression from its endogenous promoter under conditional control demonstrate a myeloproliferative phenotype that is similar to that observed with constitutively activated tyrosine kinases such as FLT3.19,20 Animals develop neutrophilia with myeloid hyperplasia of the bone marrow and extramedullary hematopoiesis comprising maturing myeloid elements. These cells do not serially re-plate, and are not efficiently transplanted into secondary recipients. These data indicate that, like activated FLT3, oncogenic RAS alleles are not sufficient to cause AML, and suggest that constitutively activated RAS is a functional equivalent of constitutively activated kinases. These findings correlate with the clinical observation that these two mutations do not occur together in the same patient with AML, and that they comprise a functional complementation group that confers proliferative and survival advantage to myeloid lineage progenitors, but does not affect differentiation, and does not appear to confer properties of self-renewal as assessed by serial re-plating and transplantation assays.
Activating mutations in PTPN11 (SHP2)

Acquired mutations in the protein tyrosine phosphatase PTPN11 (SHP2) have recently been identified in several hematologic malignancies, including both sporadic cases of juvenile myelomonocytic leukemia (JMML) and JMML arising in individuals with Noonan’s syndrome, as well as rare cases of AML and MDS. The molecular mechanisms of leukaemogenesis associated with activating mutations in SHP2 are not well understood, but provide another example of signal transduction mutations associated with myeloid leukemias. Murine models of activated PTPN11 alleles knocked-in to the endogenous promoter also demonstrate that these mutant alleles, when expressed alone, result in a myeloproliferative phenotype.

References

The treatment of acute myeloid leukemia (AML) faces a number of challenges if improvement is to be made. In younger patients improvement has taken place over the last 20 years with an increase in the proportion of patients who become long term survivors. It is doubtful, however, whether further dose intensification is either feasible or likely to ameliorate the main problem, which is relapse of disease. In older patients who are fit for an intensive treatment approach the ability to achieve remission is limited to 50-60% of cases but 80% will relapse over the subsequent 3 years. Most collaborative groups have not observed significant improvement in the survival of older patients. A significant proportion of older patients are not considered suitable for, or decline, conventional chemotherapy. New treatment approaches are therefore required.

A limitation of dose escalation is the associated collateral damage. Antibody-directed chemotherapy offers the potential to deliver more treatment to the leukemic clone in the expectation that there will be proportionately little extra toxicity. The requirements of such an approach are reliable targeting of a relatively specific antibody, a suitably effective chemotherapeutic agent which is appropriately linked to the antibody, and the ability to administer repeated doses without inducing antibodies. The CD33 antigen has emerged as the favored target in AML. It is expressed on the majority of AML blasts, and has been thought to be limited to hematopoietic precursors. Being substantially humanized, the antibody can be repeatedly administered. The rapid internalization of the CD33 antibody-antigen complex makes it a convenient delivery system.

HuM195 (lintu-zumab) is a humanized unconjugated monoclonal antibody directed against CD33.1 As a single agent it has been effective in achieving molecular negativity in patients with acute promyelocytic leukemia who were persistently polymerase chain reaction (PCR)-positive following extensive conventional treatment approaches.2 As a single agent in relapsed disease it had disappointing results.3 A randomized trial was conducted in combination with chemotherapy for the treatment of refractory or relapsed disease, versus chemotherapy alone. No significant differences were seen between the arms in either response or survival.4

Treatment of relapsed disease

Evaluation of new treatments in the setting of relapsed disease is complicated by the major influence which factors unrelated to treatment can have on the achievement of complete remission and subsequent survival. These factors include age, duration of first remission, cytogenetics at diagnosis, and whether the relapse occurred post-transplant. It is, therefore, difficult to be sure about the superiority of one or other treatment which is not assessed in the context of a randomized trial.

Gemtuzumab ozogamicin (GO) was evaluated in three studies involving 142 patients conducted for approval purposes.5,6 The eligibility for study entry differed slightly between trials. All patients were required to be CD33 positive and in untreated first relapse. In two studies the required minimum duration of first complete remission was 6 months whereas in the third study it was 3 months. Two studies had a lower age limit of 18 years, one of which permitted relapse after stem cell transplant. The third study which required a first complete remission duration of 3 months, did not permit prior stem cell transplantation and was restricted to patients over 60 years old. The dose and schedule (9 mg/m² day 1 and 15) was derived from a phase 1 study which showed that the receptor was maximally saturated at that dose level.7 The results showed that marrow blasts could be cleared in 30% of patients of all ages. Half of these failed to regenerate platelets, a response which became known as CRp. The significance of this response in terms of survival has only recently been clarified as being inferior to complete remission, but better than PR.
These data permitted approval of GO for the treatment of relapse in older patients in the USA and Japan.

**Toxicity**

The side effect profile was reported to be acceptable. Myelosuppression was inevitable, but infections occurred in under one third of patients. Grade 3 or 4 liver toxicity occurred in less than 25% of patients and was usually transient. Several patients were able to receive treatment as an outpatient.

Subsequent to these studies, with wider use of the drug the more significant complication of veno-occlusive disease (VOD) has emerged. This complication has been particularly associated with the use of GO together with stem cell transplantation or intensive chemotherapy.

In the phase II database comprising information on 277 patients, including those in the registration studies, 39% of patients above or below 60 years developed grade 3 or 4 liver toxicity, any of the three liver parameters (as reflected by alterations of aspartate transferase, alanine transferase or bilirubin) with an elevated bilirubin being the most frequent abnormality.

The overall response rate was 26%; half the patients achieved a complete response and half a CRp. CRp emerged from these studies as a new category of response which is identical to CR except that the platelet count failed to reach 100x10^9/L, all patients are, however, transfusion independent. Now designated as CRi, the implications for survival have recently been described as inferior to complete response, but superior to response failure. The responses seen do not appear to be different in younger patients or those over 60 years, but tend to be better in patients with longer complete remissions, as would be expected. The incidence of VOD varies widely between studies (12-64%) in series that have relatively small numbers. The phase II database of relapsed disease documented 16 cases in 277 patients giving an incidence of 5.4%. The incidence was significantly higher in patients who received a stem cell transplant (17% vs 1%) and tended to be more frequent when GO was given after SCT – mostly for post transplant relapse (20%) rather than to treat relapse and consolidate with SCT (15%). The frequency in autologous-SCT is lower because of liver toxicity, and delayed hematological toxicity, (iii) it was not possible to give GO with two sequential courses because of liver toxicity, and delayed hematological toxicity; (iv) a dose of 3 mg/m² combined with DA, FLAG-Ida MACE or high dose Ara-C was feasible, (v) the administration of GO in course 1 and course 3 was feasible, (vi) the complete remission rate with 3 mg after course 1 was 86%.

Based on this experience the MRC AML15 trial was initiated (www.aml15.bham.ac.uk/trial/). Patients, most of whom are under 60 years old are randomized to receive two courses of either DA, FLAG-Ida, or ADE (Ara-C, daunorubicin, etoposide) as induction. In each combination patients are randomized to receive, or not, GO 3 mg/m² on day 1 of the first course. In consolidation patients are randomized to receive either one of two doses of high dose Ara-C (3 g/m² or 1.5 g/m²) for two courses or MACE plus MidAc. In the first allocated course GO is given, or not, at a dose of 3 mg/m². This design will therefore evaluate the role of GO together with stem cell transplantation or intensive chemotherapy.

**Evaluation in clinical trials**

The concept of selective targeting of an effective chemotherapeutic agent has considerable appeal in AML, particularly if its potential efficacy is achieved with a proportionately modest toxicity profile. It has become clear that combined treatment with chemotherapy or stem cell transplantation could expose patients to unexpected toxicities and therefore a feasible dose needs to be chosen. Monotherapy as first line treatment has not been encouraging although it may have a role in maintenance phase. This has lead to assessments of the contribution that GO could make in a number of contexts in several major phase III trials (Table 1).
of GO in combination in induction only, in consolidation only, in both or in neither. It is expected that 1000 patients will be randomized in induction by mid 2006 and 750 in consolidation by the end of 2006. Patients with acute promyelocytic leukemia are eligible only for GO in consolidation. The median age of all patients randomized is 50 years and the remission rate is 84%. In adults under 50 years old the complete remission rate is 90% and in 125 patients > 60 years old it is 81%. The overall survival rate in the randomized patients is 55% at 2.5 years.

The SWOG S0106 study ([www.cancer.gov/clinicaltrials/SWOG-S0106](http://www.cancer.gov/clinicaltrials/SWOG-S0106)) has a similar approach to that of the MRC AML15 trial, and followed phase II studies which combined GO (6 mg/m²) on day 4 of a DA (3+7) regimen which has an acceptable toxicity profile and an encouraging remission rate of 82%. In a phase 1 study it was not possible to combine GO at a dose of 9 mg/m². The S0106 trial randomizes patients < 60 years old to receive either DA + GO 6 mg/m² on day 4 or DA alone, however each dose of daunorubicin in the GO arm is 45 mg/m² whereas in the no GO arm it is per 90 mg/m². All patients then receive three courses of high dose Ara-C (3 g/m² days 1, 3 and 5) and are then randomized to receive or not three courses of GO (5 mg/m²), as maintenance at intervals of 1 month. The recruitment target is 684 patients in induction and 342 patients in maintenance.

### Antibody directed chemotherapy before autologous stem cell transplantation

The Eastern Co-operative Oncology Group trial ([www.cancer.gov/clinicaltrials/ECOG-1900](http://www.cancer.gov/clinicaltrials/ECOG-1900)) (E1900) is assessing dose intensification in induction by comparing, in younger patients, daunorubicin 45 mg/m² with 90 mg/m². After two courses of high dose Ara-C patients are randomized, to receive, or not, a single dose of GO (6 mg/m²) on day 1 of conditioning for autologous SCT. The target accrual for induction randomization is 747, patients and for the GO randomization 338 patients.

### GO as maintenance treatment

The SWOG S0106 trial, as described, is one study that will evaluate GO as maintenance by giving 5 mg/m² doses at three-monthly intervals. The HOVON-SAKK AML-43 trial is aimed at patients over 60 years old including patients with refractory anemia with excess blasts (RAEB) and RAEB in transformation. The induction question is similar to that being addressed in younger patients in the ECOG E1900 trial, i.e. a comparison of daunorubicin 45 mg/m² with daunorubicin 90 mg/m² in a 3+7 schedule. This is followed by consolidation with high dose Ara-C (1.0 g/m²) after which patients are randomized to receive, or not, three doses of GO (6 mg/m²) on day 1 of conditioning for autologous SCT. The target accrual for induction randomization is 747, patients and for the GO randomization is 538 patients.

### Table 1. Antibody directed chemotherapy in AML: current trials.

<table>
<thead>
<tr>
<th>Treatment Context</th>
<th>Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>In combination with induction chemotherapy in younger patients</td>
<td>MRC AML 15</td>
</tr>
<tr>
<td>In consolidation in younger patients in combination with chemotherapy</td>
<td>SWOG S0106</td>
</tr>
<tr>
<td>As augmentation to autograft conditioning</td>
<td>MRC AML 15</td>
</tr>
<tr>
<td>As maintenance after chemotherapy</td>
<td>ECOG E1900</td>
</tr>
<tr>
<td>In combination with induction chemotherapy in older patients</td>
<td>HOVON 43 SWOG</td>
</tr>
<tr>
<td>in combination with intensive chemotherapy</td>
<td>S0106 EORTC</td>
</tr>
<tr>
<td>in combination with non-intensive treatment</td>
<td>GIMEMA AML-19</td>
</tr>
<tr>
<td>As total therapy in older patients who are not fit for intensive therapy</td>
<td>NCRI AML 16</td>
</tr>
<tr>
<td>As first line treatment of APL</td>
<td>EORTC-GIMEMA AML-17</td>
</tr>
<tr>
<td>as monotherapy</td>
<td>NCI-AML14</td>
</tr>
<tr>
<td>as maintenance as retinoid</td>
<td>NCI-AML16</td>
</tr>
<tr>
<td>Treatment of relapsed APL</td>
<td>GIMEMA AML-19</td>
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</tbody>
</table>

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The third major study of GO in maintenance is the EORTC-GIMEMA AML-19 trial which will be described in detail below. This will accrue patients who are not considered fit for intensive chemotherapy (i.e. aged 61-75 with a performance score of >2, or patients > 75 years old). As maintenance patients will
receive eight doses of low dose GO (2 mg/m²) at monthly intervals. This maintenance is given to patients who have only received prior treatment with GO monotherapy.

**Antibody directed chemotherapy in combination with induction treatment in older patients**

Based on an exploratory phase II trial (AML15A) the GIMEMA Study Group (www.cancer.gov/clinicaltrials/EORTC-06012) developed a randomized trial in patients aged 61-75 years old with good performance score (PS 0-2) which compares the sequential administration of GO 6 mg/m² on days 1 and 15 followed by MICE chemotherapy (mitoxantrone, ara-C, etoposide) with MICE chemotherapy alone. As consolidation patients will receive two further courses of mini-ICE with or without GO (3 mg/m²). This approach will enable patients to enter remission with GO alone followed by MICE, which will achieve complete remission in some of the patients who do not respond fully to GO. In the pilot study, AML15A, of 57 evaluable patients 25% achieved complete remission with GO alone and an additional 12% achieved CR with incomplete platelet recovery (CR1) gving an overall response to the single agent of 35%(16). When the patients completed the induction MICE treatment complete remission was seen in 35% with an additional 19% achieving CR1, giving an overall response rate of 54%. This response is broadly similar to what would be expected with conventional chemotherapy. Five patients developed VOD of liver either after GO alone (n=5) or subsequent MICE (n=2).

As a result of the recent experience in the MRC AML15 trial in patients over 60 years, in whom a remission rate of 81% was observed among 125 patients, the UK NCRI Group has opened AML16 in which patients over 60 years who are considered fit for intensive chemotherapy will be randomized to receive low-dose Ara-C (20 mg bid days 1-10) versus hydroxyurea in patients not considered fit for intensive treatment (NCRI AML14). The low-dose Ara-C treatment was significantly superior principally because more patients (18%) achieved CR. Based on this a subsequent phase II study of 100 patients has been undertaken in which patients are randomized to receive low-dose Ara-C or low-dose Ara-C with GO (5 mg) on day 1 of each treatment course. In the first 70 evaluable patients the overall remission rate is 17%. Depending on the final evaluation of this phase II study a phase III powered comparison may be incorporated within the NCRI AML16 trial.

**Special situations acute promyelocytic leukaemia**

Acute promyelocytic leukemia represents an ideal candidate for treatment with GO notwithstanding the excellent prospects that current treatment offers. The features which suggest this are the high level of CD33 expression, and the sensitivity to anthracycline-type drugs. Largely because of the success of current and emerging treatments this potential has not been fully exploited. Studies with the humanized antibody HuM195 were effective in obtaining PCR negativity in half of patients who remained persistently positive after retinoid treatment. Subsequently molecular remissions were reported with GO monotherapy in a high proportion of patients with more advanced disease in whom retinoids, anthracyclines and arsenic trioxide had failed. The median duration of these remissions was 15 months (range 7-31 months).

The MD Anderson Group used a combination of ATRA and GO as first line treatment, combined when necessary (initial high white count or persistent PCR positivity). After remission patients received ATRA for 2 weeks at 2 weekly intervals and GO (9 mg/m²) four or five weekly. The rate of complete remission was high (84%) and all patients had achieved PCR negativity by 4 months.

The MRC AML15 trial includes patients with acute promyelocytic leukemia, who are entered in the GO randomisation as described earlier.
Special situations: treatment in children
The experience in children with relapsed or refractory disease mimics that in adults, with very similar response rates and toxicities. Children are now being included in combination trials. The possibility of activity in acute lymphoblastic leukemia has been raised by in vitro studies, a recognition that approximately 20% of cases express CD33, and some case reports of efficacy including complete remissions.

Conclusion
Antibody-directed chemotherapy has the potential to improve treatment in several situations in AML. The licensed indication is of little interest, since with the possible exception for acute promyelocytic leukemia, most investigators see its potential as an augmentation of existing chemotherapy. This use does, however require care in defining the appropriate dose in order to avoid serious toxicity. Early developmental work suggested that there was activity at lower doses which have, in general, turned out to be safe in combination. It is remarkable that for an agent that has created so much interest, there are no data of significant benefit from randomized trials. However, as summarised here, several large studies are underway which may provide such evidence.

References
Emerging therapies for older adults with acute myeloid leukemia

Acute myeloid leukemia (AML) is primarily a disease of the elderly, with the median age at presentation being approximately 68 years. Advanced age is the single most important prognostic factor in AML. This is undoubtedly related to increased co-morbidity in the aging population, but also reflects the differing biology of the disease, which often presents in the elderly with a constellation of poor risk features including associated myelodysplasia, adverse cytogenetic abnormalities and expression of the multidrug resistance phenotype. Many older patients are not offered intensive chemotherapy because of personal choice or frailty. However, even for those who receive conventional treatment the prognosis remains very poor, with a 5-year survival well below 10%. Some of the limitations of conventional therapy have been intrinsic drug resistance and excess toxicity. The discouraging outcome of the disease in older patients has spurred major efforts to develop novel agents and innovative treatment strategies. Most recently, advances in the understanding of the biology of AML have resulted in the identification of new potential targets for more effective and less toxic antileukemia therapy (Table 1). This report provides an overview of some of the emerging therapies for older adults with AML.

Monoclonal antibodies

Monoclonal antibody therapy for patients with AML is currently based on targeting cell surface antigens selectively expressed on myeloid cells. Gemtuzumab ozogamicin (GO) is the prototypical and most investigated agent of this class. GO is an immunoconjugate that targets leukemic cells expressing the CD33 antigen by means of a humanized monoclonal antibody attached to a DNA-damaging toxin, a calicheamicin derivative. Combined phase II studies of GO, administered at the dose of 9 mg/m² on days 1 and 15, demonstrated activity in relapsed AML with roughly 30% of the patients achieving complete remission (half of responses met all standard criteria except for incomplete platelet recovery). The drug has shown a largely acceptable safety profile, although it can be associated with veno-occlusive disease (VOD) of the liver in a small fraction of patients (<5%). Stem cell transplantation has been identified as a risk factor for this complication, especially when performed in close association with administration of GO.

The current focus of clinical studies is to combine GO with conventional front-line therapy. A recent study suggests that GO may be associated with a high complete remission rate when combined with intensive induction chemotherapy in younger adults, and major co-operative groups are now conducting large studies to determine whether GO combined with standard induction and/or consolidation chemotherapy might lead to an improved treatment outcome. The sequential combination of GO and conventional induction chemotherapy has recently been shown to be a feasible and active treatment strategy for older individuals with untreated AML. On the other hand, attempts to offer GO as single-agent treatment for frail patients older than 61 years of age have not been encouraging, and trials with reduced doses and alternative schedules of administration in this patient population are underway.

Multidrug resistance inhibitors

Multidrug resistance (MDR) is a significant obstacle to successful treatment of AML. Multifactorial in etiology, classic MDR is associated with the overexpression of P-glycoprotein (P-gp), resulting in increased efflux of chemotherapeutic agents such as anthracyclines and epipodophyllotoxins from leukemia cells. Inhibiting P-gp as a method to reverse MDR has been studied extensively in AML, but the results have generally been
Table 1. Emerging therapies in AML.
<table>
<thead>
<tr>
<th>Class</th>
<th>Agents under evaluation (examples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal antibodies</td>
<td>Gemtuzumab ozogamicin</td>
</tr>
<tr>
<td>MDR modulators</td>
<td>Cyclosporine, PSC-833, Zosuquidar</td>
</tr>
<tr>
<td>FLT3 inhibitors</td>
<td>PKC-412, CEP-701, MLN-518, SU-11248</td>
</tr>
<tr>
<td>Farnesyl transferase inhibitors</td>
<td>Tipifarnib, Lonafarnib</td>
</tr>
<tr>
<td>Antiangiogenesis agents</td>
<td>SU-5416, PTK-787, Bevacizumab</td>
</tr>
<tr>
<td>Histone deacetylase inhibitors</td>
<td>Depsipeptide, Valproic acid, SAHA</td>
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<tr>
<td>Proteasome inhibitors</td>
<td>Bortezomib</td>
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<td>Apoptosis modulators</td>
<td>Oblimersen</td>
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<tr>
<td>Cell cycle inhibitors</td>
<td>UCN-01</td>
</tr>
<tr>
<td>Hypomethylating agents</td>
<td>Decitabine, 5-azaacytidine</td>
</tr>
<tr>
<td>Other</td>
<td>Clofarabine, Tranacabine, As2O3</td>
</tr>
</tbody>
</table>

disappointing. With few exceptions, most of the trials combining the so-called reversing agents (verapamil, cyclosporine and its analog PSC-833, quinine) with conventional chemotherapy have shown no benefit or have been terminated prematurely due to excess toxicity, possibly related to the reduced clearance of the cytotoxic drugs induced by the MDR modulators. Newer agents (zosuquidar, laniquidar), more selective for P-gp and with minimal impact on drug pharmacokinetics, are currently being investigated.

**FLT3 inhibitors**

FLT3 is a transmembrane receptor tyrosine kinase that is expressed at high levels in 70% to 100% of patients with AML. Approximately 25% of adults with AML have an internal tandem duplication in the juxtamembrane domain and another 7% have an activating loop mutation, both of which result in constitutive receptor activation. This defect appears to portend a worse prognosis. Activating mutations of the FLT3 tyrosine kinase confer leukemic cell lines growth factor independence and cause a fatal myeloproliferative syndrome in mice. Small molecules capable of inhibiting FLT3 activity can selectively kill such transformed cell lines and improve survival in the murine model. There are four FLT3 inhibitors that are currently being tested in clinical trials: PKC-412, CEP-701, MLN-518, and SU-11248. These compounds are well tolerated at doses that achieve inhibition of the target FLT3, and have shown activity in relapsed AML with activating mutations. However, responses have been quite modest and characterized by a transient reduction in peripheral blood blasts. In a recent review, a complete remission was reported to have occurred in 1/42 patients. A number of investigators have begun to explore optimizing the use of FLT3 inhibitors in combination therapy.

**Farnesyl transferase inhibitors**

Activating mutations in one of the RAS family proteins are frequently observed in hematological malignancies and might have a pathogenetic role in the development of myeloid leukemias. Farnesyl transferase inhibitors (FTI) were initially developed to interfere with RAS processing and localization to the cell membrane, thereby preventing transduction of mitogenic signals. However, it is likely that the inhibition of prenylated proteins other than RAS may be more relevant to the activity of FTI. In a phase II study in high-risk AML, tipifarnib (R115777) was investigated as single-agent therapy. AML was defined as high risk when it occurred in a patient older than 65 years, in a patient older than 18 years in the presence of adverse cytogenetics, or when it was secondary AML. In an interim report, the overall response rate (complete and partial response) to therapy was 34% in the general study population, and 30% in patients older than 75 years of age. The median duration of response in complete responders was 6.4 months. Complete responders had a median survival of 14.4 months, with 63% being alive at 12 months. Grade 3 tipifarnib-related non-hematological adverse events occurred in 45% of patients and consisted mainly of infectious and gastrointestinal complications. Based on these encouraging findings, several groups have activated prospective comparative trials evaluating tipifarnib as front-line therapy in older adults with AML who are not felt to be suitable for conventional chemotherapy. This agent is also being investigated in younger patients in combination with intensive chemotherapy. Two other FTI, Lonafarnib and BMS-214662, are currently undergoing clinical trial testing.

**Anti-angiogenic agents**

Angiogenesis is an appropriate therapeutic target in AML because increased levels of angiogenic growth factors and increased bone marrow vascular density are correlated with poor prognosis in myeloid leukemias. Furthermore, vascular endothelial growth factor (VEGF) plays a role in stimulating growth and proliferation of the malignant clone in myelodysplastic syndromes and AML. Receptor tyrosine kinase inhibitors of VEGF are under active evaluation. Preliminary data suggest that SU-5416, a small molecule inhibitor of phosphorylation of VEGF receptors 1 and 2, C-KIT, the stem cell factor (SCF) receptor and FLT3, have both biological and clinical activity in AML. Vatalanib (PTK-787) is an oral inhibitor of a number of kinases, including all the members of the VEGF receptor family, as well as the platelet-derived growth factor receptor (PDGF-R). This molecule has shown single-agent activity in patients with advanced cancers and is currently being investigated in patients with high-risk AML and myelodysplastic syndromes. On the other hand thalidomide, a potential anti-angiogenic agent, combined with chemotherapy did
not show a therapeutic benefit in patients with AML. Bevacizumab, an anti-VEGF antibody, has been demonstrated to be safe in AML and is currently undergoing phase II testing.

**Histone deacetylase and proteasome inhibitors**

Histone deacetylases are a class of enzymes that remove acetyl groups from histones, ultimately creating a tightly bound DNA structure that is not accessible to the transcriptional machinery. The end result is that various genes, which may be critical to cell proliferation and differentiation, are not expressed. Several histone deacetylase inhibitors (phenylbutyrate, trichostatin A, suberoylanilide hydroxamic acid [SAHA], valproic acid, and depsipeptide) are under clinical investigation. A phase I study of depsipeptide given at a dose of 13 mg/m² infused on days 1, 8, and 15 every 28 days in nine patients with AML showed transient declines in blast counts, and tumor lysis syndrome in one patient; none of the patients achieved a clinical response.

The 20S proteasome is an ATP-dependent multicatalytic protease. Increased proteasome-mediated degradation of proteins (e.g. p53, p21, p27) that lead to apoptosis and/or cell cycle arrest has been described in leukemic cells. Bortezomib, a 20S proteasome inhibitor approved for use in multiple myeloma, has been used as single-agent therapy in 15 patients with acute leukemia and myelodysplastic syndrome. Sustained clinical activity was not demonstrated, but transient decreases in blood or bone marrow blasts were reported. Preclinical synergistic activity with several antileukemic agents has led to its evaluation of bortezomib in combination therapy.

**Apoptosis modulators**

Anti-apoptotic proteins, such as bcl-2, have been shown to play an important role in the regulation of the mitochondrial apoptosis pathway, and a high level of expression of these molecules in AML is associated with drug resistance and a poor prognosis. Thus, pharmacologic downregulation of bcl-2 might restore chemosensitivity to leukemic cells. A direct approach to inhibit bcl-2 is through the use of bcl-2 antisense oligodeoxynucleotides. Oblimersen (G3139) is an 18-mer phosphorothioate oligodeoxynucleotide antisense that was designed to target bcl-2 mRNA. A phase I trial of oblimersen in combination with fludarabine, Ara-C, and granulocyte colony-stimulating factor (FLAG) showed a response in nine of 20 patients with relapsed or refractory acute leukemia. A complete remission was observed in six cases, while in three patients there was no evidence of disease but failure to recover normal neutrophil and/or platelet counts, or remissions lasted less than 30 days. In a subsequent phase I trial the agent was administered in combination with induction and consolidation chemotherapy to untreated older adults. Of the 29 treated patients, 14 achieved complete remission. With a median follow-up of 12.6 months, seven patients had relapsed. Side effects of this combination were similar to those expected with chemotherapy alone. A CALGB randomized phase III study evaluating oblimersen as an adjunct to conventional induction and consolidation chemotherapy is currently ongoing.

**Cell cycle inhibitors**

DNA damage-inducible cell cycle checkpoints are complex signal transduction networks that integrate the cellular responses to genotoxic insults by arresting cell cycle progression during the repair of DNA damage or the induction of apoptosis. The integrity of these checkpoint pathways is critical for the maintenance of genomic stability and for cellular recovery from genotoxic damage. UCN-01 (7-hydroxystaurosporine) is a protein kinase C inhibitor that abrogates both the S- and G2-phase cell cycle checkpoints, preventing cell cycle arrest. Down-regulation or inhibition of protein kinase C by UCN-01 has been shown to circumvent Ara-C resistance. The impact of UCN-01 on the efficacy of Ara-C at concentrations used clinically has been determined in a phase I trial. The use of UCN-01 in combination with Ara-C decreased Chk1 phosphorylation, inhibited the Akt survival pathway and activated JNK during the course of therapy. Clonogenic survival assays of primary AML samples showed that leukemia colony formation was decreased 5-fold relative to Ara-C alone when samples were simultaneously incubated with UCN-01. In contrast, the clonogenic viability of normal myeloid progenitor cells was minimally affected by either Ara-C alone or Ara-C in combination with UCN-01. This selective action of the combination suggests a biological basis for a favorable therapeutic index, offering a rationale for evaluation of the combination in AML patients.

**Hypomethylating agents**

Epigenetic changes in DNA involving methylation of cytosine residues have recently become recognized as major contributors to gene silencing in human cancers, including leukemias. Hypomethylation of promoters of genes, such as p15, have been associated with disease progression and with a worse outcome in patients with myeloid malignancies. Unlike gene inactivation by mutation or loss of heterozygosity, epigenetic changes are potentially reversible and therefore represent a possible target for therapy. DNA methylation inhibitors have shown activity in the treatment of hematological malignan-

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cies. The cytosine analog 5-azacytidine was recently approved by the FDA for the treatment of myelodysplastic syndromes. 5-aza-2'-deoxycytidine (decitabine), a compound chemically related to 5-azacytidine but a more potent inducer of demethylation, in vitro, is also being evaluated in clinical trials both in myelodysplastic syndromes and AML. Early studies demonstrated the efficacy of decitabine in patients with recurrent or refractory leukemia. The dose-limiting toxicity of decitabine in these trials was prolonged myelosuppression. However, dose-response experiments using multiple cell lines as well as specimens from patients with AML, suggest that low doses of the drug (e.g. 5-20 mg/m² daily) will produce hypomethylation without the myelosuppression that occurs at higher doses. The recommended dose for phase II trials is 15 mg/m² for 10 days, because in a phase I study this regimen was associated with the best response rate. Studies in North America and Europe are ongoing and have demonstrated encouraging response rates. Further developments of low-dose decitabine in AML may take into account the in vitro synergy between hypomethylating agents and histone deacetylase inhibitors, as well as combinations with differentiating agents such as retinoic acid.

Other agents

Clofarabine is a new adenosine nucleoside analog that was designed with the specific intention of overcoming the limitations of fludarabine and cladribine, while keeping their favorable therapeutic attributes. This agent has shown significant activity in patients with relapsed or refractory AML in phase I and II studies, in which a complete remission rate of 42% was recorded. In a phase II study in older patients with untreated AML, who were not felt to be suitable for intensive chemotherapy, single-agent clofarabine (30 mg/m² on days 1-5) yielded a complete remission rate of 60% with acceptable toxicity. Ongoing studies are investigating the combination of clofarabine with conventional chemotherapy for patients with AML as front-line and salvage therapy. Troxatubine is the first L-enantiomer nucleoside analog shown to have anticancer activity and, unlike Ara-C, is not a substrate for the deactivating deoxycytidine deaminase. In a phase II study, Giles et al. used an intravenous infusion of troxatubine at a dose of 8 mg/m² on days 1-5 in 42 patients with refractory or recurrent hematologic malignancies, and reported two complete remissions and one partial remission among 16 evaluable patients with AML. Ongoing studies are further exploring the activity of this drug alone or in combination in AML.

Although best known for its use in the treatment of acute promyelocytic leukemia, arsenic trioxide also induces hyperacetylation of histones, indicating the difficulty in categorizing some agents as modulators of apoptosis rather than chromatin remodeling agents. These in vitro results have prompted trials of arsenic trioxide in myeloid leukemias other than acute promyelocytic leukemia.

Conclusions

Several classes of novel agents are currently available and have shown promising activity in early clinical trials for AML. The generally favorable toxicity profiles and the good oral bioavailability of at least some of these drugs (e.g., farnesyl transferase inhibitors and FLT3 antagonists) make them particularly attractive treatment options in the older population or in patients not considered fit enough for conventional chemotherapy. Although these drugs have documented antileukemic activity, meaningful clinical responses must still be defined. It is anticipated that future studies will serve to define the optimal role of these molecules in the overall treatment strategy for AML, and provide insights on how best to combine them with other targeted and conventional therapies.

References

9. Cortes J, Kantarjian H, Albitar M, et al. A randomized trial of liposomal daunorubicin and cytarabine versus liposomal daunorubicin and topotecan with or without thalidomide as initial therapy for patients with poor prognosis acute myel-
Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood. Major advances have been made in the treatment of childhood ALL in the last decades, based on the identification of prognostic markers and the development of risk-adapted treatment strategies. However, therapy still fails in about 25% of patients and surviving patients often suffer from significant toxicities. Therefore, an improved assessment of an individual’s risk of relapse is necessary in order to adapt the treatment and thus to enhance the chance of survival.

Several clinical and biological factors at diagnosis can be used to assess the relapse risk and to adapt the intensity of therapy accordingly. In recent years, the in vivo response to chemotherapy was identified to be a very strong prognostic parameter. This response can be assessed by cytology of bone marrow or peripheral blood smears, by immunophenotyping or molecular genetic analysis of appropriate bone marrow or blood samples. Such an in vivo response assessment will reflect factors characteristic for that specific leukemia subtype and factors related to the individual’s genetic predisposition (host factors).

Despite the strong prognostic power of response evaluation in ALL therapy, one disadvantage that cannot be overcome is the lack of such information at the onset of therapy. Robust genetic signatures of response and remission are, therefore, urgently sought for.

In the ALL-BFM 2000, trial a risk-adapted treatment stratification (standard-, intermediate-, or high-risk) was made based on cytogenetic markers, t(9;22), t(4;11) or their molecular equivalents, BCR-ABL and MLL-AF4, and the in vivo response to treatment. Response was assessed cytologically by the initial cytoreduction (blast reduction in peripheral blood after 7 days of treatment; blast clearance from the bone marrow after induction therapy on day 35 of treatment), or molecularly by measurement of minimal residual disease (MRD) on day 33 of treatment and after induction consolidation at week 12. Measurement of MRD was based on the detection of clone-specific immunoglobulin and T-cell receptor gene rearrangements by polymerase chain reaction (PCR) amplification. The level of sensitivity reached in most cases was $10^{-4}$-$10^{-3}$ (detection of one leukemic cell in $10^4$-$10^5$ cells).

We hypothesized that response to induction therapy and induction consolidation is determined by factors present already at onset of treatment, and that these are reflected in the pattern of gene expression in ALL cells. Furthermore, we hypothesized, that treatment-resistance can be predicted prior to treatment, which would allow early treatment adjustment. To test these hypotheses, we compared leukemic gene expression profiles in a set of childhood ALL patients homogeneous with regard to relevant prognostic factors (such as age, white cell count, genetics, immunophenotype) and only differing by their molecular treatment response (high MRD load vs. without measurable MRD during treatment).

Even though patients showed similar clinical characteristics at diagnosis, and despite the fact that the leukemic cells appeared biologically and phenotypically homogeneous, and that patients received the same initial induction and consolidation therapy, some of the patients had no detectable MRD on day 33 of treatment and at week 12 (MRD-standard-risk, MRD-SR) while others still had a high MRD load at week 12 ($\geq 10^{-3}$, MRD-high-risk, MRD-HR). In a third group of patients, MRD was measurable, but at a low level ($<10^{-3}$, MRD-intermediate-risk, MRD-IR). The in vivo response to initial therapy, assessed systematically by the determination of MRD at 5 and 12 weeks of treatment, has evolved as the strongest prognostic factor in pediatric ALL if patients are treated with the BFM regimen: the probability of relapse-free survival in MRD-SR patients is more than 95%,
whereas for MRD-HR patients it is only 19%. We identified 54 genes that clearly distinguished resistant from sensitive ALL samples. Genes with low expression in resistant samples are predominantly associated with cell cycle progression and apoptosis, suggesting that impaired cell proliferation and apoptosis are involved in treatment resistance. Prediction analysis using randomly selected samples as a training set and the remaining samples as a test set revealed an accuracy of 84%. We conclude that resistance to chemotherapy seems at least in part to be an intrinsic feature of ALL cells.

Interestingly, genes that appeared to be associated with in vitro drug resistance in another investigation are not identical to the ones found in our analysis. This is most likely due to the fact that genes which are strongly up- or downregulated (and thus detected in the gene expression profiling, GEP) during the in vitro resistance test are not necessarily the same as those which are relevant for in vivo resistance. In addition, a number of constitutive host factors may be essential for the heterogeneous treatment response found in patients with ALL. These may not be detected by GEP of ALL cells but through careful studies of genetic variation (single nucleotide polymorphism, SNP) in (germline) host cells.

Careful analysis of clinical study data is required, for an adequate prognostic assessment of data generated through GEP. In particular, it needs to be determined whether such profiles can indeed provide prognostic information which is specific enough to comprise the majority of recurrences and/or other adverse events. It appears that in T-ALL, a rather small number of genes may play a crucial role in determining treatment success. The genes which control cell cycle progression appear to be downregulated in resistant patients. This observation coincides with the observation made in our comparative analysis of patients with poor or adequate early response to multiagent chemotherapy, which indicated a similar mechanism in resistant cases.

The large range of data generated by GEP in ALL should be carefully analyzed with regard to specific biological subgroups, treatment subgroups, and outcome. Certainly, sampling and cell processing may also have a large impact, in particular in retrospective analyses, on the profiles found in certain subgroups.

The large heterogeneity found in treatment response and final outcome is also observed with regard to treatment-related toxicity and may be explained by genetic variation in crucial genes. The classical example is the genotype for thiopurinemethyltransferase (TPMT). This genotype was recently found to be relevant also for treatment response in childhood ALL in patients treated with BFM consolidation containing 6-mercaptopurine. Abnormalities in the karyotype of leukemia cells may interfere with the functional gene activity as shown in an interesting study in childhood ALL. Thus, cytogenetic information coincides with pharmacogenetic information.

The new technologies briefly summarized here may eventually enable better adapted treatment schedules, of which the largest benefit may be the prevention of severe toxicity.

References

Acute lymphoblastic leukemia: therapeutic strategies in children and adolescents revisited

Clinical outcome and prognostic factors

In 2000 the treatment results of trials on childhood acute lymphoblastic leukemia (ALL) run in the early 1990s by the major study groups were presented in a uniform way. The 5-year event-free survival ranged from 71% to 83% (Table 1) after having achieved complete remission rates of 98% or higher. An accurate assessment of relapse risk is important in the treatment of childhood and adolescent ALL to avoid excessive toxicity and to improve or maintain the cure rate. An overview of the clinical and biological prognostic factors is given in Table 2. Age, sex and white blood cell count have prognostic value do immunophenotype and genotype of the leukemia. Early response to prednisone in the peripheral blood after 1 week, the response to chemotherapy in the bone marrow after 1 to 3 weeks, and the achievement of complete remission after 4 to 6 weeks of therapy are also used for the stratification of patients. In several European protocols this is now extended with the determination of minimal residual disease in the first months of therapy.

Age and immunophenotype

Even nowadays age remains an independent prognostic factor of outcome. Children from 1-9 years have the best outcome; children and adolescents in the age category of 10-20 years have a slightly lower survival rate than children in the first decade of life, although this is partially related to the higher incidence of T-cell leukemia and the lower incidence of favorable genetic abnormalities. Infants (defined as < 1 year of age) have a relatively poor outcome, which is associated with a high incidence of MLL gene rearrangements. However, within patients with specific types of MLL gene rearrangements, age still influences outcome.

T-cell ALL is detected in ~15% of childhood ALL. Compared to B-lineage ALL, T-cell ALL is characterized by a relative resistance to different classes of drugs. However, with risk-adapted therapy the outcome of T-cell ALL now approaches that of B-lineage ALL (Table 1). The far majority of cases of childhood ALL are of B-lineage, mainly common or preB-ALL cases. A very immature subtype characterized by the lack of CD10 expression (proB-ALL) is associated with a high incidence of MLL gene rearrangements and an unfavorable outcome. Mature B-ALL, defined by the presence of immunoglobulins at the cell surface, has a good outcome only when treated by protocols for B-non Hodgkin’s lymphoma.

Genetics

Hyperdiploidy (a DNA index >1.16 or >50 chromosomes per leukemia cell) is present in about one quarter of cases of childhood ALL and confers a favorable outcome, especially when extra copies of chromosome 4, 10 or 17 are present. Hyperdiploid ALL cells have an increased tendency to undergo apoptosis, accumulate large amounts of metho-trexate polyglutamates and are highly sensitive to antimetabolites and L-asparaginase. Hypodiploidy (<45 chromosomes) is very rare but is associated with a poor outcome; the association is is even clearer for the low-hypodiploid (33-39 chromosomes) or near haploid cases (23-29 chromosomes).

The TEL/AML1 fusion is also found in one quarter of cases and is the most frequent genetic abnormality. This genetic abnormality is associated with a favorable outcome. It is formed by a fusion of the TEL gene on chromosome 12, encoding for a nuclear phosphoprotein of the ETS family of transcription factors, and the AML1 gene on chromosome 21, a transcription factor gene encoding for part of the core-binding factor. The TEL/AML1 fusion most probably inhibits the transcription activity of the normal AML1 gene involved in proliferation and differentiation of hematopoietic cells. The TEL/AML1 fusion is associated with a high sensitivity to L-asparaginase.
Both hyperdiploidy and TEL/AML1 mainly occur in children <10 years with common/preB-ALL and are rare above this age and in other immunophenotypes of ALL.

Abnormalities of the MLL (mixed lineage leukemia) gene on chromosome 11q23 occur in only ~2% of children above the age of 1 year while 80% of infants with ALL have MLL gene rearrangements. In infant ALL all types of MLL gene rearrangements such as MLL/AF4 created by t(4;11), MLL/ENL by t(11;19) and MLL/AF9 by t(9;11) are associated with a poor outcome while in older children this only holds true for the presence of MLL/AF4. The precise actions of the fusion products involving MLL are not known but they are associated with abnormal expression of HOX genes, which may lead to abnormal growth of hematopoietic stem cells. ALL cells with MLL gene abnormalities are highly resistant to glucocorticoids in vitro and in vivo and also to L-asparaginase. These cells do, however, show a marked sensitivity to Ara-C, which is related to a high expression of the membrane nucleoside transporter ENT1.

The translocation t(9;22) fuses the BCR gene of chromosome 22 to the ABL gene from chromosome 9. This results in an abnormal ABL tyrosine kinase activity leading to decreased apoptosis and increased proliferation. The BCR/ABL fusion is mainly found in B-lineage ALL and its incidence increases with age from 2% in children <10 years to ~25% in adults with ALL. The presence of BCR/ABL predicts a poor outcome.

The prognostic value of genetic abnormalities in T-ALL is less clear. Ectopic expression of TAL-1 is caused by the translocation t(1;14), in a low percentage of T-ALL cases, or by the more frequent SIL-TAL fusion transcript. Activation of HOX11 by the translocations t(10;14) and t(7;10) occur in ~10% of T-ALL. Two recently described abnormalities occur frequently and exclusively in T-ALL. These are the ectopic expression of HOX11L2 mainly caused by the translocation t(5;14) in ~25% of T-ALL cases and activating mutations of the NOTCH1 gene in 50% of T-ALL cases.

**Therapy**

The backbone of ALL therapy consists of the following elements:

1. **Induction.** The goal of induction therapy is to induce morphological remission and to restore normal hematopoiesis. Induction therapy is based on at least three systemic drugs, i.e. a glucocorticoid, vincristine and L-asparaginase plus intrathecal therapy. The addition of an anthracycline as a fourth drug is matter of debate. In some protocols this is done for all patients, in others only in high-risk cases.

2. **Central nervous system (CNS)-directed treatment.** This block of therapy aims to prevent CNS relapses and to reduce the minimal residual burden of systemic leukemia. This is usually done by administering three or four courses of high dose methotrexate and 6-mercaptopurine plus intrathecal therapy. Some groups add other drugs in this phase.

3. **Reinduction.** Reinduction therapy or delayed intensification most often contains comparable drugs
as those given for induction therapy and has clearly shown its value by reducing the risk of relapse.

4. Maintenance. Therapy for ALL is completed by prolonged maintenance therapy for a total therapy duration of 2 years or in some protocols even longer. Maintenance consists of 6-mercaptopurine daily and methotrexate weekly. In some protocols additional pulsed applications of a glucocorticoid and vincristine and intrathecal therapy are administered.

5. Stem cell transplantation. Allogeneic stem cell transplantation is reserved only for a small number of selected patients in first complete remission. It is important to note that the contribution of specific parts of the treatment depends on the backbone of the total therapy administered to a patient. A few important topics on which new recent data have been produced will be discussed below.

**Dexamethasone or prednisone?**

Several recent randomized studies have shown that substituting prednisone (~40mg/m²) by dexamethasone (~6 mg/m²) significantly decreases the risk of bone marrow and CNS relapses when used in what is thought to be equipotent dosages. The benefit of dexamethasone may be due to higher free plasma levels and a better CNS penetration or to the fact that the presumed equivalent antileukaemic activity for prednisone: dexamethasone is not at a 6:1 dose ratio but higher as some in vitro experiments suggest.

**What dose-intensity of which asparaginase?**

Randomized studies have revealed that at the same dosedrives, the use of L-asparaginase derived from *Escherichia coli* results in significantly better event-free and overall survival rates than the use of *Erwinia chrysanthemi* (Erwinase). This is due to differences in that half-lives of the drugs and the difference would presumably not be found if Erwinase is given in an adequate dose/intensity schedule.

The dose intensity schedule to achieve complete asparagine depletion is 5,000 U-m² every 3 days for *E. coli* asparaginase. For the pegylated type of *E. coli* asparaginase 2,500 U/m² once every 2 weeks leads to the same pharmacodynamic effects. Lower doses of PEG asparaginase (1000 U/m²) also lead to complete asparagine depletion in serum but not in the cerebrospinal fluid.

Intensive use of asparaginase in induction and reinduction has led to excellent outcome results and it was shown that asparaginase intolerance was an independent factor predicting an inferior event-free survival.

**Which CNS-directed therapy?**

To clarify the role of different CNS-directed thera-
Table 3. Outcome of adolescents treated on a paediatric or adult ALL protocol.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Patient number</th>
<th>Age category</th>
<th>5-year EFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA: pediatric CCG</td>
<td>196</td>
<td>16-21 years</td>
<td>64%</td>
</tr>
<tr>
<td>USA: adult CALGB</td>
<td>103</td>
<td>16-21 years</td>
<td>38%</td>
</tr>
<tr>
<td>Dutch: pediatric DCOG</td>
<td>47</td>
<td>15-18 years</td>
<td>69%</td>
</tr>
<tr>
<td>Dutch: adult HOVON</td>
<td>44</td>
<td>15-18 years</td>
<td>34%</td>
</tr>
<tr>
<td>French: pediatric FRALLE</td>
<td>77</td>
<td>15-20 years</td>
<td>67%</td>
</tr>
<tr>
<td>French: adult LALA</td>
<td>100</td>
<td>15-20 years</td>
<td>41%</td>
</tr>
</tbody>
</table>

maintenance to less than 2 years has led to an increased risk of relapse.\textsuperscript{11}

Who should and who should not be transplanted?

Autologous stem cell transplantation (SCT) is not effective in childhood ALL and should, therefore, never be performed. A collaborative study of several large study groups showed that patients with BCR/ABL-positive ALL benefit from allogeneic SCT from a matched related donor both in terms of disease-free and overall survival.\textsuperscript{12} For other types of donor this was not proven. A comparable analysis for children with t(4;11) did not, however, show a beneficial effect of SCT from any type of donor.\textsuperscript{13}

Recently, a comparison was performed between very high risk ALL children in first remission who were assigned by the availability of a compatible, related donor to receive SCT or to receive chemotherapy in the case no donor was available.\textsuperscript{14} Very high risk was defined by the presence of one or more of the following criteria: failure to achieve complete remission after 5 weeks of therapy, t(9;22) or t(4;11) positivity, a poor prednisone response associated with T-cell phenotype or white cell count > 100x10\textsuperscript{9}/L. The 5-year disease-free survival was better for the patients who received SCT from a matched related donor than for those who received chemotherapy (disease-free survival, 57% vs 40%, respectively). However, only ~20% of all patients in this small subset of very high risk patients had a suitable family donor. SCT from alternative donors resulted in an inferior outcome.

Treatment of adolescents

Three recent reports from the USA, France and the Netherlands suggest that the outcome of adolescents with ALL is better if they receive pediatric than adult protocols.\textsuperscript{15,16,17} A comparison of the 5-year event-free survival of patients aged 15-21 years was ~30% higher when treated according to a pediatric protocol (Table 3). The outcome differences could not be explained by differences in patients’ characteristics such as immunophenotype and genetic abnormalities but were attributed to mayor differences in dose-intensity schedules. The pediatric protocols contained more glucocorticoids, vincristine, L-asparaginase, methotrexate and 6-mercaptopurine. The French and Dutch studies also suggested that longer delays between different parts of treatment might play a role in the outcome difference. This might be related to the fact that adult hematologists have a different attitude to toxicities because they also treat older patients who do not tolerate intensive therapy. In the Dutch study, however, a relatively large number of patients on the adult protocol received SCT and the adult protocol not only resulted in a higher relapse rate but also in a higher toxic death rate. So, in practice, the pediatric approach proved less toxic and more effective than the adult approach for the same adolescent age category.

It is clear, however, that toxicity increases with age of the patient. For example, children > 10 years have a higher incidence of side effects of glucocorticoids such as avascular necrosis of bone and hyperglycemia, and of L-asparaginase such as pancreatitis and thromboembolic complications.\textsuperscript{7} About 5-15% of children and adolescents over 10 years old experience one or more of these side effects. It has been shown that for the same cumulative doses, short pulses of glucocorticoids (5 days) lead to fewer side effects than do glucocorticoids given in more continuous schedules.

Perspectives

Minimal residual disease

The detection of MRD by polymerase chain reaction (PCR) analysis and eventually also by flow cytometry enables us to identify more accurately very good responders to therapy and the poor responders, whatever the biological causes of these responses.\textsuperscript{18} In several protocols, MRD is now used to reduce therapy and also to select patients for therapy intensification. In Germany, Austria, Italy, the Netherlands and Australia/New Zealand simultaneous studies are run to reduce therapy in the 30% very low risk patients and to start moderate therapy intensification in the 55% of patients who belong to the intermediate risk group and very marked treatment intensification in the high risk group, composed of 15% of all patients.

Gene expression profiling

Gene expression profiling can be helpful not only in the classification of ALL patients but also for providing new insights into pathways involved in different genetic subtypes of ALL and identifying new pathways involved in therapy resistance and new therapeutic targets. For example, high levels of wild type FLT3 were discovered in MLL gene rearranged and hyperdiploid ALL and it was shown that espe-
Table 4. New targeted therapies for childhood and adolescent ALL.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Type of ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>ABL tyrosine kinase</td>
<td>BCR-ABL fusion, NUP214-ABL1 fusion</td>
</tr>
<tr>
<td>Dasatinib AMN107</td>
<td>ABL tyrosine kinase (also many mutations), SRC kinases</td>
<td>BCR-ABL fusion, other ALL subtypes?</td>
</tr>
<tr>
<td>PKC412, CEP701, other FLT3 inhibitors</td>
<td>Mutated FLT3, wild type overexpressed FLT3</td>
<td>MLL gene rearranged ALL, hyperdiploid ALL</td>
</tr>
<tr>
<td>Demethylating agents</td>
<td>Hypermethylation</td>
<td>MLL gene rearranged ALL, other subtypes?</td>
</tr>
<tr>
<td>Rituximab</td>
<td>CD20</td>
<td>CD20+ (B-lineage) ALL</td>
</tr>
<tr>
<td>Epratuzumab</td>
<td>CD22</td>
<td>CD22+ (B-lineage) ALL</td>
</tr>
<tr>
<td>Gemtuzumab ozogamicin</td>
<td>CD33</td>
<td>CD33+ ALL</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>CD52</td>
<td>CD52+ ALL</td>
</tr>
</tbody>
</table>

especially the *MLL* gene rearranged subtype is susceptible to FLT3 inhibitors. Gene expression patterns related to in vitro resistance to several classes of drugs and to clinical outcome have also been identified. Based on this, it was discovered that the glycolytic pathway may be involved in glucocorticoid resistance in ALL. Subsequent studies showed that by targeting this pathway, ALL cells could be sensitized to glucocorticoids. In coming years, large-scale studies will be analyzing the profile of gene polymorphisms and expression of microRNA in ALL subtypes.

**Targeted therapies**

Several new targeted therapies may contribute to a further improvement of treatment results in childhood and adolescent ALL (Table 4). The ultimate target of therapy is the leukaemogenic fusion product. The best example at the moment is the BCR/ABL fusion product leading to abnormal ABL tyrosine kinase activity. Imatinib is an effective inhibitor of this kinase but resistance rapidly occurs when this inhibitor is used as a single agent, mainly because of selection or development of leukemic subclones with BCR-ABL point mutations. Imatinib must, therefore, be combined with standard chemotherapy for BCR-ABL-positive ALL. A European randomized study (EsPhALL) is analyzing the efficacy and toxicity of the addition of imatinib to all chemotherapy blocks. A paediatric phase I-II study with dasatinib, which targets the majority of ABL mutations among others, will start in 2006. The very rare subset of T-ALL with NUP214-ABL1 fusion may also be a suitable group for targeted therapies with these compounds.

The recent finding that half of T-ALL cases have activating mutations of the NOTCH1 gene provides a rationale for targeted therapies of the NOTCH pathway. Cleavage of the transmembrane receptor NOTCH1 by gamma-secretase leads to release of the intracellular domain of NOTCH1 (ICN1), followed by translocation to the nucleus and transcription activation. Inhibitors of ICN1 production and activity appeared to be toxic to T-ALL cells in vitro, and this has led to a clinical trial of a gamma secretase inhibitor in patients with refractory T-ALL.

Overexpression of wild type FLT3, especially in *MLL* gene rearranged ALL and hyperdiploid ALL, also provides a rationale for targeted therapies with FLT3 inhibitors. Another rationale may be found in the hypermethylation state of certain leukemias, especially *MLL* gene rearranged ALL, in which for example the tumor suppressor gene *FHIT* is silenced by hypermethylation. Re-expression leads to cell killing in infant *MLL* gene rearranged ALL cells and demethylating agents lead to the same effect.

Finally, monoclonal antibodies, directed against different antigens (CD20, CD22, CD33 and CD52) with or without conjugated toxins, are being investigated in early clinical studies in childhood ALL.

**References**

Therapy of adult acute lymphoblastic leukemia according to prognostic factors and minimal residual disease

Acute lymphoblastic leukemia (ALL) is not a uniform disease but consists of subtypes defined by immuno-phenotype and molecular aberrations. These subtypes have a different clinical courses, treatment responses and prognoses.

One of the major aims of clinical research in adult ALL is to identify patients who will have a poor outcome in response to chemotherapy alone and who might, therefore, be candidates for a stem cell transplant (SCT) in first complete remission. In most large prospective trials in adult ALL the 5-year survival rate does not exceed 30-40%. However, there is a wide range in outcomes resulting in overall survival rates between 70% in subtypes of T-ALL down to 20% in ALL patients with complex cytogenetic aberrations. The results of SCT vary widely being best (50%) for patients in first complete remission receiving an allogeneic transplant from a sibling donor, 40% in those whos allogeneic donor is unrelated and a similar or inferior outcome with autologous transplants. Thus the question of which patients are candidates for transplantation in first complete remission is not solved and is differently defined in the various ongoing adult ALL trials.

The diagnostic armentarium for defining of subgroups with different prognoses has been substantially enlarged in ALL in recent years. Earlier conventional parameters used to define risk groups were clinical characteristics, such as age, white blood cell count, organ involvement (e.g. central nervous system, mediastinum, extramedullary involvement), immunophenotype and cytogenetic aberrations. New prognostic features such as evaluation of minimal residual disease, (MDS) and biological markers such as molecular aberrations are not only of prognostic relevance but also lead to targeted therapies such as antibody treatment or the use of tyrosine kinase inhibitors for Philadelphia chromosome (Ph)/BCR-ABL positive ALL.

Prognostic factors

In adult ALL the still most important prognostic factors are determined at diagnosis and include age, white blood cell (WBC) count, immunophenotype, cytogenetics and molecular genetics (Table 1).

White blood cell count

A WBC count greater than 30,000/µL at the time of diagnosis is associated with a higher relapse risk, even if this is the only risk factor. In a recent study by the German Multicenter Study Group for Adult ALL (GMALL) it turned out to be the most deleterious prognostic factor in B-precursor ALL. In T-ALL, at least according to the data of the GMALL study group, no predictive cut-point for WBC could be defined since the prognostic impact of immunophenotype was overwhelming in multivariate analysis.

Immunophenotype and corresponding cytogenetic/ molecular markers

A complete immunologic characterization at diagnosis is required not only to identify subtypes with different clinical presentations and prognoses but also to assess surface markers as potential targets for antibody therapy. Furthermore distinct cytogenetic and/or molecular aberrations (reviewed in ref. 1) occur in association with certain immunophenotypes and will, therefore, be discussed together.

Patients with the formerly unfavorable subgroup of CD10-negative pro-B-ALL, which is characterized by a high proportion of t(4;11)/ALL1-AF4-positive ALL (70%) and high WBC at presentation (>100,000/µL in 26%), now reach leukemia-free survival rates above 50% and particulary favorable results are achieved with SCT, as reported from the GMALL studies.2 Other study groups however observed unfavorable results, at least for the subtype of t(4;11) positive ALL.3 These variable results probably reflect the prognostic impact of applied therapy. Recently the lack of CD10
expression, also within pre-B-ALL, was identified as a poor prognostic feature. Furthermore it was demonstrated that 20% of MLL-rearranged samples have activating mutations of the FLT3 gene which is a potential therapeutic target. Common/pre-B-ALL include a high incidence of Ph/BCR-ABL-positive ALL (40-50%). In children there are two prognostically favorable subgroups of common/pre-B-ALL, the TEL-AML1-positive ALL with an incidence of 25% and hyperdiploid ALL (50-50%), which are partly overlapping. In adults however these groups account for a very small proportion of cases of ALL. Some groups consider the translocation t(1;19)/PBX-E2A, which has an incidence of around 5% in common/pre-B-ALL, as an unfavorable prognostic feature. Also -7, -8 and hypodiploid ALL have been reported to be unfavorable prognostic subgroups. Common/pre-B-ALL can be subdivided into standard and a high-risk groups with significantly different outcomes. Unfortunately this subtype is prone to late relapses even after 2 or more years. Beside antibody therapy, new approaches result from molecular targeting and MRD analysis.

It is state of the art that mature B-ALL is treated according to different concepts with short intensive cycles without maintenance therapy (see below). T-lineage ALL comprise the subtypes early T-ALL, thymic (cortical T-ALL) and mature T-ALL. The expression of HOX11, HOX11L2, SIL-TAL1 and CALM-AF10 is associated with different maturation states of thymocytes. T-ALL is characterized by a high WBC at diagnosis, mediastinal tumors (50%), central nervous system (CNS) involvement (8%) and a higher rate of CNS relapses (10%). T-ALL patients often have a large tumor mass and show rapid disease progression at diagnosis and at relapse. There are, however, only a few relapses 2 or more years after diagnosis. In the GAML studies the most relevant prognostic factor in T-ALL was the immunologic subtype, with an inferior leukemia-free survival (<50%) for early T-ALL and mature T-ALL compared to thymic (cortical) T-ALL (>50-60%). Microarray analysis identified several new molecular prognostic factors for T-ALL. HOX11 appears to be associated with a favorable prognosis. This aberration, however, correlated with the favorable karyotype thymic T-ALL and it remains open which of the two features represents an independent prognostic factor. A very unfavorable prognosis was observed for HOX11L2. NOTCH1 mutations were identified in up to 50% of T-ALL cases. These mutations have been correlated with a more favorable prognosis and also identify target structures for a therapeutic approach with γ-secretase inhibitors. Furthermore a small proportion of T-ALL patients show the NUP214-ABL1 aberration which may identify a target population for imatinib therapy.

With current treatment regimens complete remission rates of more than 80% and a leukemia-free survival of 50% or more can be achieved in adults with T-ALL. Even in high-risk T-ALL subtypes, such as early and mature T-ALL, the outcome could be improved by SCT in first complete remission.

**Minimal residual disease as a prognostic factor**

Response to treatment is an important prognostic factor since on the one hand it may indicate that the leukemic blasts have a primary drug resistant phenotype, and on the other hand it also reflects the individual realization of therapy which may for example be hampered by dose reductions or delays due to complications. Response to treatment may be evaluated as time to achievement of complete remission - mostly analyzed after 2-4 weeks. A more accurate approach to assessment of individual response is, however, evaluation of MRD. MRD is an independent prognostic factor which can also be predicted accurately by microarray analysis.

In adult ALL a considerable number of mainly retrospective MRD studies have been performed with different methods (overview in ref. 9). Prospective studies with MRD based risk stratification are ongoing. In adults, as in children, a very good response, as indicated by an early and rapid decrease of MRD already during induction, may be associated with a very low relapse risk. However in general the decrease of MRD occurs slowly in adults and fewer patients reach a negative MRD status. This applies particularly for patients with low MRD immediately

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**Table 1. Prognostic factors for risk stratification of adult ALL.**

<table>
<thead>
<tr>
<th>Good</th>
<th>Adverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC &lt; 30.000/µL</td>
<td>WBC &gt; 30.000/µL B-Lineage</td>
</tr>
<tr>
<td>WBC &gt; 100.000/µL T-lineage (?)</td>
<td></td>
</tr>
<tr>
<td>Age &gt; 35 ? &gt; 55 ?</td>
<td></td>
</tr>
<tr>
<td>Early CR</td>
<td>Late CR (&gt;3-4 wks)</td>
</tr>
<tr>
<td>Poor prednisone response (?)</td>
<td></td>
</tr>
<tr>
<td>Thymic T</td>
<td>Pro B (B-lin., CD10-)</td>
</tr>
<tr>
<td>Early T (T-lin., CD1a, sCD3)</td>
<td>Mature T (T-lin., CD1a+, sCD3+)</td>
</tr>
<tr>
<td>TEL-AML1 (?)</td>
<td>t(9;22) / BCR-ABL</td>
</tr>
<tr>
<td>HOX11 (?)</td>
<td>t(4;11) / ALL-AF4</td>
</tr>
<tr>
<td>NOTCH1 (?)</td>
<td>t(1;19) / E2A-PBX (?)</td>
</tr>
<tr>
<td>HOX11L2 (?)</td>
<td>CALM-AF4 (?)</td>
</tr>
<tr>
<td>Complex aberrations ?</td>
<td></td>
</tr>
<tr>
<td>Negative / &lt; 10^-4</td>
<td>Positive &gt; 10^-4</td>
</tr>
</tbody>
</table>

* Generally accepted factors are printed in bold.
after induction who still show a considerable relapse rate of approximately 50%. Thus in adult ALL MRD analysis immediately after induction provides a good tool for identifying patients at high relapse risk but not those at low risk. The longitudinal course of MRD may be more important in adults than in children. High MRD at any time-point after induction is associated with a higher relapse risk and the predictive value increases at later time-points (months 6-9). The evaluation of MRD based on the detection of BCR-ABL is significantly more sensitive and proved to be highly predictive for response to imatinib therapy.\(^1\)

These results underline that the predictive value of MRD evaluation depends on the technical quality such as sensitivity (10\(^{-4}\) for negative results), number of targets (at least two) and on the frequency of evaluations (3 monthly) in individual patients. In the GMALL studies a combination of several time-points for MRD evaluation during the first year and a cut-off point of 10\(^{-4}\) yielded the highest discriminative value for relapse risk. Using MRD levels to make treatment decisions is still not established practice and should be restricted to clinical studies (see below).

**Targeted and individualized treatment according to prognostic factors**

Risk-adapted strategies, particularly for identifying candidates for SCT in first complete remission are already integrated in the majority of trials for adult ALL. However with the availability of new targeted drugs, specific treatment options for subtypes of ALL need to be considered for risk stratification do individualized approaches considering the molecular response in single patients. Targeted therapy in adult ALL mainly includes: 1. subtype-adjusted treatment; 2. treatment according to MRD; 3. risk-adapted indications for SCT; 4. molecular targeting of the signal transduction cascade e.g. with tyrosine kinase inhibitors; 5. antibody therapy; 6. evaluation of new cytostatic drugs.

Examples for these strategies will be discussed below.

**Subtype-adjusted treatment: mature B-ALL**

Mature B-ALL is generally treated according to a different concept, with short intensive cycles of chemotherapy without maintenance therapy. This subtype of B-ALL is characterized by a high rate of organ and CNS involvement. The regimens include fractionated cyclophosphamide or ifosfamide, high-dose methotrexate and high-dose cytarabine in conjunction with the conventional drugs for remission induction in ALL given at frequent intervals over 6 months. With these regimens complete remission rates range from 60%-100% compared to the former

40% and leukemia-free survival from 20%-65% compared to <10% previously.\(^2\) Similar regimens are successfully administered in Burkitt’s lymphoma and other high-grade lymphomas.

It has recently become evident that immunotherapy is a very promising approach in mature B-ALL since >80% of the patients are CD20-positive. Thus additional application of rituximab before each chemotherapy cycle has yielded a significant further improvement of treatment results in B-ALL and Burkitt’s non-Hodgkin’s lymphoma in several studies.\(^3\)\(^4\)

**Treatment according to MRD**

There are different approaches to the integration of MRD analysis in risk stratification of adult ALL. The differences are related to: 1. the time-point of risk stratification; 2. the selection of patients placed to MRD risk stratification; 3. the combination of MRD-based and conventional risk factor-based stratification; and; 4. the aims of MRD risk stratification regarding treatment decisions.

The GMALL has defined a sequential approach with a first risk stratification according to conventional factors followed by a second stratification according to MRD, which is at present focused on patients with a standard risk according to conventional factors. Patients are allocated to a MRD-based low risk (MRD-LR), high-risk (MRD-HR) or intermediate-risk (MRD-IMR) group. Treatment options are to stop therapy after one year for MRD-LR, SCT or experimental therapy for MRD-HR. The recommendation for the MRD-IMR group is intensified maintenance therapy with six further consolidation cycles during the second year of therapy.\(^5\)\(^6\) Other study groups submit all patients to risk stratification according to MRD, independently of whether they are at high- or low risk according to conventional prognostic factors.\(^7\) The aim in these studies is mainly to identify patients with a high risk of relapse MRD in order to offer them treatment intensification. The modulation of indications for SCT according to MRD is a very important issue. One the one hand patients with a low level of MRD before SCT have a very good prognosis after SCT but are at risk of transplant-related mortality. On the other hand patients with high MRD levels before SCT have a high risk of relapse and could probably benefit from additional conventional therapy to reduce tumor load. The best strategy remains open to question.

**Risk-adapted indications for SCT**

At present there is no general agreement on indications for SCT in first complete remission. In some studies SCT in first complete remission is recommended for all patients with a sibling donor, inde-
pendently of risk factors, whereas in the remaining patients a randomized comparison of autologous SCT and chemotherapy consolidation is performed. Matched, unrelated SCT is reserved for Ph/BCR-ABL positive ALL in these studies. The GMALL and other groups define indications for SCT in first complete remission based on prognostic factors and include matched related and unrelated SCT. The high-risk group accounts for half of the patients in the GMALL study group. Standard risk patients reach a survival of 50% with chemotherapy consolidation. In these patients the indication for SCT in first complete remission is based on MRD.

Recently an evidence-based review on the role of SCT in adult ALL was published by the American Society for Blood and Bone Marrow Transplantation. Based on studies published from 1980 to 2005 it was concluded that SCT in first complete remission is recommended in high risk but not in standard risk patients. For patients in second complete remission the outcome after SCT is superior to that after chemotherapy. Related and unrelated SCT yield comparable results whereas allogeneic SCT overall is probably superior to autologous SCT. Autologous SCT is not superior compared to chemotherapy. Regarding conditioning regimens there is apparently an advantage for based on total body irradiation regimens. This comprehensive review also elucidates the urgent need for prospective well-defined studies of SCT in ALL.

For older high risk patients and patients with contraindications for conventional SCT, non-myeloablative SCT is a reasonable treatment alternative.

**Molecular targeting: treatment of Ph/BCR-ABL-positive ALL**

New molecular therapeutic strategies are being evaluated, particularly in Ph/BCR-ABL-positive ALL such as the specific Abl-tyrosine kinase inhibitor imatinib (former STI571). Ph/BCR-ABL-positive ALL in adult ALL is 20-25%, increasing with age to >40% in patients above 50 years, and until recently it was the worst prognostic subgroup with a survival <20%. The current use of imatinib in Ph/BCR-ABL-positive ALL is reviewed in ref. #24.

**De novo ALL in younger patients.** In younger patients imatinib is being explored concomittant to induction chemotherapy and in several multicenter trials the complete remission rate was >90%. In addition with parallel application of induction chemotherapy and imatinib, the rate of molecular remissions could be increased to 50%.

**After SCT.** After allogeneic SCT imatinib can be successfultly administered to patients with MRD, which is probably due to the combined effect of targeted therapy and immunological mechanisms of graft-versus-leukemia effects. Persistent detection of MRD after SCT is associated with a high relapse risk, whereas patients with negative MRD status have a favorable prognosis.

**De novo ALL in elderly patients.** A randomized trial of the GMALL study group comparing dose-reduced chemotherapy with Imatinib monotherapy for remission induction, followed by consolidation therapy combined with imatinib in both arms, showed a complete remission rate of more than 90% in patients receiving imatinib as a single-drug induction therapy over a period of 4 weeks. Other study groups also observed high complete remission rates in elderly patients with Ph/BCR-ABL-positive ALL and imatinib monotherapy. However the long term outcome requires further improvement and the development of resistance should be a consideration in treatment adaptations.

In the future it needs to be evaluated whether the molecular complete remission rate and thereby the duration of response can be improved by combination therapies e.g. with chemotherapy but also with other molecular drugs or very different approaches such as monoclonal antibodies, interferon-α or donor lymphocyte infusions after SCT.

Besides Imatinib for Ph/BCR-ABL-positive ALL, new tyrosine kinase inhibitors are under early phase investigation, particularly for imatinib-resistant cases. These new inhibitors include AMN107 and dasatinib. The measurement of MRD enables the efficacy of new targeted therapies to be monitored closely.

**Antibody therapy**

ALL blast cells express a variety of specific antigens, such as CD20, CD19, CD22, CD33, and CD52, which may serve as targets for treatment with monoclonal antibodies. A generally accepted prerequisite for monoclonal antibodies therapy is the presence of the target antigen on at least 20% of the blast cells. Antibody therapy is an additional attractive treatment approach in ALL since it is targeted, subtype-specific and, compared to chemotherapy, has different mechanisms of action and side effects. The highest expression has been shown for CD20, CD52, CD22 and CD38.

CD20 (>20%) is expressed on more than one-third of B-precursor ALL blasts, particularly in elderly patients (40-50%), and on the majority of mature B-ALL blast cells (80-90%). Rituximab in combination with specific chemotherapy regimens have given very promising results in mature B-ALL and Burkitt’s non-Hodgkin’s lymphoma (see above).

Rituximab is now also being explored in several pilot studies, in B-lineage CD20+ ALL in the elderly, in standard-risk patients and as in vivo purging for adult high-risk B lineage CD20+ ALL. In a GMALL
protocol for elderly patients with CD20 positive B-precursor ALL, rituximab is added at a conventional dose before chemotherapy cycles starting from induction phase I and for a total of eight applications. In this study preliminary results showed that in 19 evaluable patients with a median age of 66 (55-79) years, the complete remission rate was 63% and the survival after 1 year was 54%. The combination of the Hyper-CVAD regimen with rituximab in B-precursor ALL was also feasible and a favorable outcome of CD20-positive ALL was reported. Thus in B-precursor ALL, the combination of chemotherapy and rituximab is feasible, but long-term results are awaited.

The CD52 antigen is expressed in most lymphatic cells and to a higher degree in T-compared to B-lymphoblasts. The humanized antibody campath-1H showed clinical activity in chronic lymphocytic leukemia, T-cell prolymphocytic leukemia and other T-cell non-Hodgkin’s lymphomas. In a few cases limited clinical effects were observed in patients with relapsed adult ALL. Nevertheless several studies with campath-1H in ALL are ongoing, in relapsed patients (also in combination with chemotherapy), in patients with of MRD and in combination with other antibodies. The CALGB has integrated MabCampath as consolidation therapy in the front-line therapy of adult ALL and some of these drugs support the concept of targeted therapy.

Nelarabine is a purine analog with greater cytotoxic effects on T-lymphatic cells than on B-lymphoblasts. In early studies the complete response rate in adult and childhood T-ALL was 44%. The GMALL observed remission rates of 40-50% in T-ALL refractory to other regimens. Treatment is generally well tolerated. The drug has recently obtained FDA registration. Further evaluation in combination and in front-line therapy is warranted.

BCX1777 is another T-cell specific drug which acts as inhibitor of the enzyme purine nucleoside phosphorylase. A clinical phase II trial is ongoing.

Clofarabine is a purine analog which shows no subtype-specific effects. In a phase I trial no responses were obtained in the few adult ALL patients. In pediatric patients the response rates were more favorable.

Apal pidine is a marine depsipeptide which showed in vitro activity against ALL. Due to its alternative mechanism of action it may be of interest and is now being tested in phase II studies.

Other new drugs for ALL do not fit in the concept of targeted therapies but may improve treatment intensity and / or tolerability, such as liposomal vincristine or daunorubicin, PEG asparaginase and a long-acting liposomal cytarabine (Depocytes) for intrathecal application.

**Future risk stratification and treatment concepts for adult ALL**

Future risk stratification and treatment concepts will probably not be based on conventional prognostic factors but also on individual response and on availability of targeted therapies is subgroups of ALL (Figure 1) Altogether the new risk stratification, integrating several approaches such as MRD, microarray analysis, should result in a more complex but patient-specific treatment approach and therapy improve the outcome in adult ALL.

**Evaluation of new cytostatic drugs**

Several new drugs have been proposed for use in ALL and some of these drugs support the concept of targeted therapy.

**References**

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Clinical impact of gene expression profiling in aggressive lymphoma

In recent years, gene expression profiling has become a powerful tool for elucidating molecular features of cancer subtypes that are characterized by pronounced biological and clinical heterogeneity. In diffuse large B-cell lymphoma (DLBCL) this heterogeneity has been evident for more than twenty years, since a significant subset of these patients can be cured using conventional chemotherapeutic approaches, while the remainder suffers from relapses or progressive disease. In diffuse large B-cell lymphoma (DLBCL), this heterogeneity has been evident for more than twenty years, since a significant subset of these patients can be cured using conventional chemotherapeutic approaches, while the remainder suffers from relapses or progressive disease. Gene expression profiling using DNA microarray technology has begun to shed light on varying molecular features in DLBCL that may account for these differences in the clinical behavior. In particular, three major DLBCL subtypes can be distinguished on the molecular level; these subtypes differ fundamentally in their global transcriptional profiles, in their underlying genetic alterations and with regard to the oncogenic pathways that are active in the tumor cells. The germinal center B-cell like type of DLBCL (GCB DLBCL) is the most common subtype and expresses many of the genes that are characteristically expressed in normal germinal center B-cells, such as CD10 and BCL6. The germinal center B-cell signature in DLBCL was associated with superior clinical outcome in retrospective studies that included patients receiving adriamycin-based chemotherapy. Among the germinal center B-cell signature genes, BCL6, SERPINA9 and GCET2 (HGAL) were found to be most strongly associated with a more favorable clinical outcome. The second major DLBCL subgroup is termed activated B-cell like DLBCL (ABC DLBCL) and shows a gene expression profile that bears resemblance to mitogenically stimulated (activated) blood B-cells. Importantly, many transcriptional targets of the potent oncogenic NFκB-pathway are highly expressed in ABC DLBCL. A third molecular DLBCL subgroup is constituted by primary mediastinal large B-cell lymphomas (PMBL), which are currently defined by a combination of pathologic and clinical features. Two gene expression profiling studies demonstrated that PMBL are characterized by a transcriptional profile that is distinct from the profiles of both GCB DLBCL and ABC DLBCL and that shares significant overlap with the gene expression profile of Hodgkin's lymphoma. From these studies, however, it also became clear that the current approach to defining PMBL on clinical and pathologic grounds is imprecise, since in approximately 25% of cases the conventional diagnosis of PMBL is not supported by the underlying gene expression profile. Instead, these cases correspond to GCB DLBCL or ABC DLBCL cases that happen to predominantly involve the mediastinum. Conversely, a PMBL-specific gene expression profile can occasionally be found in lymph node samples from outside the mediastinum, allowing the diagnosis of PMBL to be made even in cases in which primary mediastinal involvement cannot be proven clinically. Furthermore, these observations suggest that the PMBL-specific expression signature can be maintained outside the mediastinum and thus may not depend on a specific mediastinal environment. Using a slightly different approach, with a different DNA microarray platform (Affymetrix), gene expression profiling was applied to define DLBCL comprehensive clusters to capture the molecular heterogeneity of this aggressive lymphoma. By using three different clustering algorithms and by determining the most stable groups of co-regulated and variably expressed genes, three robust biological clusters were identified that are variably expressed among DLBCL cases. The oxidative phosphorylation signature comprises genes that are involved in mitochondrial function and regulation of...
apoptosis. Another gene expression signature derived from neoplastic DLBCL cells was termed the B-cell receptor/proliferation (BCR) signature. In this group of genes, proliferation-associated and B-cell receptor-associated genes were overrepresented as well as important B-cell transcription factors such as BCL6. A third cluster, the host response (HR) signature, included genes expressed by T cells, macrophages, cytokines and adhesion molecules which are not derived from the neoplastic B-cell population, but instead from infiltrating bystander cells. While these three DLBCL comprehensive clusters may reflect variable molecular features in DLBCL tumors, it is important to note that they were not associated with the clinical course of DLBCL patients in this study.

**Oncogenic events associated with gene expression-based DLBCL subgroups**

Besides fundamental differences in their global gene expression profiles, GCB DLBCL, ABC DLBCL and PMBL are distinct in their underlying oncogenic events and cytogenetic alterations. The translocation t(14;18)(q32;q21), which constitutes the hallmark translocation of follicular lymphoma and involves the BCL2 oncogene, is also present in approximately 20% of DLBCL. Strikingly, the vast majority of t(14;18)-positive DLBCL belong to the GCB DLBCL subgroup, in which this cytogenetic alteration is observed in 45% of cases.4,5 The oxidative phosphorylation comprehensive DLBCL cluster, as defined by Shipp and colleagues, also shows enrichment of DLBCL cases carrying the t(14;18)6 suggesting that there may be a partial overlap between the gene expression-based GCB DLBCL and the oxidative phosphorylation DLBCL subgroups. While almost half of the GCB DLBCL cases are characterized by this cytogenetic alteration leading to deregulated expression of BCL2, it is important to realize that ABC DLBCL constitutively express high levels of BCL2 mRNA and BCL2 protein, which is probably due to constitutive activation of the NFκB-pathway in ABC DLBCL (see below). Therefore, different molecular mechanisms account for deregulated expression of BCL2 in DLBCL and efforts to use BCL2 mRNA or protein expression as a clinical marker, e.g. to predict outcome, will have to take this into account. Genomic amplifications of the short arm of chromosome 2 (2p) involving the c-REL locus are observed in 16% of GCB DLBCL and in 25% of PMBL cases; in contrast, c-REL amplifications are never observed in ABC DLBCL.10 A recent study using conventional comparative genomic hybridization (CGH) in a large series of DLBCL previously characterized by gene expression profiling revealed an association of additional genetic events with the GCB DLBCL and ABC DLBCL subgroups.16 In particular, gains of 3q and 18q12-q22 were a frequent finding among ABC DLBCL, whereas genomic material in 12q12 was frequently gained in GCB DLBCL.10 These observations provide further support for the concept that GCB and ABC DLBCL represent biologically distinct diseases (Table 1). Besides the overlap in overall gene expression between PMBL and Hodgkin’s lymphoma, these two lymphoma subtypes also share genomic gains/amplifications in the chromosomal region 9p24. This genetic event is present in 40-50% of cases of PMBL and the tyrosine kinase JAK2 is one of the genes in the amplicon that may be aberrantly activated by this alteration.11 However, additional genes neighboring the JAK2 locus also show altered expression patterns (e.g. PD L1, PD L2 and SMAR CA2) and may also deserve attention, since they may be involved in regulating the interaction between neoplastic B cells and bystander (medias tinal) T-cells or affect the chromatin structure.

In addition to profound differences regarding their underlying genetic alterations, GCB DLBCL, ABC DLBCL and PMBL also show differences in their functional properties. ABC DLBCL and PMBL are characterized by high expression of NFκB target genes on the transcriptional level, and cell lines representative of these entities do indeed also display functional constitutive activation of NFκB. The finding that blocking the NFκB-pathway is toxic to ABC DLBCL and PMBL cell lines, but not to GCB DLBCL cell lines suggests that this important oncogenic pathway may represent an attractive therapeutic target in ABC DLBCL and PMBL, whereas no benefit would be expected clinically in GCB DLBCL patients, in whom NFκB is not active.12,13 The development of small molecules inhibiting the IkB kinase, the upstream regulator of NFκB, may therefore provide a promising therapeutic approach in future phase I clinical trials. Why the IkB kinase, and consequently NFκB, is constitutively active in ABC DLBCL and PMBL is currently not known.

<table>
<thead>
<tr>
<th>Table 1. Differences between GCB DLBCL and ABC DLBCL with regard to cell of origin, oncogenic mechanisms and clinical behavior.</th>
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<tbody>
<tr>
<td>Cell of origin</td>
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<td>Oncogenic mechanisms</td>
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<td>Clinical outcome</td>
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Amsterdam, The Netherlands, June 15-18, 2006
Outcome prediction in DLBCL using gene expression profiling

The clinical course and survival rates of DLBCL patients are influenced by differences in global gene expression, genetic alterations and the utilization of different oncogenic pathways such as NFκB-activation. In a retrospective analysis, GCB DLBCL, ABC DLBCL and PMBL patients had 5-year survival times of 59%, 31% and 64% following anthracycline-based chemotherapy. Using a Bayesian-type gene expression-based classifier, in an independent series of DLBCL patients GCB and ABC DLBCL patients again had different survivals (5-year survival rates of 62% and 26%, respectively).

The germinal center B-cell signature that in part picks up the distinction between GCB and ABC DLBCL cases and includes typical germinal center-associated genes such as BCL6 and CD10 constitutes one major component of a gene expression-based survival predictor for DLBCL patients. The variable expression of this signature suggests that GCB DLBCL and ABC DLBCL are derived from B-cells at different stages of differentiation (germinal center B-cell vs. post-germinal center B cell) and it is probably the germinal center B cell associated phenotype that accounts for a more favorable prognosis rather than individual genes of this signature. Nevertheless, individual germinal center-associated genes (BCL6, LMO2) as well as genes that are more highly expressed in ABC DLBCL (PRKCB1, PDE4B, CCND2 and BCL2) are powerful components in survival prediction for of DLBCL patients, as described by Shipp and colleagues and Lossos et al.

In addition to the germinal center B-cell signature, the lymph node signature is associated with a more favorable outcome in DLBCL patients. This signature may reflect the host response to the lymphoma and cannot be simply attributed to the contribution of non-malignant bystander cells as these are also present in normal, reactive lymph nodes. Prominent genes from this signature that are associated with survival include genes expressed by fibroblasts (CTGF), by histiocytes (ACTN4) and by components of the extracellular matrix (FN1). One gene expression signature associated with inferior survival in DLBCL patients is the proliferation-associated signature. This signature comprises a large number of genes that are associated with the biological features of cell growth and cell cycle progression/regulation, and, interestingly, only a subset of the genes is predictive of survival in DLBCL. In particular, cMYC and some of its target genes, which are key regulators of cell growth, are associated with inferior survival, while genes that are involved in cell cycle progression are not predictive of outcome. This is, for example, in sharp contrast to gene expression profiling studies in mantle cell lymphoma in which cell cycle-associated molecules are strongly correlated with outcome.

Finally, MHC class II expression in the neoplastic B-cells of DLBCL patients is associated with better survival and this feature can also be reliably measured by gene expression profiling. Loss of MHC class II expression in DLBCL, which occurs by transcriptional mechanisms and not by chromosomal deletions of the MHC class II locus, may participate in preventing an effective immune response against the tumor cells. In summary, various gene expression signatures, which reflect different biological features in the tumor samples, can be combined in a mathematical model that predicts survival of DLBCL patients at the time of diagnosis. Using this approach, subgroups with particularly favorable or poor 5-year survival rates (73% vs. 15%) can be determined among DLBCL patients and this model is independent from the clinically used International Prognostic Index (IPI).

The value of gene expression-based survival predictors can be further improved by adding genetic features to the predictor. In a study that combined gene expression profiles of DLBCL with alterations on the genomic level, as determined by conventional CGH, gains of 3p11-p12 provided prognostic information that was independent of the information yielded by gene expression-based outcome predictor alone. Moreover, certain chromosomal alterations were associated with some of the gene expression signatures described above suggesting the presence of key regulatory genes in these loci that orchestrate particular biological features of the tumor sample (e.g. host response or amount of tumor-infiltrating T cells).

Clinical translation of gene expression profiling studies in DLBCL

What conclusions can be drawn from gene expression profiling studies in DLBCL and how can the results be translated into clinical practice?

First of all, future clinical trials in DLBCL will have to address the important question of which molecular subsets of this heterogeneous lymphoma subtype have been studied in a particular trial. The response to the therapy under investigation will have to be interpreted within the context of the molecular subgroups GCB DLBCL, ABC DLBCL and PMBL, since, given the enormous differences in their biology, it does not appear likely that one therapeutic approach will be equally beneficial in all DLBCL patients. For example, small molecules inhibiting NFκB will only be effective in DLBCL cases in which this pathway is active (ABC DLBCL and PMBL), while few effects can be expected in the GCB DLBCL subgroup. While gene expression profiling would be the most reliable
tool to provide a molecular diagnosis of DLBCL subsets, it is clear that this technology is not suitable for application in a routine clinical setting at present. However, every effort should be made to incorporate expression profiling into clinical trials and initial steps towards this goal are under way. In the meantime, more feasible approaches to picking up the molecular distinction between GCB DLBCL and ABC DLBCL can be applied, e.g. by immunohistochemical staining for surrogate markers such as CD10, BCL6 and MUM118 (Figure 1). Using the proposed classification scheme by Hans and colleagues, survival differences between patients with GCB and non-GCB DLBCL have become evident, and this approach has been validated by others. It should, however, be noted that algorithms based on immunohistochemistry can only mirror and approximate classifications based on global transcriptional profiles of the tumor samples, since the number of proteins investigated is limited and since immunohistochemistry is only semiquantitative in nature.

Mathematical models that integrate various gene expression signatures, which reflect tumor cell characteristics as well as microenvironmental factors, can be powerful predictors of the clinical course in DLBCL patients at the time of diagnosis. Likewise, the quantitative measurement of RNA levels of a few selected genes may prove useful for assessing the aggressiveness of the lymphoma and for guiding treatment decisions. However, there are also caveats. First, molecular markers with prognostic impact (e.g. gene expression signatures or immunohistochemical markers providing relevant prognostic information) may lose their prognostic value in the presence of a different therapy. A precedent has been provided in a recent study, in which BCL6 protein expression - widely accepted as a prognostic marker in DLBCL - loses its predictive power when rituximab is added to an anthracycline-based chemotherapy. It is, therefore, conceivable that certain gene expression signatures (e.g. the lymph node signature) may lose significance when treatment approaches that alter the microenvironment of the tumors (e.g. vaccination strategies) are applied.

Secondly, the prognostic impact of individual markers in DLBCL will have to be tested within the molecularly defined subgroups. As described earlier, BCL2 expression in DLBCL occurs by two distinct mechanisms: through the t(14;18) in the GCB DLBCL subset and constitutively in the majority of the ABC DLBCL subset. These different mechanisms may provide an explanation for the finding that BCL2 expression is associated with inferior outcome in DLBCL in some studies, but not in others. These contradictory data may have been reconciled in a recent study in which BCL2 expression was shown to have a prognostic impact in the subset of ABC DLBCL, but not in GCB DLBCL. These examples illustrate the need to incorporate ancillary molecular studies (gene expression profiling, immunohistochemistry, etc.) in future clinical trials of DLBCL patients to determine molecular markers specific for a given therapy, which may guide future treatment decisions.

Finally, the detailed molecular characterization and classification of DLBCL will likely lead to new molecular targets. Initial steps to inhibit the NFκB-pathway, which is constitutively active in ABC DLBCL and PMBL, by using small molecules have been taken. The impact of a molecular diagnosis of DLBCL will gain importance in the clinical setting, as ever more drugs for targeted therapy become available.

References


Response criteria in malignant lymphomas revisited - a proposal of the international harmonization project of the German competence network of malignant lymphomas

Standardized criteria for response evaluation are necessary for the interpretation and comparison of clinical studies and trials in lymphomas. Previous attempts have been made to achieve consensus on response criteria in lymphomas. For Hodgkin’s lymphoma (HL) the Cotswold criteria have been used by some. In 1999, the International Working Group (IWG) published standardized response criteria for non-Hodgkin’s lymphomas (NHL), which have been widely used, although often with modifications. However, limitations of the IWG definitions have been identified. Moreover, recent advances in imaging with 18 fluorodeoxyglucose positron emission tomography (FDG-PET) and in pathology with immunohistochemistry and flow cytometry have made a thorough revision of the response criteria necessary.

In order to incorporate new methods and to clarify misinterpretations and ambiguities of the existing guidelines a new international project, the International Harmonization of Trial Parameters in Malignant Lymphoma, was launched in 2002 by the Competence Network Malignant Lymphoma, the organization of German trial groups in malignant lymphoma.

International Harmonization Project

In 2002 the project International Harmonization of Trial Parameters in Malignant Lymphoma was initiated, chaired by Bruce Cheson, Volker Diehl, and Beate Pfistner. The objective was to develop recommendations that would be transparent among study groups and lymphoma subtypes. Subcommittees with international expert members were organized on the topics of Response Criteria (chair Bruce Cheson), Endpoints for Clinical Trials (chair Lena Specht), Imaging (chairs Sigrid Stroobants and Malik Juweid), Clinical Features (chair Steve Rosen), and Pathology/Biology (chair Randy Gascoyne).


Definitions currently used by trial groups for malignant lymphomas

An analysis of 20 randomized clinical trials on malignant lymphoma published in 2003 showed large variability of the trial parameters employed. A number of synonyms were used, e.g., freedom from treatment failure, failure-free survival, time to treatment failure, and event-free survival for the primary survival end-point. Different definitions for the measurement of lesions, response assessment, and time of assessment were found. Although the IWG seemed to have had a large impact, modifications had evidently been adopted, and differences were found between nine large trial groups (Cancer and Leukemia Group (CALGB), German Study Group for high-grade NHL (DSHNHL), Eastern Cooperative Oncology Group (ECOG), European Organisation for Research on Treatment of Cancer (EORTC), Groupe d’Etude des Lymphomes de l’Adulte (GELA), German Hodgkin Study Group (GHSG), Gruppo Italiano Studio Linfomi (GISL), National Cancer Institutes (NCI), and the Southwest Oncology Group (SWOG)). For confirmation of complete remission some groups required that it should last 4 weeks, last 1 month, or last 3 months, but in many cases the necessary duration of status was not specified. The method for imaging residual masses for determining unconfirmed complete remission (CRu) could be gallium scanning or magnetic resonance imaging (MRI), or no method was specified. The definitions for partial remission
were in accordance with the IWG (50% decrease and no new lesions). However the method of measurement of lesions varied, most groups using the sum of the products of the greatest diameter (SPD) of the six largest nodes, as specified by the IWG, but sometimes a decrease in all lesions was required, and sometimes the method of measurement was not specified.

Proposal for new definitions of response criteria

FDG-PET has recently emerged as a powerful functional imaging tool for staging and restaging lymphomas as well assessing response to treatment. Nevertheless the value of FDG-PET has not yet been proven for response evaluation and post-treatment assessment in HL and aggressive NHL. Recent publications give an indication of the power of FDG-PET, but the number of patients examined is small. The role of FDG-PET is less clear for response assessment of indolent NHL and it cannot be recommended for routine use at present. A pre-therapy (baseline) FDG-PET is not necessary for HL, most aggressive NHL (such as diffuse large B-cell lymphoma), or follicular lymphomas, but it is strongly encouraged as it is of great value in interpreting the post-therapy scan. However, other lymphoma entities, e.g. marginal zone, peripheral T-cell, and small lymphocytic, reportedly exhibit low FDG avidity. If FDG-PET is to be used for response assessment of patients with these subtypes, there must be documentation that they were FDG-PET positive before treatment. Details regarding the timing of FDG-PET in relation to therapy and regarding the interpretation of the images will be discussed in the guidelines.

IF FDG-PET is used the definitions for remission criteria should be as follows:

Complete remission (CR) is proposed to be defined as follows:
1. Complete disappearance of all evidence of disease as well as of all disease related symptoms.
2. For typical FDG-avid lymphoma and/or positive FDG-PET prior to treatment, residual node or mass of any size is possible if post treatment PET is negative.
3. For variable FDG-avid lymphoma and negative FDG-PET prior to treatment, computed tomography (CT) scans must be normal.
4. Spleen and liver should be of normal size by physical examination.
5. Bone marrow must be negative by morphology. Immunohistochemistry should be negative in cases with indeterminate involvement.

Complete remission unconfirmed (CRu) will no longer exist for patients who are evaluated by FDG-PET, except for those patients who have an indeterminate bone marrow after therapy.

Partial remission (PR) is proposed to be defined as follows:
1. Decrease of at least 50% in SPD of up to six masses. For only a single mass the decrease should be at least 50% in the greatest transverse diameter (GTD).
2. No new mass and no increase in any mass.
3. For typical FDG-avid lymphoma and/or positive FDG-PET scan prior to treatment FDG-PET scan is positive post treatment, at any previously involved site.
4. For variable FDG-avid lymphoma and negative FDG-PET prior to treatment, CT scans are positive after treatment.
5. Bone marrow results are not relevant if assessment was positive prior to treatment.

Stable disease (SD) is proposed to be defined as follows:
1. Less than PR (see above) but no progressive disease (see below).
2. A performed FDG-PET scan is positive at previously involved sites.

Progressive disease (PD) for patients in PR or SD is proposed to be defined as follows:
1. Increase of at least 50% in SPD of previously involved nodes or increase of at least 50% in a single involved node or
2. Appearance of any new lesion even if other lesions have decreased in size.
3. Lesions are FDG-PET positive if typical FDG-avid lymphoma and/or FDG-PET scan was positive prior to treatment-unless the size of the lesion is below the resolution of the FDG-PET scan.

Relapsed disease (RD) for patients in CR is proposed to be defined as follows:
1. Appearance of any new lesion or disease symptoms or
2. Increase of at least 50% in GTD of any previously involved site or in SPD of multiple nodes.
3. Lesions are FDG-PET positive if typical FDG-avid lymphoma and/or FDG-PET scan was positive prior to treatment-unless the size of the lesion is below the resolution of FDG-PET scan.

Criteria for node sizes in the definitions of PD and RD:
A node must be greater than 1 cm in its shortest
diameter and nodes previously between 1 cm and 1.5 cm in GTD must increase by 50% and must be larger than 1.5 cm in GTD. Nodes previously larger than 1.5 cm in GTD must increase to more than 2.0 cm.

Discussion

The evidence for the use of FDG-PET scanning in response evaluation is fairly well documented for HL and aggressive NHL, both potentially curable diseases, in which the attainment of a CR is the primary goal of therapy. Trials are now being implemented by several trial groups, e.g., the German Hodgkin Study Group and the EORTC, test the value of FDG-PET for treatment modification in these diseases. For other lymphoma entities the evidence is less clear. For disseminated indolent lymphomas other endpoints such as progression-free survival are more important than response rates. In this situation a CT scan is usually quite sufficient. However, if response rates are considered important in these patients, a pre-treatment FDG-PET scan is recommended. For patients with FDG-PET negative lymphomas or patients who are not evaluated with FDG-PET, response the definition of response will rely on the proposed response criteria without the use of FDG-PET.

Conclusion

The definitions proposed above are still being discussed in detail among the experts of the International Harmonization Project, as are the definitions of the different end-points (overall survival, disease-specific survival, progression-free survival, event-free survival, duration of response, and duration to a defined specific event). The preliminary proposals are being presented at international conferences with the aim of stimulating discussion in order to reach consensus in the international scientific community. Only by doing this can we hope to achieve full compliance and implementation of the final criteria.

It is hoped that the new harmonized response criteria will increase the transparency and comparability of clinical lymphoma research in the future.

References

Therapeutic groups

According to the International Prognostic Index (IPI) four prognostic subgroups each for young and elderly patients can be distinguished: low risk, low-intermediate, high-intermediate and high-risk. However, for practical reasons, many cooperative groups lump young low and low-intermediate risk patients together into the therapeutic group of young good-prognosis patients who are distinguished from young poor-prognosis patients (comprising patients with high-intermediate and high risk) and develop differential therapeutic strategies for the two groups. A young patient is clinically defined as a patient who is considered fit for high-dose chemotherapy requiring hematopoietic stem-cell support, which is usually the case up to something between 60 and 65 years of age. Thus, it is up to the treating physician to decide on an individual basis whether a middle-aged patient between 61 and 65 years fits better into a protocol designed for young or elderly patients. In contrast to good-prognosis as defined above, limited-stage disease, early-stage disease, low-stage disease, and localized disease are less well defined and often used exchangeably. Based on the results of the MinT trial a subdivision of good-prognosis patients into a very favorable and less favorable subgroup (see below) appears to fit best the therapeutic needs of these patients if treated with a rituximab-CHOP combination.

While some groups use a differentiated approach for patients in stage I (e.g. a reduced number of chemotherapy cycles), in the DSHNHL trials there was no significant difference in prognosis between patients in stage I and stage II, if they were balanced for other risk factors according to the IPI or bulky disease. Similarly, the prognosis of very old patients (>70, >75, or >80 years of age) is not significantly worse than that of patients between 65 and 70, as long as their increasingly frequent comorbidities do not compromise the consequent adherence to the therapeutic regimen. Therefore, we recommend a careful initial and follow-up evaluation of patients >70 years of age for co-existing morbidity that is perceivable before the commencement of therapy or might evolve during the therapy. Based on these considerations, the therapeutic strategies for three groups of patients are discussed:

1. Young good-prognosis patients (age-adjusted IPI=0,1)
2. Young poor-prognosis patients (age-adjusted IPI=2)
3. Elderly patients

Perspectives/ongoing all-inclusive trials

There are ongoing trials for which most patients with diffuse large B-cell lymphoma (DLBCL) are eligible, irrespective of age, stage and risk factor profile. All these trials, however, stratify patients into different age and risk groups. One such trial is organized by the British NCRI and is comparing eight cycles of R-CHOP-21 and six cycles of R-CHOP-14 in two arms of 540 patients each with stratification according to IPI, age and treatment center. Similarly, the CALGB trial, which is comparing R-CHOP-21 with the infusional and dose-adjusted approach of DA-EPOCH-R in a randomized fashion, is open to all DLBCL patients.

Young good-prognosis patients

Background

After radiotherapy alone, disease-free survival rates of 70 to 80% have been observed only in single center and mostly retrospective studies of patients with small lymphomas (<2.5 cm). Radiotherapy alone has been widely abandoned for patients with aggressive lymphoma, except for rare cases with contraindications against chemotherapy. The data supporting a combined modality approach consisting of chemotherapy plus radiotherapy are conflicting. Combinations of three to five cycles of chemotherapy and

Non-Hodgkin's lymphoma
radiotherapy with 35 to 45 Gy achieved survival rates between 75 to 80% and 5-year survival rates of 80 to 90% in limited stage disease. However, follow-up studies show that relapse rates are significant even after 5 years, if four or fewer cycles of chemotherapy are given, suggesting that abbreviated chemotherapy is unable to eradicate the malignant clone.

In the late 1990s, a SWOG study set the benchmark for the treatment of limited stage disease. While an early analysis of this study had shown an advantage for the combined approach (3× CHOP followed by involved-field radiotherapy), longer follow-up revealed crossing of the event-free and overall survival curves after 7 and 9 years, respectively. Moreover, the LNH 93-1 trial of the French GELA that included patients with stage I and II disease without a risk factor, according to the IPI, showed the superiority of three cycles of the ACVBP regimen followed by sequential consolidation chemotherapy over the combination of three cycles of CHOP with involved-field radiotherapy with 30 to 40 Gy.

Radiotherapy in addition to full-cycle chemotherapy in the ECOG 1484 study resulted in a border-line significance in favor of the combined approach with respect to disease-free, but not overall survival. Support for limiting additional radiotherapy to areas of primary bulky disease was derived from a small Mexican study, in which radiotherapy to initial bulky disease resulted in prolonged relapse-free and overall survival. Finally, there are no data from prospective trials supporting additive radiotherapy to sites of residual masses. In summary, the data supporting the use of radiotherapy in a combined modality approach or in addition to full-dose chemotherapy are being scrutinised carefully, and some co-operative groups such as the French GELA have totally eliminated radiotherapy from their therapeutic armamentarium.

In the pivotal American Intergroup trial, the intensified m-BACOD, ProMACE-CytaBOM and MACOP-B regimens were not superior to CHOP with respect to complete remission rates, event-free or overall survival, but proved to be more toxic. However, in the NHL-B1 trial of the DSHNHL, CHOEP was significantly better than CHOP with respect to the primary end-point of event-free survival, while the reduction of treatment intervals from 3 to 2 weeks resulted in a significantly better overall survival. Therefore, CHOEP-14 is the preferred chemotherapy only regimen for young good-prognosis patients if rituximab is not available or indicated (e. g. rare CD20-negative cases).

In the Mabthera International Trial (MInT), 824 patients with an age-adjusted IPI (aaIPI) of 0 or 1 (excluding patients with stage I non-bulky disease) from 18 countries with stages II to IV and stage I with bulky disease were randomized to receive six cycles of a country-specific CHOP-like regimen (CHEMO: CHOP-21, CHOEP-21, MACOP-B, PMitCEBO) or the same regimen plus rituximab given on day 1 of each chemotherapy cycle (R-CHEMO). Patients with bulky disease received additional radiotherapy to the respective areas. After a median observation time of nearly 2 years, the addition of rituximab increased event-free survival from 61 to 80% ($p=0.000000007$) and overall survival from 86 to 95% ($p=0.0002$). The

### Table 1. Results of randomized trials comparing continual with high-dose chemotherapy in the primary treatment of aggressive lymphomas.

<table>
<thead>
<tr>
<th>Author</th>
<th>Study population</th>
<th>Randomized population</th>
<th>EFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haioun et al., 2000</td>
<td>≥1 RF, Bulk</td>
<td>CR</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>≥2 RF</td>
<td>CR</td>
<td>0.02*</td>
<td>0.04*</td>
</tr>
<tr>
<td>Verdonck et al., 1994</td>
<td>I-IV</td>
<td>&lt;CR</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Gianni et al., 1997</td>
<td>I/Ib/III/IV</td>
<td>all</td>
<td>0.004</td>
<td>n.s.</td>
</tr>
<tr>
<td>Santini et al., 1998</td>
<td>Ia/Ib/III/N</td>
<td>≥2 RF*</td>
<td>0.008*</td>
<td>n.s.</td>
</tr>
<tr>
<td>Kluin-N. et al., 2001</td>
<td>all</td>
<td>CR</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Kaiser et al., 2002</td>
<td>LDH &gt;UNV</td>
<td>CR, PR</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Gisselbrecht et al., 2002</td>
<td>≥1 RF</td>
<td>all</td>
<td>-0.01</td>
<td>-0.009</td>
</tr>
<tr>
<td>Martelli et al., 2003</td>
<td>≥2 RF</td>
<td>CR, PR</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Sertoli et al., 2003</td>
<td>Ia/Ib/III/N</td>
<td>all</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Milpied et al., 2004</td>
<td>1 &amp; 2 risk factors</td>
<td>all</td>
<td>0.01*</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

* Studies in Italic found an advantage of high-dose over conventional-dose chemotherapy; the study in bold found the high-dose approach to be inferior; the remainder studies found no differences; * Retrospective subgroup analysis.
superiority of CHOEP over CHOP was confirmed, but disappeared after the addition of rituximab.

Conclusions

Based on the results of the MInT trial, six cycles of CHOP with rituximab can be considered the standard and reference approach that novel concepts will have to be compared to. Additional radiotherapy (e.g. to sites of initial bulky disease or extranodal involvement) should not be given outside clinical trials.

Perspectives/ongoing trials

Following the demonstration that ACVBP is superior to 3×CHOP plus involved-field radiotherapy, the GELA is currently comparing this regimen alone vs. ACVBP plus rituximab in the LNH 03-1B trial for patients aged 18 to 65 years with aIPI=0. For the same age group, but with aIPI=1, the GELA is comparing R-ACVBP with eight cycles of R-CHOP-21 in the LNH 03-2B trial.

The current DSHNHL approach for this patient subgroup is based on a multivariate analysis of the MInT results which identified a very favorable subgroup with a 2-year event-free survival of 90%, and a less favorable subgroup with a 2-year event-free survival of only 77%. The respective figures for 2-year overall survival are 94% and 98%. In the very favorable subgroup (IPI=0, no bulky disease) further improvement will be difficult to achieve and demonstrate, but the results in the less favorable subgroup definitely warrant further improvement. Therefore, the FLYER trial of the DSHNHL, which includes patients in the very favorable subgroup (stage I&II, no bulky disease, aIPI=0), is comparing six cycles of CHOP-like regimen plus six cycles of rituximab with four cycles of the same CHOP-like regimen combined with six applications of rituximab. In the UNFOLDER trial for the less favorable group (aIPI=0 with bulky disease and all aIPI=1) six cycles of CHOP-21 with rituximab are being compared with a dose-dense regimen of six cycles of CHOP-14 with rituximab, because a retrospective analysis of the NHL-B1 trial showed that bulky disease is very sensitive to interval reduction (Figure 1).

Young poor-prognosis patients

Background

For young poor-prognosis patients (aIPI=2,3) CHOP-21 is formally still the standard conventional chemotherapy regimen, because approaches shown to be superior to CHOP-21 in young good-prognosis patients, such as CHOEP-14 or the combination of a CHOP-like regimen with rituximab have not been tested in a randomized fashion in young poor-prognosis patients. Because the majority of young high risk patients present with elevated pre-treatment lactate dehydrogenase (LDH) and/or bulky disease, two clinical parameters that profited most from dose densification in the NHL-B1 and NHL-B2 trials, respectively, CHOEP-14 or the even more intensive ACVBP of the French GELA might be the preferred conventionally dosed chemotherapy regimens for these patients. The results of high-dose chemotherapy in the primary treatment of aggressive lymphomas are contradictory. A summary of randomized trials addressing this question is shown in Table 1. In general, the results of these trials are difficult to interpret because the conventional and high-dose arms in these trials differ in more than the dose of chemotherapy.

Like the dose-dense regimen CHOEP-14, the monoclonal anti-CD20 antibody rituximab has never been tested in a randomized trial in young poor-prognosis patients with aggressive lymphomas, and such trials are warranted. The overview on primary high-dose chemotherapy in aggressive lymphoma con-
firms that the statement of the Lyon 1997 consensus conference is still valid in 2005. There is no justification for high-dose chemotherapy in the primary treatment of aggressive lymphomas outside clinical trials. This should hold even more true in the era of combined chemo-immunotherapy with rituximab, which equalizes differences of efficacy of different chemotherapy regimens. Moreover, it should be kept in mind that more pronounced myelosuppression of aggressive chemotherapy regimens might interfere with the effector mechanisms of rituximab, in particular antibody-dependent cellular cytotoxicity, and might therefore compromise the efficacy of rituximab when combined with high-dose chemotherapy. Nevertheless, novel approaches of high-dose chemotherapy are still warranted and justified. A non-contaminated testing of the value of high-dose chemotherapy is possible if the high-dose chemotherapy regimen and the conventional arm use the same cytotoxic drugs, which should include those with the highest efficacy in aggressive lymphomas, in particular alkylating agents and anthracyclines. In the high-dose arm these drugs should be given at the maximum tolerated doses and the high-dose regimen should be given in addition to, and not as a substitute for, fully-dosed conventional chemotherapy.

Conclusions

There is no formal proof that any of the more recent approaches that have been successful in elderly patients or young good-prognosis patients, can also improve the outcome of young poor-prognosis patients. In the light of the experience in young low-risk patients, for whom the interval reduction from two 3 to 2 weeks and the addition of etoposide (CHOEP-14) improved complete response, event-free and overall survival rates, and the addition of rituximab further improved the outcome of these patients, it is problematic to randomize young poor-prognosis patients into a control arm with CHOP-21. Due to the low toxicity of rituximab and its efficacy in young good-prognosis patients it is difficult not to give this antibody to young poor-prognosis patients, even though its efficacy has not been demonstrated in this subpopulation and is less pronounced in elderly high-risk than elderly low-risk patients. Because there is no standard therapy for young poor-prognosis patients, all these patients should be treated within randomized prospective trials that compare dose-dense regimens (e.g. CHOEP-14) with maximally escalated high-dose regimens containing the most efficacious drugs for the treatment of aggressive lymphomas (e.g. Mega-CHOEP). For young poor-prognosis patients who are not candidates for or who are not willing to participate in clinical trials, a full-cycle dose-dense regimen (eight cycles of CHOEP-14 or four cycles of ABCVP) in combination with rituximab appears to be a viable choice.
Perspectives/ongoing trials

In the Mega-CHOEP trial the DSHNH is comparing the R-Mega-CHOEP approach (one cycle of dose-escalated R-CHOEP followed by three cycles of maximally dose-escalated R-CHOEP necessitating triple stem cell support) with 8 cycles of dose-dense R-CHOEP-14 (Figure 2). The GOELAMS is comparing eight cycles of CHOP-14 with the winner of their recently published trial (dose-dense CEEP-15, followed by high-dose cytarabine plus methotrexate followed by high-dose BEAM with stem cell support), both combined with rituximab. The designs of the current trials of the Italian IIL group and the American Intergroup Study for these patients are shown in Figures 3 and 4. The GELA is currently running the LNH 03-3B study, a phase-II trial evaluating rituximab in combination with ACVBP followed by high-dose methotrexate and BEAM with autologous stem cell support, while in their LCB-04 trial, the Nordic Lymphoma group is evaluating the role of early salvage therapy in patients with a positive positron emission tomography scan after six cycles of CHOEP-14 followed by high-dose methotrexate and high-dose cytarabine (Figure 5).

Elderly patients

General considerations for the treatment of elderly patients

The curative intention of treatment is also valid for elderly patients with aggressive lymphomas, although the prognosis worsens with increasing age. Only in cases where a careful examination of the patient and his concomitant diseases indicates unacceptable risks for a full-dose therapy, a palliative treatment approach can be justified. However, the definitive decision about the treatability of a patient should only be made after a so-called pre-phase therapy, which consists of a single injection of 1 mg vincristine and 100 mg prednisone daily for one week. This pre-phase treatment leads to a considerable improvement of the patient's performance status and helps in ameliorating the so-called first cycle effect, the phenomenon that side effects of a chemotherapy regimen are most pronounced after the first cycle of chemotherapy causing most therapy-associated...
Background

Because patients in the GELA trial LNH 93-4 above 70 years of age with IPI=0 who received radiotherapy in addition to 4 cycles of CHOP-21 had a worse overall survival than those who did not, radiotherapy should be used cautiously and not outside clinical trials for elderly patients.

In the NHL-B2 trial of the DSHNHL in 689 elderly patients, three intensified regimens (CHOP-14, CHOEP-21, CHOEP-14) significantly improved outcome compared to CHOP-21, with CHOP-14 not only inducing the highest remission rate (CR: 76% vs. 60%; p<0.001), but also the best 5-year event-free survival (44% vs. 33%; p=0.003) and 5-year overall survival (53% vs. 41%; p=0.0001) compared to CHOP-21, with no differences in toxicity between CHOEP-14 and CHOP-21 and a treatment related mortality of 2.9% after CHOP-14 versus 3.4% after CHOP-21. The double-intensive CHOEP-14 proved to be too toxic in the elderly population of the NHL-B2 trial.

In the LNH-98.516 trial in 399 patients aged 60 to 80 years (median: 69 years) the addition of rituximab to CHOP-21 improved the CR rate from 65% to 76%, and event-free survival from 38% to 57% as well as overall survival from 57% to 70% after 2 years, respectively, compared to classical CHOP-21 alone. There were no differences in toxicity with a treatment related mortality of 6 % in both arms. The ECOG 4494 trial with 632 elderly patients confirmed the results of the GELA trial favouring the addition of rituximab to CHOP, even though this could be demonstrated only after the biometrical rescue measure of a so-called weighted analysis. Patients who had already received rituximab together with CHOP during induction therapy did not profit from rituximab maintenance therapy. An interim analysis of the RICOVER-60 trial of the DSHNHL with 828 patients demonstrated that the addition of 8 applications of rituximab significantly improve EFS of patients receiving CHOP-14 with no differences between 6 and 8 cycles of CHOP-14 chemotherapy. Overall survival (78% after 2.5 years) after R-CHOP-14 was not (yet) significantly better than after CHOP-14 alone (74%). A HOVON and Nordic Group trial comparing 8 cycles of CHOP-14 with the same chemotherapy plus 6 applications of rituximab showed also a significant improvement for the combined immunochemotherapy, both with respect to EFS and overall survival; however, adherence to and results of the CHOP-14 protocol were very bad in this trial. The problems of the HOVON trial with CHOP-14 demonstrate how critical a consequence adherence to the CHOP-14 protocol is: While the median relative dose intensity in the RICOVER-60 trial was >90%, less than 50% of the patients in the HOVON trial completed their chemotherapy. A mandatory prephase treatment (1 mg vincristine day -7, 100 mg prednisone days -7 to 0), consequent application of G-CSF (starting no later than day 4 of each cycle), strict adherence to intensity reduction rules (dose reductions only allowed, if treatment delays exceed one week in an individual patient), and intercycle administration of hydrocortisone (20 mg p.o in the morning, 10 mg in the afternoon) for patients complaining about fatigue might be responsible for the excellent results after CHOP-14 in the German compared to the HOVON trial (e. g. CR/CRu: 76% in the German trial, 51% in the HOVON trial). The experience with CHOEP-14, which proved to be worse than CHOP-14 in the elderly population of the NHL-B2 trial, demonstrates that increasing dose intensity (and hence toxicity) beyond a certain point can be counterproductive in elderly patients, pointing to the direction of future strategies for the improvement of treatment results in the elderly.

Conclusions

The results reported with 6 cycles of CHOP-14 with rituximab are the best reported to date for elderly patients.

Perspectives/ongoing trials

Both the GELA (LNH 03-6B trial) and the British NCRI are currently comparing 8 cycles of R-CHOP-21 with 8 cycles of R-CHOP-14 in elderly patients. While data from the RICOVER trial suggest that dose densification (R-CHOP-14) can be expected to result in a 10% improvement in overall survival over R-CHOP-21, this must be confirmed in these ongoing randomized trials to definitely establish R-CHOP-14 as the standard for elderly patients. The experience with the double intensive CHOEP-14 in the NHL-B2 trial of the DSHNHL teaches us that intensification of chemotherapy might not be feasible in elderly patients; however, intensification of immunotherapy or introduction of novel therapeutic strategies might hold promise. The DSHNHL is currently testing R-CHOP-14 with a densified application of rituximab (4 additional applications of rituximab during the first month of treatment) in their
phase-II DENSE-R-CHOP-14 study, while the SWOG is evaluating the combination of R-CHOP-21 and anti-VEGF receptor antibody bevacizumab (R-A-CHOP) in this population.

References

Treatment of stage I and II Hodgkin's lymphoma

The treatment of patients with early stages (Ann Arbor stage I and II) of Hodgkin's lymphoma (HL) has evolved from extensive radiation alone to combined modality treatment in which chemotherapy and radiotherapy are given in close connection. Over 90% of patients with early stage disease can be cured. The popularity of the combined modality approach stems not only from its remarkable efficacy. Initially, chemotherapy was added to radiotherapy to increase cure rates in patients with adverse prognostic factors. But, long-term observation of patients treated with wide-field irradiation such as mantle field, subtotal or total nodal irradiation in the 1960s and 1970s revealed a 10-15% excess death rate due to causes other than HL, especially second malignancies. A higher incidence of cardiovascular events has also been attributed to previous irradiation. These data highlighted the need for less extensive radiation fields and doses, preferably without jeopardizing tumor control. Facilitated by the recognition of polychemotherapy schedules - such as the combination of adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) - with great efficacy and a relatively moderate toxicity profile as compared to the classic methotrexate, vincristine, procarbazine, prednisone (MOPP)-scheme, patients with early stages of disease increasingly frequently receive chemotherapy. Most often it is given in combined modality approaches, incorporating fixed combinations of chemotherapy and a reduced radiation burden. Nevertheless, the optimal balance between high cure rates and acceptable toxicity has not been established yet. For patients with advanced stages (III/IV), chemotherapy alone is an accepted standard treatment provided an unambiguously complete remission is reached after six to eight cycles of modern chemotherapy. For patients with incomplete remission additional radiotherapy is still recommendable. For patients with the early stages I and II, several important questions remain to be answered. How much or how little radiotherapy is indicated? How much or how little chemotherapy is required? Can RT be omitted completely? What combined modality schedule shares the best of both worlds: high efficacy and low toxicity? These issues and some directions for future clinical research are discussed here.

Favorable stages I and II

In the past, staging laparotomy was developed and used in order to achieve a more accurate definition of the extent of
radiological occult abdominal disease. Through this routine screening procedure, clinical prognostic factors could be identified that proved to be predictive of occult abdominal disease. In addition, these factors identified patients with a more favorable stage I/II disease (early stages) and an unfavorable stage I/II disease (referred to by some as intermediate stages). In general, age < 50 years, fewer than four involved nodal areas, absence of B-symptoms, and absence of bulky mediastinal disease indicate the more favorable group. (Table 1). Bulky mediastinal disease is associated with a high risk of relapse when treated with radiotherapy alone. The number of involved areas was found to be independently significant for disease-free and overall survival. Older age is generally considered to be associated with a higher risk of abdominal disease and a poorer overall survival. Elevated erythrocyte sedimentation rate (ESR) indicates a higher risk of relapse and poorer survival. These factors have been quite consistently used by several independent groups. For current and future clinical management, we have to bear in mind that the factors were identified predominantly in studies of patients treated with radiotherapy alone. Whether combined modality treatment strategies or chemotherapy-alone programs, require adaptation of these clinical prognostic indices is yet to be determined.

In the landmark H6-study of the Lymphoma Group of the European Organization for Research and Treatment of Cancer (EORTC), the concept of using clinical prognostic factors for tailoring treatment was tested by randomly comparing clinical staging supplemented by prognostic factors with pathologic staging through staging laparotomy. Patients with favorable characteristics were randomized between staging laparotomy or immediate treatment with subtotal nodal irradiation (STNI). No differences in disease-free or overall survival were noted between the two approaches. Since then, staging laparotomy has been abandoned. Despite the high complete remission rates, the 6-year disease-free survival was only about 70-75% for the wide-field radiotherapy. Although many of the relapsed patients can be salvaged with chemotherapy, the high relapse rates are now deemed unacceptable: not only because of the psychological burden for the patient but also because of the increase in toxicity by adding intensive chemotherapy to the already delivered wide-

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field radiotherapy. These considerations were the hallmark of the start of combined modality programs in early stage disease, which aimed to combine the efficacy of chemotherapy and radiotherapy while reducing toxicity. In the H8 trial of the EORTC and the Groupe d’Etude des Lymphomes Adulte (GELA), the standard STNI was randomly compared with combined modality treatment consisting of three cycles of MOPP/ABV hybrid followed by involved-field radiotherapy (IF-RT). The combined modality approach produced a significantly better event-free survival (EFS) than did the STNI: 4-year results of 99% vs. 80%. Importantly, possibly thanks to a more effective chemotherapy schedule, a significant overall survival advantage for the combined modality arm emerged. These results support the use of combined modality as the new standard treatment instead of wide-field radiotherapy alone. The data demonstrate that the radiation fields can be substantially reduced when combined with adequate chemotherapy. This conclusion is corroborated by the long-term results of the Milan trial comparing four cycles of ABVD followed by either IF-RT or by STNI. The 12-year freedom-from-treatment-failure rates are 94% and 93% respectively; neither is there a difference in overall survival. Although the sample size was small and both favorable and unfavorable stages I/II patients were included, the data support the use or more restricted radiotherapy fields after adequate chemotherapy. In the German Hodgkin Study Group (GHSG) HD7 trial the combined modality treatment consisted of only two cycles of ABVD but it was followed by extended-field radiotherapy (EF-RT). The combined modality produced a significantly better freedom from treatment failure than did EF-RT alone: 91% vs. 75%, without, however, there being a benefit on overall survival. Comparable results were reported by the Southwestern Oncology Group (SWOG) trial comparing three cycles of adriamycin, vinblastine + STNI vs. STNI alone. Therefore, we can reliably conclude that the standard treatment for early favorable stage I/II disease is the combination of a restricted number of cycles of a relatively non-toxic chemotherapy with a restricted target volume of radiotherapy. In the HD10 trial, the GHSG evaluated the use of only two cycles of ABVD vs. four cycles and the use of 20 Gy vs. 30 Gy of IF-RT. The 4-year interim results show no significant difference in the event-free survival between the four groups but follow-up is still rather short. In the EORTC/GELA H9 trial preliminary data show no significant difference in outcome between patients treated with epiadriamycin, bleomycin, vinblastine, prednisone (EBVP) + 56 Gy of IF-RT and those receiving the same chemotherapy but 20 Gy of IF-RT: the 4-year event-free survival rates were 88% vs. 85%, respectively. If these results hold with prolonged follow-up, two to three cycles of modern chemotherapy, such as ABVD, followed by 20 Gy IF-RT might well be the new standard treatment. Anyway, there is no doubt that with a limited amount of chemotherapy and widely reduced fields and doses of radiotherapy, a highly effective treatment is available. Meta-analysis data on the role and extent of radiotherapy are in accordance with this. Long-term follow-up should also clarify whether this treatment approach combines excellent tumor control with the – anticipated – significant reduction in late toxicity.

**Can radiotherapy be omitted in favorable stages I and II?**

The issue of whether patients with early favorable stages of disease can be treated safely with chemotherapy alone is far from settled. There are clear advocates and opponents. A few early studies evaluated MOPP chemotherapy, typically six cycles, alone versus combined modality or radiation treatment. While the one claimed a significantly better outcome for the MOPP-alone arm, in the other the patients in the MOPP-alone arm fared significantly worse. It is difficult to judge these trials on current merits because of the inclusion of other than clinical early favorable stages and because of the use of MOPP, which is no longer standard chemotherapy. Straus et al. concluded that six cycles of ABVD are as effective as six cycles of ABVD + extensive radiotherapy in terms of remission rates, freedom from progression and overall survival. The study was powered to detect a difference between the two treatment arms of at least 20% which precludes the detection of probably, more realistic smaller differences in outcome. In the study by Laskar et al. patients in complete remission after six cycles of ABVD were randomized to receive IF-RT or no further treatment. The authors found a significant improvement in survival for the combined modality arm. However, patients with advanced stages and children were also included. The recently published National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG) and Eastern Cooperative Oncology Group (ECOG) trial provides us with valuable new information. Patients without adverse prognostic characteristics were randomized to receive either STNI and four cycles of ABVD alone when a complete remission was already reached after two cycles or six cycles when complete remission was reached after four cycles. After long-term follow-up, no differences were noted in freedom from treatment failure or overall survival between the two treatment arms. The study suggests that for
favorable stage I/II patients with an early complete remission, a total of four cycles of ABVD can be sufficient treatment. Finally, inclusion in the EORTC/GELA H9, trial comparing EBVP chemotherapy with 36 Gy, 20 Gy or no IF-RT, had to be closed prematurely because of an excess of relapses in the arm not including radiotherapy, though without detrimental effects on overall survival. Therefore, at present there is no compelling evidence that patients with early favorable stages I/II disease should receive chemotherapy alone. The provocative data from Meyers et al. on the significance of an early rapid complete remission, in line with findings in advanced stage disease, strongly suggest that there is probably a large subgroup of patients who can be spared the toxicity of additional radiotherapy after being treated by a restricted number of cycles of ABVD. However, we need a more reliable and early identification of this particular good-risk group.

Fluorodeoxyglucose positron emission tomography (FDG-PET) scanning is expected to provide more reliable response measurements at the end of treatment, differentiating between true complete responders and those with residual active disease. The Lymphoma Clinical Studies Group from the National Clinical Research Institute in the UK is exploring in their current randomized study of patients with early stages disease whether RT can be omitted after three cycles of ABVD in patients with a negative FDG-PET scan assuming that this group of patients have no active residual disease. Exciting data from Hutchings et al. suggest that an early complete remission established by a negative FDG-PET scan after two cycles of chemotherapy, predicts an excellent freedom from treatment failure whereas persistent FDG-PET positivity after two cycles heralds a significantly higher risk of treatment failure. A concept of early adaptation of treatment based on the early response to chemotherapy as determined by FDG-PET is now being prospectively and randomly tested in the EORTC/GELA H10 trial. The results of trials incorporating FDG-PET scan results as a guidance to adapt treatment might more reliably identify the patients who can be treated with chemotherapy alone and those who need additional radiotherapy.

Patients with unfavorable stages I and II
Combined modality treatment is less controversial for patients with unfavorable stage I/II disease. First, this group of patients was already identified as the group with the higher risk of relapse after radiotherapy alone. Second, patients with mediastinal bulky disease are included in this category. These patients most often do not achieve an unambiguously complete remission on chemotherapy and thus are candidates for additional radiotherapy. Third, the improvement in both freedom from treatment failure but also in overall survival for this group of patients, is impressive thanks to the wide spread use of combined modality treatment. Nevertheless, for the early unfavorable stages the need for reduction of toxicity in large subgroups may be beneficial and improvement of tumor control in a small subset is definitely needed. In the EORTC/GELA H8 trial using MOPP/ABV hybrid as standard chemotherapy, patients treated with four cycles + IF-RT had similar event-free and overall survival as those with six cycles + IF-RT, both survival rates exceeding 90% after 4 years. The early results of the EORTC/GELA H9 trial show that the same holds true for four cycles of ABVD as compared to six cycles of ABVD, both followed by IF-RT. These observations are in line with the results of the GHSG HD8 trial in which IF-RT proved to be as sufficient as EF-RT when combined with adequate chemotherapy. These data indicate that even in the prognostically unfavorable stages I and II, treatment burden can be substantially reduced: a combination of four cycles of adequate chemotherapy followed by IF-RT is at present considered standard treatment. Since the mediastinum is often involved and included in the radiation field, the reduction of dose and field of RT could have major implications on long-term cardiac complications. A dose of 30 Gy IF-RT with a boost of 6-10 Gy to residual abnormalities is still widely employed. Whether reduction of the dose of IF-RT to 20 Gy or even less is feasible has yet to be demonstrated. Early results from the GHSG HD11 trial suggest that a dose of 20 Gy IF-RT can be sufficient for early unfavorable stages after adequate chemotherapy. Evidently, when substantiated after more prolonged follow-up, one may reasonably expect that the long-term toxicity will decrease significantly with reduced radiation fields and size. In this respect new developments in radiation through three-dimensional conformal radiotherapy and applying involved-node radiotherapy instead of the involved-field principle, will further refine the target size thereby reducing the risk of untoward events.

The 10-15% of patients who fail to benefit from the current standard treatment, deserve special attention. In their current study, the GHSG is randomizing patients with intermediate stages (roughly unfavorable stages I/II) between increasing the intensity of chemotherapy using two cycles of escalated bleomycin, etoposide, adriamycin, cyclophosphamide, vincristine, procarbazine, prednisone (BEACOPP) + two cycles of ABVD and the standard of four cycles of ABVD, both treatment arms followed by IF-RT of 30 Gy. In the ongoing North American Intergroup trial, six cycles of ABVD are being compared with Stanford V during 12 weeks followed by
radiotherapy to involved bulky sites. It will be a major challenge to conclude from these trials which subset (if any) of patients benefit from the intensified treatment and which subset is being overtreated. The EORTC/GELA H10 trial addresses these issues. An early FDG-PET scan response after two cycles of ABVD is used to adapt treatment. The standard treatment is four cycles of ABVD + IF-RT 30 Gy (+ boost of 6 Gy to residual abnormalities). In the experimental arm those with a negative FDG-PET scan after two cycles of ABVD (the presumed good-risk subgroup), will receive another four cycles of ABVD (a total of six cycles) but no further radiotherapy; those with a positive FDG-PET scan after two cycles of ABVD (the presumed poor-risk subgroup) will receive intensification of chemotherapy with two cycles of escalated BEACOPP followed by IF-RT. Although it is certainly worthwhile to try to reduce toxicity by reducing or even omitting radiotherapy for patients with stages I/II disease, we should keep in mind that the serious long-term events are encountered after the rather old-fashioned wide field radiotherapy.\(^3\) Comparable data after IF-RT at lower doses are simply lacking or just beginning to emerge. Further, more chemotherapy instead of radiotherapy has its own toxicity. Among others, such as cardiotoxicity, infertility, neuropathy, and secondary leukemias, the pulmonary toxicity of bleomycin is likely to be observed more often when increasing numbers of cycles are administered.

**References**

New approaches for patients with advanced-stage and relapsed disease

Current strategies for patients in advanced stages of Hodgkin’s lymphoma (HL) are not only aimed at further improving treatment outcome. Simultaneously, they are intended to minimize or prevent therapy-induced complications, such as infertility, cardiopulmonary toxicity, and secondary malignancies. New drug combinations with higher dose density and intensity have been developed recently and are currently being evaluated in randomized trials. Furthermore, ongoing studies are examining the use of positron-emission-tomography (PET) with 6-fluorodeoxyglucose as a new diagnostic tool to detect early response during chemotherapy or a satisfactory response after chemotherapy, eventually rendering consolidating radiation unnecessary.

Approaches for patients with relapsed HL consist of radiotherapy, chemotherapy and high-dose chemotherapy followed by autologous stem cell transplantation (SCT). In recent years, the introduction of effective salvage high-dose therapy and a better understanding of prognostic factors have remarkably improved the management of relapsed HL. Allogeneic transplantation may become an appropriate strategy in selected subgroups of young poor-risk patients who relapse after autologous bone marrow transplantation. For multiply pretreated patients, radioimmunoconjugates, monoclonal antibodies or small molecules targeting signal transduction pathways have demonstrated some clinical efficacy; these approaches, however, are still experimental.

Advanced stages of HL

Choice of patients and prognostic factors

Most centers and study groups assign patients with stage III and IV disease to the advanced-stage risk group. Some IIB patients with certain risk factors are additionally included in this category. Besides stage and B-symptoms, most groups have used larger tumor burden as a relevant prognostic factor including bulky disease >10 cm or a large mediastinal mass >1/3 of thoracic diameter. The differences in the allocation of patients to the advanced stage risk group and in the definition of risk factors among the different study groups in Europe and the USA are shown in Table 1. In the USA, patients are usually allocated to early or advanced stages. This results in more patients even with small tumor burden being included in the advanced stage group. These patients then receive more therapy than in other groups, which must be considered when comparing the data.

For the clinician, the most important use of prognostic factors is to select appropriate treatment strategies. In an attempt to define the risk of patients with advanced HL, a variety of clinical and laboratory parameters were analyzed to construct a prognostic index. The International Prognostic Score (IPS) consists of seven factors that were significantly related to an unfavorable prognosis when present at initial diagnosis of HL: serum albumin < 4 g/dL, hemoglobin < 10.5 g/dL, male sex, age > 45 years, stage IV disease, leukocytosis > 15,000/mm³, lymphocytopenia < 600/mm³ and/or < 8% of white cells.

Treatment strategies and current trials

Before the introduction of combination chemotherapy, more than 95% of patients with advanced HL succumbed to their disease within 5 years. Thus, the remission rates in excess of 50% achieved with MOPP were a major breakthrough in oncology. MOPP was successfully used for many years for advanced-stage disease, resulting in long-term remission rates of nearly 50%. The regimen was then replaced by ABVD, after a series of large multicenter trials had proven the superiority of ABVD and alternating MOPP/ABVD over MOPP alone. Hybrid regimens such as MOPP/ABV were only equally effective when compared with alternating MOPP/ABVD and even rapidly
Table 1. Definition of the advanced stage treatment group according to the EORTC/GELA, GHSG, and NCIC/ECOG.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>EORTC/GELA</th>
<th>GHSG</th>
<th>NCIC/ECOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced stage</td>
<td>CS III-IV</td>
<td>CS IIb with A/B; CS III-IV</td>
<td>High risk group: CS I or II with bulky disease; intraabdominal disease; CS IIuv</td>
</tr>
<tr>
<td>Risk factors</td>
<td>A large mediastinal mass</td>
<td>A large mediastinal mass</td>
<td>A ≥40 years</td>
</tr>
<tr>
<td></td>
<td>B age ≥50 years</td>
<td>B extranodal disease</td>
<td>B not NLPHL or NS histology</td>
</tr>
<tr>
<td></td>
<td>C elevated ESR*</td>
<td>C elevated ESR*</td>
<td>C ESR ≥50 mm/h</td>
</tr>
<tr>
<td></td>
<td>D ≥4 involved regions</td>
<td>D ≥3 involved areas</td>
<td>D ≥4 involved nodal regions</td>
</tr>
</tbody>
</table>

Abbreviations: GHSG, German Hodgkin Lymphoma Study Group; EORTC, European Organisation for Research and Treatment of Cancer; GELA, Groupe d’Etude des Lymphomes de l’Adulte; ECOG, Eastern Cooperative Oncology Group; NCIC, National Cancer Institute of Canada; NLPHL, nodular lymphocyte predominant Hodgkin’s Lymphoma; NS, nodular sclerosing subtype; * erythrocyte sedimentation rate (≥ 50 mm/h without or ≥30 mm/h with B-symptoms).

Alternating multidrug regimens such as COPP/ABV/IMEP did not result in better outcome. However, more acute toxicity and a higher incidence of leukemia were reported after MOPP/ABV hybrid than after ABVD.

Currently, in many countries, six to eight cycles of ABVD are still regarded as the standard regimen for advanced stages of HL. However, a long-term follow-up report of 123 patients who were previously treated with ABVD for advanced HL revealed a failure-free survival of only 47% and an overall survival of 59% after 14.1 years. Different study groups tried to improve these rates by developing new regimens with additional drugs and by increasing dose intensity and dose density with the support of colony-stimulating factors and modern antibiotics. New approaches, including multidrug regimens such as Stanford V, MOPP/VBVCAD, VAPEC-B, CHIVPP/EVA and BEACOPP and the corresponding trial results are listed in Table 2.6,7

Stanford V seemed to be a promising strategy when used at a single center.6 However, a prospectively randomized multicenter comparison with MOPP/VBVCAD and ABVD showed that Stanford V was clearly inferior.6 These conflicting results might be partially explained by the use of less radiotherapy in the randomized setting and the better treatment quality of single-center reports. The HD9 trial of the German Hodgkin Study Group (GHSG) compared COPP/ABVD, BEACOPP baseline and BEACOPP-escalated. Results from 1195 randomized patients showed a clear superiority of escalated BEACOPP over BEACOPP-baseline and COPP/ABVD at 5 years. The follow-up data at 7 years confirm these results: with a median follow-up of 82 months, the freedom from treatment failure (FFTF) and overall survival (OS) rates were 67% and 79% in the COPP/ABVD group, 75% and 84% in the BEACOPP-baseline group, and 85% and 90% in the BEACOPP-escalated group.6 The subsequent GHSG HD12 trial aimed at de-escalating chemotherapy and radiotherapy by comparing eight courses of BEACOPP-escalated with four courses of escalated and four courses of baseline BEACOPP, with or without consolidating radiation to initial bulky and residual disease. In the latest interim analysis of HD12 at a median follow-up of 30 months, the freedom from treatment failure was 88% and overall survival 94% for the whole cohort. At that point, there was no significant difference between the different arms.7 In the ongoing HD15 trial, patients are randomized between eight courses of BEACOPP-escalated, six courses of BEACOPP-escalated, or eight courses of BEACOPP-14, which is a time-intensified variant of BEACOPP-baseline. Additional radiotherapy is only applied to residual lesions ≥2.5 cm positive by positron emission tomography (PET). The question whether escalated BEACOPP is superior to ABVD alone in a randomized setting is currently being evaluated in an intergroup trial initiated by the EORTC (#20012). Here, eight cycles of ABVD are being compared with four cycles of BEACOPP-escalated plus four cycles of BEACOPP-baseline.

Strategies and study results in young patients with advanced stages of HL do not automatically apply to elderly patients. Patients older than 60 years often show a poorer risk profile, more treatment-associated toxicity, a lower ability to maintain dose-intensity and higher mortality resulting in a poorer outcome.8 In the HD9-elderly trial of the GHSG, patients between 66 and 75 years with advanced stage HL were treated with either COPP/ABVD or BEACOPP baseline. Tumor control appeared to be better with the BEACOPP regimen, but toxicity was higher, resulting in no differences in terms of freedom from treatment failure or overall survival.9 Thus, in phase I/II trials of the GHSG two new regimens are currently being evaluated for elderly patients: PVAG (prednisone, vinblastine, doxorubicin and gemcitabine) and BACOPP (bleomycin, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisone). Other topics of current interest, especially in patients...
with advanced HL undergoing intensive chemotherapy, include the maintenance of fertility after chemotherapy and the prevention of long-term toxicities and secondary malignancies.

### Role of radiotherapy and PET scans

The role of consolidating radiotherapy after effective chemotherapy in the treatment of patients with advanced HL is still subject to clinical research. Meta-analyses comparing combined modality approaches and chemotherapy alone reported equal tumor control and even better overall survival in patients treated with chemotherapy alone. Therefore, randomized trials currently evaluate the impact of radiotherapy after effective chemotherapy for advanced HL. A study conducted by the EORTC indicated that consolidating involved field radiotherapy (IF-RT) did not result in better outcome in patients who had already achieved a complete remission after six to eight cycles of MOPP/ABV, although it may be beneficial to patients with partial remissions.\(^{10}\) Longer follow-up of the recently terminated GHSG HD12 trial and the ongoing HD15 trial may help to define the role of radiotherapy for residual disease. In the HD15 trial, PET scans are utilized and investigated as a tool to analyze tumor activity.

### Table 2. Selected trials for advanced Hodgkin's lymphoma.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Therapy regimen</th>
<th># Pts.</th>
<th>Outcome Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stanford</td>
<td>Stanford V (12 weeks) (+ RT to initial mediastinal bulk+hilar+supracl. nodes)</td>
<td>108</td>
<td>95% (OS) 4</td>
</tr>
<tr>
<td>Intergroup</td>
<td>A. ABVD (6 cycles)</td>
<td>98</td>
<td>83% (FFS); 86% (FFP); 90% (OS) 5</td>
</tr>
<tr>
<td>Italy</td>
<td>B. Stanford V (12 weeks)</td>
<td>89</td>
<td>67% (FFS); 76% (FFP); 83% (OS) &amp;</td>
</tr>
<tr>
<td></td>
<td>C. MOPPEBVCAD (six courses) (+ RT initial bulk/ residual mass)</td>
<td>88</td>
<td>85% (FFS); 93% (FFP); 90% (OS) &amp;</td>
</tr>
<tr>
<td>Intergroup</td>
<td>A. ChlVPP/EVA hybrid (6 cycles) (+/- RT initial bulk/ residual mass)</td>
<td>144</td>
<td>82% (FFP); 78% (EFS); 89% (OS) 6507a</td>
</tr>
<tr>
<td>GB &amp; Italy</td>
<td>B. VAPEC-B (11 weeks)</td>
<td>138</td>
<td>62% (FFP); 58% (EFS); 79% (OS)</td>
</tr>
<tr>
<td>GHSG</td>
<td>A. COPP/ABVD (4 cycles)</td>
<td>260</td>
<td>67% (FFTF); 79% (OS) 6</td>
</tr>
<tr>
<td>HD9</td>
<td>B. BEACOPP baseline (8 cycles)</td>
<td>469</td>
<td>75% (FFTF); 84% (OS) 7</td>
</tr>
<tr>
<td></td>
<td>C. BEACOPP escalated (8 cycles)</td>
<td>466</td>
<td>84% (FFTF); 90% (OS)</td>
</tr>
<tr>
<td>GHSG</td>
<td>A. 8 BEA esc. (A.+C.: +RT initial bulk/residual mass)</td>
<td>348</td>
<td>4° interim analysis [2 years] 7</td>
</tr>
<tr>
<td>HD12</td>
<td>B. 8 BEA esc.</td>
<td>345</td>
<td>all pts:</td>
</tr>
<tr>
<td></td>
<td>C. 4 BEA esc. + 4 BEA baseline (A.+C.: +RT initial bulk/residual mass)</td>
<td>351</td>
<td>88% (FFTF); 94% (OS)</td>
</tr>
<tr>
<td>GHSG</td>
<td>A. 8 BEA esc.</td>
<td>352</td>
<td></td>
</tr>
<tr>
<td>HD15</td>
<td>B. 6 BEA esc.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. 8 BEA-14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intergroup</td>
<td>8 x ABVD (+RT to PET+ residual mass &gt; 2.5 cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#20012</td>
<td>4 BEA esc. +4 BEA baseline</td>
<td></td>
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</table>

**Abbreviations:** SWOG, Southwest Oncology Group; EORTC, European Organization for Research and Treatment of Cancer; GELA, Groupe d’Etude des Lymphomes de l’Adulte; GHSG, German Hodgkin Lymphoma Study Group; ECOG, Eastern Cooperative Oncology Group; EF/IF-RT, extended/involved-field radiotherapy; STNI, subtotal nodal irradiation; FFS, failure free survival; FFP, freedom from progression FTTF, freedom from treatment failure; RFS, relapse-free survival; EFS, event-free survival; OS, overall survival; ABVD, Adriamycin, Bleomycin, Vinblatistin, Dacarbazine; Stanford V, Mechlorethamine, Adriamycin, Vincristine, Bleomycin, Etoposide, Prednisone; MOPPEBVCAD, Mechlorethamine, CCNU (Lomustine), Vindesine, Alkeran, Prednisone, Epidoxorubicin, Vincristine, Procarbazine, Vinblastine, Bleomycin; VAPEC-B, Vincristine, Adriamycin, Prednisolone, Etoposide, Cyclophosphamide, Bleomycin; CHIVPP/EVA, Chenammbucil, Vinblastine, Procarbazine, Prednisolone, Etoposide, Vincristine, Adriamycin; COPP, Cyclophosphamide, Vincristine, Procarbazine, Prednisone; BEACOPP, Bleomycin, Etoposide, Adriamycin, Cyclophosphamide, Vincristine, Procarbazine, Prednisone.
in residual masses after chemotherapy. In addition, there are some data suggesting that early PET scans during chemotherapy may discriminate between responders and non-responders and thus have a potential role for use in response-adapted strategies.11,12

**Relapsed HL**

The majority of patients achieve complete remission with current first-line treatment. Patients who relapse still have a chance of being cured with adequate salvage treatment. Depending on first-line therapy, there are various treatment options. Conventional chemotherapy is usually the treatment of choice for patients who relapse after initial radiotherapy only. In contrast, options for those who relapse after prior chemotherapy include salvage radiotherapy for strictly localized relapse in previously non-irradiated areas, salvage chemotherapy, or high-dose chemotherapy (HDCT) followed by autologous stem cell transplantation (SCT). Other experimental options, such as allogeneic SCT and monoclonal antibodies are being evaluated for multiply pretreated patients. Depending on the duration of remission after first-line treatment, most study groups categorize failures into three subgroups: early and late relapses of HL and primary progressive HL. General strategies and trial results for relapsed and refractory HL have been reported by our group.13

**Salvage radiotherapy and salvage polychemotherapy**

Salvage radiotherapy alone offers an effective treatment option for patients with localized relapses in previously non-irradiated areas. In a recent retrospective analysis from the GHSG database including 624 relapsed or refractory HL patients, 100 patients were eligible to receive salvage radiotherapy alone: the 5 year freedom from second failure and overall survival rates were 28% and 51%, respectively. Prognostic factors for overall survival were B-symptoms, stage at relapse, performance status and duration of first remission in limited stage relapses.14

Conventional chemotherapy is the treatment of choice for patients who relapse after initial radiotherapy for early-stage disease. The prognosis of these patients has been shown to be equal to that of patients with advanced stage HL initially treated with chemotherapy. The best treatment for recurrent HL after primary chemotherapy is high-dose chemotherapy. Overall response rates with conventional salvage therapy in this setting ranged between 60 and 80%, but less than 30% of patients achieve a lasting remission. Long term follow-up data from conventional salvage protocols are scarce since most of the patients not achieving complete or partial remission immediately proceeded to HDCT plus autologous SCT. Patients relapsing after more than two cycles of chemotherapy should be treated with HDCT at relapse. The best treatment for patients who relapsed after two cycles of chemotherapy and radiotherapy remains to be defined. A recent analysis from the GHSG database including patients relapsing after two cycles of ABVD plus involved field radiotherapy showed best but equal results for application of either eight cycles of BEACOPP escalated or HDCT plus autologous SCT at relapse.15

**HDCT followed by autologous SCT**

Younger patients relapsing after initial chemotherapy are usually treated with HDCT and peripheral blood SCT, a strategy that has shown to produce 30-65% long-term disease-free survival. Thus far, two randomized trials demonstrated the superiority of HDCT followed by autologous SCT over conventional chemotherapy. The British National Lymphoma Investigation (BNLI) reported that patients with relapsed or refractory HL receiving high-dose BEAM with autologous SCT fared significantly better than those treated with conventional dose mini-BEAM. The 3-year event-free survival was 53% for the HDCT group and 10% for those treated with conventional chemotherapy. In the HD-R1-trial of the GHSG, chemosensitive patients who relapsed after initial chemotherapy were randomized between four cycles of Dexam-BOEAM and two cycles of Dexam-BOEAM followed by BEAM and autologous SCT. The final results at 5 years demonstrated a higher freedom from second failure in the transplanted group than in the group receiving conventional salvage-chemotherapy (55% vs. 34%). The follow-up data at 7 years confirm these results.17

The HDR2 pilot study conducted by the GHSG evaluated the feasibility and efficacy of sequential HDCT in 102 patients with relapsed or refractory HL. Treatment consisted of two cycles of DHAP (dexamethasone, ara-C, cisplatin) followed by a sequential high-dose chemotherapy with cyclophosphamide, methotrexate, and etoposide. The final myeloablative course was BEAM followed by peripheral blood SCT. With a median follow-up of 30 months, freedom from second failure and overall survival rates were 59% and 78%, respectively. In multivariate analysis response after DHAP and duration of first remission were prognostic factors for both freedom from second failure and overall survival.18 Based on the promising results of this study, the GHSG started a prospective European intergroup trial (HD-R2) that is still ongoing. The rationale is to compare the effectiveness of two courses of DHAP followed by BEAM with the intensified sequential strategy in a randomized setting.
Primary progressive and refractory HL

For patients with primary progressive disease during induction treatment or relapse within 3 months after the end of first-line therapy, conventional salvage chemotherapy has given disappointing results in the vast majority of patients. Reasons for not proceeding to HDCT include insufficient stem cell harvest, poor performance status and older age. The effectiveness of HDCT and autologous SCT for patients with primary refractory HL was shown in a study of 75 consecutive patients who were treated with HDCT and autologous SCT. At a median follow-up of 10 years for surviving patients, the event-free survival, progression-free survival, and overall survival rates were 45%, 49% and 48%, respectively. Chemosensitivity to standard-dose second-line chemotherapy was predictive of a better survival.19

Allogeneic stem cell transplantation

Allogeneic SCT cannot yet be considered an alternative standard treatment in patients with relapsed HL. So far, the advantages of a potential graft-versus-lymphoma effect were offset by a substantial transplant-related mortality of more than 50%. As shown by a matched-pair analysis, transplant-related mortality might be significantly reduced by employing reduced-intensity conditioning.20 Furthermore, a recent study using reduced intensity conditioning in 49 HL patients indicates the potential for durable responses in patients who have previously had substantial treatment for HL. The low non-relapse-related mortality suggests that allogeneic transplants should be considered earlier in the course of disease.21 Allogeneic SCT following reduced intensity conditioning might thus become an appropriate strategy in selected subgroups of young poor-risk patients, however, the number of patients treated is still small, requiring further clinical studies and information in order to define clear indications.

Experimental strategies

Experimental strategies in the treatment of HL include passive immunotherapy based on monoclonal antibodies to specifically target malignant cells and active immunotherapy with modulation of cellular response by cytokines, tumor vaccines or cells and active immunotherapy with modulation of clonal antibodies to specifically target malignant cells. Approaches involving antibody-based agents have given promising results in experimental HL models and have demonstrated some clinical efficacy in patients with advanced refractory HL. Clinical phase I/II trials with unmodified humanized or human monoclonal antibodies are ongoing.

These antibodies either induce target cell death by direct interaction or antibody-dependent cellular or complement-dependent cytotoxicity. Different approaches evaluated clinically include bispecific immunotoxins constructs, and radioimmunoconjugates. More recently, fully human antibodies against CD30 have given promising results.22

However, it seems unlikely that patients with resistant disease and with larger tumor masses can be cured by either of these approaches. Future strategies aim at combining conventional chemo- and/or radiotherapy for debulking, and experimental therapies with biological agents to kill residual Hodgkin and Reed-Sternberg cells and thus prevent relapses.

Acknowledgments

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References


Late toxicity in patients treated for Hodgkin's lymphoma

Treatment results for patients with Hodgkin's lymphoma have improved dramatically over the last decades. In general, between approximately 1960 and 1990 treatment was aimed at improving survival rates through intensification of treatment, as before this period most patients died from this disease. In the 1980s and 1990s, with higher survival rates for the majority of the patients, the long-term toxicity of treatment became recognized.

There is a large spectrum of late effects of treatment for Hodgkin's lymphoma including decreased fertility, hormonal disturbances, pulmonary toxicity, soft tissue damage, second cancers and cardiovascular diseases leading to considerable morbidity and mortality. Mortality data showed that in the first 10 years after treatment for Hodgkin's lymphoma 15-30% of patients died with Hodgkin's lymphoma being the cause of death in 70-85%. Mortality from Hodgkin's lymphoma is, however, negligible after a follow-up of 20 years or more, whereas risk of death from second cancers and cardiovascular disease continues to increase even after a follow up of 10-15 years.1 Risks of mortality from second cancers and cardiovascular diseases in large cohorts of patients treated for Hodgkin's lymphoma are summarized in Tables 1 and 2.

Second cancers

After prolonged follow-up, 20- to 70-fold increased standardized incidence ratios of leukemia and two- to five-fold increased risks of solid cancers, especially of the breast, lung, stomach and bladder, have been observed, leading to absolute excess risks of approximately 17 per 10,000 patients per year for leukemia and 29 per 10,000 patients per year for solid tumors.2 Table 1 shows risk of death from second cancers in large cohorts of patients treated for Hodgkin's lymphoma. The highest risks of leukemia are observed between five to ten years after treatment, whereas the increased risks of solid tumors remain elevated even after prolonged follow-up.1,3,12,13 It is important to realize that even if standardized incidence ratios remain constant over time, this leads to increasing absolute excess risks, because of higher background cancer incidence rates with increasing age.

An elevated risk of acute non-lymphocytic leukemia is consistently reported in patients treated with alkylating agents. No statistically significant effect of radiation for Hodgkin's lymphoma on risk of leukemia has observed. The increased risk of second solid tumors, however, is clearly related to radiation. It has been consistently been observed that the risk of second solid tumors is strongly increased with younger age at treatment. Hypothetically, these young patients might be at greater risk of side effects because immature tissues and organs are more vulnerable to the effects of ionizing radiation, or because these individuals might have genetic alterations also influencing their susceptibility to develop malignancies at an early age.

Smoking significantly increases the risk of lung cancer attributable to radiotherapy. A radiation dose-response relation has been shown for second lung cancer and breast cancer in women treated at a young age for Hodgkin's lymphoma. Whether there is an effect of irradiated volume in second breast cancer remains to be determined.

Chemotherapy, however, may also play a role in the increased risk of second malignancies. Statistically significantly elevated risks of lung cancer were reported after treatment with alkylating agents for Hodgkin's lymphoma (relative risk [RR] = 4.2; 95% confidence interval [CI] = 2.1 to 8.8), whereas a decrease has been observed in breast cancer risk especially when the chemotherapy caused premature menopause. In the latter study patients who received both chemotherapy and radiotherapy had a statistically signif-
significantly lower risk than those treated with radiotherapy alone (RR = 0.45, 95% CI = 0.22 to 0.91).18

Cardiovascular toxicity
Cardiovascular toxicity can arise after both radiation and chemotherapy. Radiation-induced heart disease includes a wide spectrum of cardiac pathologies, such as coronary artery disease, valvular heart disease, myocardial dysfunction, pericardial disease, and electrical conduction abnormalities. Damage of the vascular endothelium, leading to accelerated atherosclerosis, and, in the long term, to an increased risk of vascular stenosis and thromboembolism of the arteries of the heart muscle is probably important in explaining of radiation-induced heart disease. Radiation-induced damage to the myocardium might also be caused by damage to the microvasculature of the myocardium, leading to fibrosis. The fibrosis of the heart valves cannot be explained in a similar way because the valves do not have blood vessels. The mechanism of radiation damage to the valves is not clear; one could hypothesize that in some cases the vascular problems are secondary to other damage to the heart. Conduction abnormalities and arrhythmias are also frequently observed.20 Whether elevated heart rates are related to autonomic dysfunction or compensate for decreased cardiac output is unclear.

Cardiotoxicity is clearly related to cumulative anthracycline dose21 and radiation dose to the heart.22 Whereas cardiotoxicity following radiotherapy is usually observed from 5-10 years of follow-up, anthracycline-related toxicity may be observed at different intervals after therapy. Anthracycline-associated cardiotoxicity is caused by direct damage to the myoepithelium. The occurrence of anthracycline-associated cardiotoxicity is strongly related to the cumulative dose.21,23 Doses below 500 mg/m2 are usually well tolerated. The total dose of anthracyclines during first line therapy for Hodgkin’s lymphoma is relatively low compared to that in treatment regimens for breast cancer and pediatric malignancies; the cumulative dose of eight cycles of MOPP-ABV or eight cycles of BEACOPP-escalated is 280 mg/m2 and that of eight cycles of ABVD 400 mg/m2. In addition the majority of the patients will be treated with less than eight cycles of anthracycline-containing chemotherapy.

Effects of anthracycline-containing chemotherapy

Table 1. Risk of death from second cancers in large cohorts of patients treated for Hodgkin’s lymphoma (adults or no age limit).

<table>
<thead>
<tr>
<th>Authors</th>
<th>Number in cohort</th>
<th>Years of treatment</th>
<th>Age range at treatments in years (median)</th>
<th>Follow-up time in years</th>
<th>Type of treatment</th>
<th>Mortality end-point</th>
<th>SIR* (95% CI)</th>
<th>AER per 10,000 person years *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Henry-Amar M. (65) (1190)</td>
<td>1449 (826 males)</td>
<td>1963-86</td>
<td>31.2 mean for all</td>
<td>-</td>
<td>Mantle or TNI or STNI±chemotherapy</td>
<td>Second cancers</td>
<td>4.2</td>
<td>2.9-5.9</td>
</tr>
<tr>
<td>Ng A. (5)(2002)</td>
<td>1080</td>
<td>1969-97</td>
<td>3-50 (25)</td>
<td>Median 12.0</td>
<td>97% RT±CT</td>
<td>Second tumors</td>
<td>11.2</td>
<td>8.6-14.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.2-20 (17.0)</td>
<td>Median 19.5 (0.1-35.6)</td>
<td>Leukemia (not including MDS)</td>
<td>37.7</td>
<td>10.3-96.6</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>97% RT±CT</td>
<td></td>
<td></td>
<td></td>
<td>6.7</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1.2-40 (26.0)</td>
<td>Median 17.8 (0.1-35.6)</td>
<td>Solid tumors</td>
<td></td>
<td>6.6</td>
<td>5.2-8.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.2-40 (26.0)</td>
<td>Median 17.8 (0.1-35.6)</td>
<td>Leukemia (not including MDS)</td>
<td>28.9</td>
<td>16.2-47.6</td>
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</table>

*SIR* Standardized Incidence Ratio as the ratio of the observed (O) and expected (E) numbers of cancers in the cohort. The expected numbers are calculated based on general population rates. *AER* Absolute Excess Risk as O minus E, divided by number of person-years at risk, times 10,000. *60% of patients estimated to be ≤40 years.

*Standardized Incidence Ratio (SIR) as the ratio of the observed (O) and expected (E) numbers of cancers in the cohort. The expected numbers are calculated based on general population rates. *Absolute Excess Risk (AER) as O minus E, divided by number of person-years at risk, times 10,000. *60% of patients estimated to be ≤40 years.
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<tr>
<th>Authors</th>
<th>Number in cohort</th>
<th>Years of treatment</th>
<th>Age range at treatment (median)</th>
<th>Follow-up time in years</th>
<th>Type of treatment</th>
<th>Mortality end-point</th>
<th>SIR* (95% CI)</th>
<th>AER per 10,000 person years</th>
</tr>
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<tr>
<td>Adult or no age limits</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Henry-Amar M (69) (1190)</td>
<td>1449 (826 males)</td>
<td>1963-86</td>
<td>31.2 median for all</td>
<td>-</td>
<td>Mantle or TNI or STNI±chemotherapy</td>
<td>Myocardial infarction</td>
<td>8.8 for males (5.1-14.1)</td>
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<tr>
<td>Boivin M (68)(1992)</td>
<td>4665</td>
<td>1940-85</td>
<td>All ages</td>
<td>Average 7 (-)</td>
<td>Mediastinal irradiation±chemotherapy</td>
<td>Coronary artery disease</td>
<td>3.2 (1.4-8.0)</td>
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<tr>
<td>Boivin M (68)(1992)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mediastinal irradiation±chemotherapy</td>
<td>Myocardial infarction</td>
<td>4.1 (1.5-10.9)</td>
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<tr>
<td>Hancook S (22)(1993) and Hoppe R (66)(1997)</td>
<td>2232</td>
<td>1960-91</td>
<td>1.82 (average 29)</td>
<td>Average 9.5 (-)</td>
<td>89% with mediastinal irradiation</td>
<td>Heart disease</td>
<td>3.1 (2.4-3.7)</td>
<td>28.0</td>
</tr>
<tr>
<td>King V (69)(1996)</td>
<td>326</td>
<td>1954-89</td>
<td>5.72 (mean 25.6)</td>
<td>Mean 13.3 (3.37)</td>
<td>Mantle irradiation±chemotherapy</td>
<td>Myocardial infarction</td>
<td>2.8 (0.7-4.9)</td>
<td>10.4</td>
</tr>
<tr>
<td>Glanzmann C (70) (1998)</td>
<td>352</td>
<td>1964-92</td>
<td>4.0-81 (mean 33.8)</td>
<td>11.2 (1.0-31.5)</td>
<td>Mediastinal irradiation±chemotherapy</td>
<td>Myocardial infarction</td>
<td>4.2 (1.8-8.3)</td>
<td></td>
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<tr>
<td>Brierley JD (71)(1998)</td>
<td>611</td>
<td>1973-84</td>
<td>17-90 (31)</td>
<td>Median 11.0 (0.7-18.0)</td>
<td>97% RT±CT</td>
<td>Myocardial infarction</td>
<td>1.5 (0.7-3.0)</td>
<td>5.4</td>
</tr>
<tr>
<td>Aviles A (67)(2000)</td>
<td>2980</td>
<td>1970-95</td>
<td>&gt;18+</td>
<td>Median 14.6 (5.0-24.0)</td>
<td>63% RT±CT</td>
<td>Cardiac disease</td>
<td>29.8 (15.6-46.8)</td>
<td>16.8</td>
</tr>
<tr>
<td>Ng A (6)(2002)</td>
<td>1080</td>
<td>1969-97</td>
<td>3-50(25)</td>
<td>Median 12.0</td>
<td>97% RT±CT mainly extended field in cases of RT only</td>
<td>Cardiac disease</td>
<td>3.2 (1.9-5.2)</td>
<td>9.0</td>
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<tr>
<td>Young ages</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hancook S (72)(1993)</td>
<td>635</td>
<td>1961-91</td>
<td>&lt;21 (mean 15.4)</td>
<td>Mean 10.3</td>
<td>89% with mediastinal irradiation mainly ≥40 Gy</td>
<td>Myocardial infarction</td>
<td>41.5 (18.1-82.1)</td>
<td>10.4</td>
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<tr>
<td>Hudson MM (73)(1998)</td>
<td>387</td>
<td>1968-90</td>
<td>3-25(14.4)</td>
<td>Median 15.1 (2.9-28.6)</td>
<td>RT (mainly extended field)±chemotherapy</td>
<td>Cardiac disease</td>
<td>22.2 (8.1-48.4)</td>
<td>10.2</td>
</tr>
<tr>
<td>Aleman BMP (14)</td>
<td>1261</td>
<td>1965-87</td>
<td>1.2-20 (17.0) 1.2-20 (17.0)</td>
<td>Median 19.5 (0.1-35.6) Median 19.5 (0.1-35.6)</td>
<td>Cardiovascular disease</td>
<td>13.6 (5.0-29.6)</td>
<td>9.5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cardiac disease</td>
<td>5.1 (0.1-28.4)</td>
<td>1.4</td>
<td></td>
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*SStandardized Incidence Ratio (SIR) as the ratio of the observed (O) and expected (E) numbers of cancers in the cohort. The expected numbers are calculated based on general population rates. °Absolute Excess Risk (AER) as O minus E, divided by number of person-years at risk, times 10,000. ±60% of patients estimated to be ≤40 years. § Calculated from the data in the paper: (Observed(7) - Expected (2.5) Person-years at risk (4335))×1000.
and radiation were evaluated in a study on cardiovascular disease in 476 patients with aggressive non-Hodgkin lymphoma (NHL) treated with doxorubicin-based chemotherapy in four EORTC trials (1980-1999). After a median follow-up of 8.4 years a significantly elevated risk of chronic heart failure was reported. Pre-existent hypertension, non-Hodgkin’s lymphoma at young age, radiation and salvage treatment increased risk for all cardiovascular events; the impact of radiotherapy was dose-dependent. The risk of coronary artery disease was not elevated, but the follow-up period was still relatively short.

The risk of cardiovascular diseases might also be increased through indirect effects of radiotherapy; for example, irradiation of the left kidney during para-aortic and spleen radiotherapy might lead to hypertension.

General risk factors for cardiovascular diseases, such as hypertension, diabetes, hypercholesterolemia, being overweight and smoking probably also contribute to the risk of cardiovascular diseases in patients treated for Hodgkin’s lymphoma. Whether the cardiovascular risk factor profile in patients treated for Hodgkin’s lymphoma differs from that of the general population is unknown.

Significantly increased risks of stroke have also been described in patients treated for Hodgkin’s lymphoma with relatively low doses of radiotherapy to the neck and with higher doses for solid tumors.

**Endocrine effects**

**Fertility**

Both chemotherapy and radiotherapy may lead to a temporary or permanent decrease in fertility in males and females and temporary loss of ovarian function or menopause in women, depending on the type of drugs, total dose of chemotherapy, radiation dose and on age at treatment.

**Men**

Testicles are more sensitive to radiation than ovaries. Testicular doses of less than 0.2 Gy had no significant effect on follicle-stimulating hormone (FSH) levels or sperm counts, whereas doses of 1.2 Gy and above are likely to be associated with a reduced chance of recovery of spermatogenesis.

The risk of infertility after chemotherapy strongly depends on the agents used and the cumulative dose of these agents. Procarbazine-containing regimens, such as MOPP, BEACOPP or cyclofosfamide, vincristine, procarbazine, prednisone (COPP) carry a much higher risk of infertility than do regimens without procarbazine, such as ABVD, which hardly ever lead to permanent sterility in men or women.

Semen cryopreservation allows subsequent artificial insemination of a female partner or *in vitro* fertilization. Semen cryopreservation may be difficult before the start of treatment because of decreased quality of the semen during illness and it is not an option for prepubertal boys. Important recent developments enabling male survivors of Hodgkin’s lymphoma to have children are intracytoplasmic sperm injection through which problems of low sperm numbers and poor motility can be circumvented.

**Women**

In women radiation toxicity is strongly dependent on age at exposure, total radiation dose and whether or not the radiation is fractionated. A dose below 1.5 Gy only rarely leads to sterility in women below the age of 40 years. Following a dose of 2.5-5.0 Gy 30-40% of the women treated between 15 and 40 years old are permanently sterilized, whereas more than 90% of the women over 40 years of age are permanently sterilized. Following a dose of more than 8 Gy all women will be sterile. The effective sterilizing dose (i.e. the dose of fractionated radiotherapy at which premature ovarian failure occurs immediately after treatment in 97.5% of patients) decreases with increasing age at treatment. The effective sterilizing dose at birth is 20.3 Gy; at 10 years it is 18.4 Gy, at 20 years 16.5 Gy, and at 30 years 14.3 Gy. Furthermore, exposure of the uterus to radiation is associated with an increased risk of miscarriage, mid-trimester pregnancy loss, preterm birth, and low birth weight.

Ovaries of prepubertal children and adolescents are less sensitive to chemotherapy-induced damage as compared to ovaries of adults. Nevertheless procarbazine and alkylating drugs used for the treatment of Hodgkin’s lymphoma may lead to acute ovarian failure and thus infertility or to depletion of ovarian reserve leading to premature menopause years after completion of chemotherapy following a period of normal functioning.

Strategies to preserve reproduction are more difficult in females. Cryopreservation of mature oocytes, immature oocytes or preimplantation embryos is usually not possible because of lack of time, lack of a male partner and doubts about the safety of the procedure. A very promising development is orthotopic transplantation of cryopreserved ovarian tissue. Recently embryo development and a live birth was reported after this procedure. Transposition of the ovaries should be considered in each case of planned irradiation exposing the ovaries in order to preserve hormonal function. Although some promising results have been published concerning preservation of ovarian function through temporary use of gonadotropin-releasing hormone agonistic analogs, the efficacy of this medication has not yet been
proven. Further investigation of the use of oral contraceptives during chemotherapy is also warranted, since the GHSG recently published data showing a statistically significant beneficial effect of the use of oral contraceptives on the return of menstrual cycle.\textsuperscript{44}

Symptoms of premature menopause may be treated with hormonal replacement therapy. In women treated with radiation including part of their breasts the risk of hormonal replacement therapy is uncertain, since hormonal replacement therapy might counter the protective effect of premature menopause on radiation-induced breast cancer.\textsuperscript{18}

\textbf{Offspring}

Although fertility may be seriously affected by treatment for Hodgkin’s lymphoma there are no indications so far of an increase in the prevalence of major congenital malformations in the offspring of patients treated for Hodgkin’s lymphoma\textsuperscript{6} nor in offspring of other cancer survivors.\textsuperscript{46}

\textbf{Thyroid function}

In the case of radiation to the lower neck, especially when both sides of the neck are irradiated, hyper- or hypothyroidism has been reported to occur in up to 50% of the population 10-15 years after radiotherapy.\textsuperscript{27-41} Replacement therapy with L-thyroxine is often necessary.

\textbf{Other effects}

Other effects that may be observed in long-term survivors are an increased risk of (lethal) infections especially from encapsulated bacteria after splenectomy,\textsuperscript{50-52} decreased pulmonary function because of radiotherapy\textsuperscript{15} and chemotherapy (especially bleomycin)\textsuperscript{16} and damage to bone and soft tissues such as muscles, salivary glands and bowels. Damage to bones may be caused by a direct radiation effect but also through induction of premature menopause in women. With regard to late radiation-related gastrointestinal toxicity, complication rates of up to 39% have been described in the case of previous laparotomies and high doses per fraction.\textsuperscript{6} Since staging laparotomy has been abandoned, fractionation schedules have changed and target volumes are generally smaller, the percentage of gastrointestinal toxicity has decreased.

\textbf{Quality of life}

Late treatment effects may significantly influence the quality of life of patients treated for Hodgkin’s lymphoma. Fatigue is a major complaint several years after treatment but the precise relationships with treatment remain to be determined.\textsuperscript{56,57}

\textbf{Clinical implications}

In general the information to the patient will depend on many patient-related factors such as age, gender, prognostic factors regarding Hodgkin’s lymphoma, co-morbidity, wish to preserve fertility, etc. Before the start of the treatment the emphasis of information concerning the first phase of the treatment is generally on control of lymphoma and acute side effects. Of course late effects must also be discussed at the beginning, especially when special measures have to be taken, such as cryopreservation in the case of a risk of infertility because of the treatment. When discussing risks of second cancers and cardiovascular toxicity, it is not easy to decide whether to use comparisons to the general population standardized incidence ratios and/or absolute excess risks. Apart from the fact that standardized incidence ratios and absolute excess risks may be difficult concepts to explain, it is also difficult to know what numbers to refer to. The interpretation of studies on late effects is always complicated by the fact that we only have the long-term results of treatments used a long time ago-treatments which have often been adjusted considerably. In addition, many authors performing studies on long-term effects have chosen, mainly for practical reasons, to study patients who have survived at least one to five years after their primary treatment. The results of these studies are valid for the majority of the patients since the 5-year overall survival nowadays is >80% for most Hodgkin’s lymphoma patients, but the data are usually not directly applicable to patients when they start their treatment and also not suitable for a risk-benefit analysis of various treatment effects. In the first period after treatment for Hodgkin’s lymphoma the main cause of death is Hodgkin’s lymphoma itself. With longer follow up, however, the long-term treatment-related morbidity and mortality become ever more important. During follow-up it is important that both the treating physicians and the patients are aware of possible long-term effects after treatment. To prevent serious morbidity and death from infections pneumococcal, \textit{Haemophilus influenzae} type b, meningococcal and influenza vaccinations are recommended after splenectomy. In addition, instructions on the use of antibiotics and the risks of traveling to areas where malaria is endemic should be given. Whether patients who might have a hypofunctional spleen due to spleen irradiation should be given the same advice as those who have had a splenectomy is difficult to assess because there are no good parameters for determining spleen function. In case of very intensive chemo- and radiotherapy patients are usually considered to have a hypofunctional spleen.

Furthermore, physicians should consider appropri-
late risk-reducing strategies such as treatment of hypertension and hypercholesterolemia and lifestyle advice to refrain from smoking, to maintain a healthy body weight, and to exercise regularly, particularly in young Hodgkin's lymphoma survivors treated with possibly cardiotoxic treatments. The role of prevention, using for instance anticoagulants, ACE inhibitors and statins remains to be determined.

Awareness of the elevated risk of second primary malignancies is also very important. In particular, women treated before the age of 30 have a strongly elevated risk of breast cancer. An actuarial risk of up to 29.0% (95% CI = 20.2% to 40.1%) has recently been described in patients treated for Hodgkin's lymphoma at young age, taking into account age and calendar year of Hodgkin's lymphoma diagnosis, Hodgkin's lymphoma treatment information, population breast cancer incidence rates, and competing causes of death. After the age of 40 years no or only slightly elevated risks of breast cancer have been observed after radiation exposure of breast. It is important to realize, however, that these risk estimates are derived from long-term follow-up of patients treated with extensive radiation, usually including a classical mantle field and with radiation doses up to 40 Gy. Nowadays the target volumes are much smaller and the radiation dose is generally lower.

Women at high risk should, in any case, be advised to undergo a yearly physical breast examination and have annual mammography from 8 years after the end of their radiotherapy, starting not earlier than the age of 25. Magnetic resonance imaging (MRI) of the breast is not used as a screening modality yet; how-ever, because of promising results of the use of MRI in women with a genetic or familial predisposition to breast cancer the possibilities of using this imaging technique as screening modality will be evaluated. Although there is also an elevated risk of several other cancers, screening for these is not yet recommended.

A common, more general problem after treatment for Hodgkin’s lymphoma is fatigue. The causes are probably related to both physical and emotional effects of the treatment. Treating fatigue is extremely difficult. Of course physical causes of fatigue (hypothyroidism, cardiovascular toxicity, etc.) must be treated optimally. In addition physical exercise appears to be beneficial.

Conclusions

In conclusion the cure rate of patients with Hodgkin's lymphomas nowadays 80% or more. The role of radiotherapy in the treatment of patients with Hodgkin’s lymphoma has changed tremendously from usually palliative care in the 1960s to mostly curative strategies in the 1980s, and from extensive radiation as a single treatment modality to limited or even no radiation in addition to chemotherapy. Effective chemotherapy combinations have been developed and possibilities to treat acute toxicity have improved significantly.

The risks of (late) toxicity of treatment must be balanced against the risk of failing to control the primary disease, since patients who have no response to initial therapy or who have an early relapse are not likely to be cured by salvage treatment, and Hodgkin's lymphoma itself is still the most important cause of death for such patients. In the future treatment will be tailored to individual patients based on prognostic factors, possibly including biological markers, and accurate evaluation of the response to treatment. Chemotherapy will be carefully chosen and radiotherapy will be applied to smaller target volumes and in a more sophisticated way requiring quality assurance even more than before. In addition, awareness of possible late effects after treatment is of the utmost importance for both patients and treating physicians.

References


The cell of origin of chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL), the most common leukemia of the western world, remains a somewhat mysterious disease. Despite the considerable progresses of cytogenetic analyses, which have led to the elucidation of the oncogenetic mechanisms of many lymphomas, unifying lesions explaining the molecular pathogenesis of CLL have still to be determined. Furthermore, the B cell subset responsible for the origin of CLL is yet matter of debate and the knowledge of the history of the cell prior to leukemic transformation is still incomplete.

Recent studies on Ig VH/VL genes utilized by the leukemic cells have provided relevant information. For example, it has been demonstrated that the Ig V region genes utilized by the neoplastic cells from 50% of cases have somatic mutations. In addition, the malignant cells exhibit a skewed usage of Ig V region genes compared to the normal B-cell repertoire. For example, leukemic cells preferentially use certain V region genes, often in combination with particular D and J segments. Moreover, these special VDJ gene segments are frequently found in association with particular VL-JL combinations. Because of these frequent associations, a substantial number of different patients share very similar or identical stereotyped receptors. The number of these patients varies in the different cohorts, but may be as high as 20% of the total CLL population and is generally higher among the CLL cases utilizing unmutated VH/VL genes. This information is useful to delineate part of the history of the malignant clone, particularly in the period preceding neoplastic transformation.

In this brief review, information on Ig V gene usage will be utilized in an attempt to trace the cell of origin of CLL.

CLL is generated by antigen-experienced B cells

The classic definition of CLL given in the past included that of an accumulation disease of immature, immuno-incompetent B cells. These concepts, based in part on clinical observations and in part on speculative grounds, were connected to the imprecise knowledge of both the immune response as well as of the biology of neoplasias. For example, confusion between the immunocompetence of the host and that of the neoplastic cells generated the notion that the leukemic cells are immuno-incompetent. Since CLL patients suffer from frequent infections because of immunodeficiency, it was hypothesized that the immuno-incompetence of CLL cells represented the starting point of this immunodeficiency. The discovery of the monoclonality of CLL helped overcome these concepts; since the leukemic cells are monoclonal, they are not obvious competent to respond to a variety of antigens irrespective of their stage of maturation and their previous history. However, their inordinate proliferation may cause lesions to the peripheral lymphoid organs and impair the capacity of normal cells to mount an immune response.

A number of studies also demonstrated that rather than immature cells, the CLL cells were cells whose past history included antigenic stimulation/selection. The concept that CLL are antigen-experienced cells is supported by several observations. The neoplastic cells from 50% or more of CLL utilize VH/VL genes that exhibit somatic mutations. Mutations of these genes occur only following antigenic stimulation. Moreover, the remaining CLL cases, which do not utilize mutated VH/VL genes, display a skewed V region gene repertoire and a preferential usage of certain stereotyped receptors. The gene expression profile of CLL cells obtained by technology is more similar to that of memory B cells than to that of virgin B cells. Finally, CLL cells express a typical memory cell marker, CD27.
Impairment of apoptosis is not the likely explanation for CLL cell accumulation

Defective apoptosis has long been believed to be a plausible cause for CLL cell accumulation. This concept was based mainly upon the observation that circulating CLL cells generally have a slow doubling time and a small proliferative compartment as assessed by immunocytochemical methods. These views changed following the observation on the leukemic cell turn-over carried out by labeling cell DNA in vivo with deuterium and by following its loss from the leukemic cells with time. This experimental approach demonstrated a relatively short half-life of the leukemic clone, spanning over intervals of months albeit with variations in the different cases. In the CLL with the most active turn-over, approximately 50% of the cells are renewed within three months with a daily rate of cell birth and death of about 2%. These values are reduced by half in the most slowly proliferating CLL cases. Proliferation of leukemic cells is observed more frequently in organized structures such as pseudofollicles, which are typical of CLL and are found in the bone-marrow and in peripheral lymphoid organs. Accessory cells present in these structures may prevent apoptosis of leukemic cells by direct cell-to-cell contact or by cytokine release. This is also supported by the finding that leukemic cells in the pseudofollicles exhibit upregulation of a number of anti-apoptotic molecules. Taking all the above into consideration, it appears that CLL expansion is related to active cell proliferation, which also likely compensates for the cell death by apoptosis. This last concept is also in agreement with the observation that CLL cells undergo spontaneous in vitro apoptosis and that no obvious lesions in either the Fas-dependent or mitochondria-dependent apoptotic pathways have so far been detected.

Antigenic stimulation occurring after neoplastic transformation may facilitate CLL cell expansion

As discussed above, considerable evidence indicates that CLL cells have undergone antigenic stimulation prior to neoplastic transformation. The next question is whether this stimulation may continue following leukemogenesis. This possibility is suggested by the observation that certain cellular features such as expression of B-cell activation markers and particular characteristics of the surface Ig, correlate with prognosis. Thus, CLL patients whose cells express ZAP-70 or CD38 or utilize unmutated VH/VL genes have a worse clinical course and outcome than patient whose cells do not express these markers and utilize mutated genes. It appears that the surface Ig signal transduction pathway is more often viable in the cases with poor prognosis, although these studies are somewhat preliminary because of the small cohorts of patients analyzed. Because unmutated VH/VL genes generally encode for antibodies with natural/polyspecific activity, it has been proposed that the neoplastic cells utilizing these genes are continuously stimulated in vivo by self-antigens that have the capacity to react with surface Ig. This stimulation, which induces the expression of activation markers such as ZAP-70 or CD38 also in normal B cells does not occur in the case of cells utilizing mutated genes. The proposed dichotomy is not absolute since certain cells with unmutated genes may be unable to bind self-antigens, while others with mutated genes may express surface Ig that retains self-reactivity. These concepts bring about self-reactivity as a major promoting factor of leukemogenesis and pose some restriction in the definition of the subset of origin of CLL cells.

Human B-cell subsets

The two major CLL subsets defined above are distinguished on the basis of certain features of the neoplastic cells. Before discussing the nature of the cell(s) giving rise to these CLL, the organization of the peripheral mature B cells must be discussed.

Immature B cells complete their process of VDJ gene rearrangement and exit from the bone-marrow as virgin, antigen-inexperienced B cells. These cells are relatively frequent in the peripheral blood or are dispersed among T cells in the T-cell-dependent areas or accumulate in the mantle of secondary lymphoid follicles of peripheral lymphoid organs. Upon encountering an antigen, the virgin B cells are activated and reach the germinal centers of the secondary lymphoid follicles, where they proliferate and accumulate VH/VL gene mutations. Because of these mutations, the progeny of proliferating cells express receptors of different specificity at the cell surface. Since continuous antigenic stimulation is required to ensure cell survival of germinal center cells, only the cells equipped with high affinity receptors for the stimulating antigen (also concentrated in the germinal centers) will survive and join the memory cell or the Ig-secreting plasma cell pool. This mechanism ensures the selection of a substantial number of cells producing high affinity antibody in a very short period of time.

Another response, different from that described above, occurs in the marginal zone (MZ). The MZ was initially defined as the outer portion of periarteriolar lymphoid sheaths of the spleen, although it is now widely accepted that the subepithelial area of tonsils, the sub-capsular area of lymph-nodes, the dome region of Peyer’s patches and mucosa-associated lymphoid tissue (MALT) all represent MZ-equivalent areas. MZ cells exhibit special properties,
including the capacity of synthesizing natural/polyspecific antibodies and of responding to polysaccharide antigens of bacteria in a T-cell-independent fashion.\(^\text{10}\) While responding to these antigens, the MZ cells may, although not necessarily, accumulate VH/VL gene mutations and be selected based upon the affinity of antibody produced.\(^\text{19,20}\) However, it is widely believed that this selection process is less efficient than that occurring in the germinal centers.

**The origin of CLL: one or two cell model?**

Based on the description provided above, there are two major subsets of CLL. One set is characterized by neoplastic cells that express surface Ig, which are encoded for by unmutated Ig V region genes and have natural/polyspecific activity. The skewed repertoire of these cells and the frequent use of stereotyped receptors indicate stimulation by antigen. These cells were probably expanding in response to T-cell-independent antigens when transformation occurred and may continue to do so following transformation. In the other CLL subset, the cells express surface Ig without natural/polyspecific activity and, although they may have expanded in response to an antigenic stimulus before or during transformation, do not appear to be affected by antigenic stimulation after the transformation process has been completed. There is evidence, albeit so far limited to small cohorts of cases, that in the unmutated CLL the slg-dependent signal transducing pathway is still efficient, whereas this pathway is faulty in the cells from mutated cases.

A possible explanation for the existence of two CLL subsets may be that they derive from two different B cell types. Unmutated CLL may derive from MZ cells and the mutated CLL from memory B cells that have exited the germinal centers at the end of their selection process.\(^\text{1}\) Although there is no compelling evidence to dismiss this model, certain assumptions do face some difficulties. For example, if the mutated CLL subset is of post-germinal center cell origin, why then do the neoplastic cells not express surface IgG or IgA rather than IgM, given the fact that virtually all B-cells that transit through germinal centers undergo isotype switches?\(^\text{5}\) Moreover, CLL cells from the two subsets present rather similar signatures when analyzed for gene expression profiles by microchip technology.\(^\text{6}\) In the case of different cellular origin of the two subsets, one would expect more marked differences at this level as well.

An alternative possibility is that both CLL subsets originate from the same cell type and namely from the MZ B cell.\(^\text{3}\) Because the Ig V gene mutation has been demonstrated to take place in the MZ, outside the canonical germinal center, it is plausible that in the mutated cases, these mutations occurred while the cells were responding to a particular T-cell-independent antigen prior to or during neoplastic transformation. Because of these mutations, the polyspecificity of the antibody itself has been lost and the cells are no longer susceptible to stimulation by self-antigen in vivo. Hence, because of the elimination of this important promoting factor in leukemogenesis, the cells of this subset will expand less rapidly and exhibit a more benign course than those of the unmutated CLL subset. It is not known whether the absence of continuous antigenic stimulation is responsible for the faulty surface Ig-dependent signal transduction pathway often observed in the cells from these cases. Alternatively, it could be that the cells have become tolerant during the response to antigen prior to transformation.\(^\text{10}\) The antibodies produced by this mutated CLL subset derive from antibodies that initially had natural/polyspecific activity. Site-directed mutagenesis tests on the VH/VL genes demonstrated that elimination of the mutated spots and reversion to the unmutated VH/VL gene configuration result in the synthesis of antibodies with natural/polyspecific activity.\(^\text{21}\) These observations reinforce the concept that the same cells gave origin to both the mutated and the unmutated CLL subsets. This concept also explains the similar surface phenotype, the general biological features and the signatures by microchips array of the cells of the two subsets (Figure 1).

**Analysis of the surface phenotype of CLL cells provides limited indications on the cell of origin**

The initial studies on the cellular origin of CLL were focused on the observation that CLL cells express CD5, a marker generally found on T cells and on a special population of B cells studied particularly in mice. These CD5-positive murine B cells, often referred to as B1 cells, produce natural/polyspecific antibodies, respond to T-cell-independent antigens, and represent a self-renewing B-cell subset, which presumably is continuously stimulated by contact with self-antigens in vivo.\(^\text{22}\) These cells share many functional features with MZ B cells and a number of investigators believe that these two cells belong to distinct subsets of the same subpopulation. B1 and MZ B cells differ from murine follicular B cells (also called B2 cells) that share all of the properties described above for human follicular (follicular mantle + germinal center) B cells.

Because of the somewhat unusual expression of CD5 by CLL cells, the studies were focused for a long time on the search for a normal CD5-positive human B cell which could represent the cell of origin of CLL. A number of investigations demonstrated the presence of CD5-positive B cells both in the circulation and in lymphoid organs. These cells had a phenotype...
similar to that of CLL cells, i.e. CD20-positive, CD23-negative, and CD5-negative with surface IgM and IgD. The major difference was that these cells had high levels of surface Ig, whereas these levels are very low, almost undetectable in CLL. Functional analyses of these normal CD5-positive cells revealed many differences from CLL cells. They do not produce polyclonal/natural antibodies, do not respond to T-cell-independent antigens and utilize unmutated (but not mutated) IgV region genes. These are virgin B cells and their preferential homing in the follicular mantle of lymphoid follicles is consistent with this notion. Because of all these features and despite their phenotype, these CD5-positive B cells cannot be considered as the cells of origin of CLL cells.1

The surface phenotype of MZ B cells is CD20-positive, CD23-negative, CD22-positive, and CD5-negative with high levels of surface IgM and low levels of surface IgD.1 This phenotype is similar to that of memory B cells that home in the MZ, except that the memory cells express surface IgG.1 This phenotype is different from that of CLL cells. However, before dismissing the hypothesis that CLL cells derive from MZ or memory B cells or both, there are important considerations to be made. First, CLL cells are activated cells as shown by the expression of typical activation markers such as CD69 or CD71. Upon activation, B cells often undergo phenotypic changes which make them considerably different from the cells of origin. For example, activation of MZ B cells in vitro induces the expression of CD23 which is not normally expressed by MZ B cells (but is found on CLL cells).21 Second, CD5 may also represent a B-cell activation marker, which cannot be used for the assessment of the lineage of origin of B cells. In connection with this, it is of interest that in vivo activation of resting MZ B cells induces the expression of both CD5 and CD38.22 Third, in vivo analyses of MZ B cells show that they can express CD5, presumably because of cell activation.23 Therefore, even if the phenotypic analyses cannot lead to the definition of CLL cell origin, the existing data do not invalidate the notion of a MZ B cell origin of CLL cells, since most of the noted differences between the phenotype of normal and malignant cells can be explained by the activation status of the latter.

References


Chronic lymphocytic leukemia: genetics for predicting outcome

C hronic lymphocytic leukemia (CLL) is characterized by a highly variable clinical course. Treatment of early stage patients with chlorambucil without risk stratification has not been shown to prolong survival. Therefore, therapeutic procedures were traditionally aimed at palliation and instituted only for advanced stage or symptomatic disease. Over recent years highly effective and potentially curative approaches such as antibody-chemotherapy and autologous or allogeneic stem cell transplantation have been developed. In parallel to this, there has been dramatic progress in our understanding of the pathogenesis of CLL and the prediction of patient’s outcome, making risk-stratified treatment a reasonable goal.

Prognostic factors in CLL
A number of markers of prognostic relevance have been identified including clinical characteristics (age, stage, gender, and performance status), as well as laboratory parameters reflecting the tumor burden or disease activity (lymphocyte count, lactate dehydrogenase (LDH) elevation, bone marrow infiltration pattern or lymphocyte doubling time). More recently, prognostic markers related to the biology of the disease have been identified: serum parameters such as soluble CD23 (sCD23), β2-microglobulin or thymidine kinase and genetic markers of the CLL cells, such as genomic aberrations, gene abnormalities (p53 and ATM), the mutation status of the variable segment of immunoglobulin heavy chain genes (VH), or surrogate markers for these factors. Over recent years the focus of research has moved towards the molecular genetic level which may not only provide insight into the biology and transforming events of CLL but may also allow the definition of mechanisms directly responsible for the course of the disease with regard to clinical progression, response to treatment and overall survival.

Clinical stage according to Binet and Rai
The standard clinical procedures to estimate prognosis in CLL are the staging systems developed by Rai and Binet. These systems define early (Rai 0, Binet A), intermediate (Rai I/II, Binet B) and advanced (Rai III/IV, Binet C) stage disease with median estimated survival times of >10, 5-7, and 1-3 years, respectively. However, there is heterogeneity in the course of the disease between individual patients within a single stage group. Furthermore, in a multivariate analysis, clinical staging was not retained as an independent prognostic marker when VH mutation status and genomic aberrations were included in the model. Importantly, biological risk factors such as the VH mutation status and genomic aberrations may have the power to identify subgroups of patients with poor prognosis among early stage patients (see below).

Markers of tumor burden: lymphocyte count, lymphocyte doubling time, serum LDH bone marrow infiltration pattern
 Elevated LDH levels and high lymphocyte counts have been associated with disease activity. In multivariate analyses including clinical (age, stage, etc.) and genetic (VH status, genomic aberrations, etc.) variables, these parameters were independent prognostic factors. Whereas a doubling time of 12 months or less identified a population of patients with poor prognosis, a doubling time longer than 12 months was indicative of good prognosis as substantiated by a long treatment-free period and survival. In addition, a short lymphocyte doubling time predicts rapid disease progression in patients in early clinical stages. Several studies showed that cases with diffuse bone marrow infiltration had a worse prognosis than cases presenting with a nodular pattern. There is a strong association between the bone marrow infiltration pattern and both clinical stage and absolute lymphocyte counts.
Serum parameters: β2-microglobulin thymidine kinase and SC2D3

Thymidine kinase levels correlate with the proliferative activity of CLL cells and elevated levels predict disease progression in CLL. In a recent study thymidine kinase levels appeared to identify a subgroup of patients with early, non-smoldering CLL at risk of rapid disease progression and provided independent prognostic information on progression-free survival. In the prospective CLL1 trial of the GCLLSG, elevated serum thymidine kinase levels were shown to be a strong predictor for high individual risk of rapid disease progression among Binet A patients. High levels of the soluble form of CD23 at initial diagnosis were linked with disease progression in early stage B-CLL. β2-microglobulin serum levels show a positive correlation with the clinical staging systems according to Binet and Rai. β2-microglobulin is associated with adverse prognostic features at presentation and higher values have been found in CLL patients with a shorter survival. This study also showed that the serum β2-microglobulin level is more powerful than clinical staging in predicting survival. Furthermore, patients with low β2-microglobulin serum levels had a significantly higher complete response rate to frontline chemoimmunotherapy with fludarabine, cyclophosphamide and the anti-CD20 antibody, rituximab.

VH mutation status

One of the most important molecular genetic parameters for dissecting the pathogenic and prognostic subgroups of CLL is the mutation status of the VH genes. Since somatically mutated VH genes can be observed in about half of all CLL cases, a separation was made into two different groups: one with unmutated VH genes and another with mutated VH genes. Most importantly, it has been demonstrated that the VH mutation status is clinically highly relevant. Furthermore, and independently of the mutation status, the usage of specific VH genes such as V3-21 may be associated with an inferior outcome. In addition, patients with rearranged V3-21 show highly restricted molecular characteristics in most cases. These findings indicate striking similarities in the B-cells receptors of V3-21-using CLL cases and suggest a stimulatory influence from an unknown antigen in the development of the disease. In addition, other frequently used and highly restricted VDJ combinations have been identified.

Genomic aberrations and their relation to the VH mutation status

Genomic aberrations can be identified in about 80% of cases of CLL by fluorescence in situ hybridization (FISH) of interphase cell nuclei (interphase-cytogenetics) with a disease-specific probe set (Table 1). Specific genomic aberrations have been associated with disease characteristics such as marked lymphadenopathy (11q deletion) and resistance to treatment (17p deletion, see below). Moreover, the rate of disease progression, as determined by the time from diagnosis to first treatment, and the overall survival time of CLL subgroups defined by specific genomic aberrations are significantly different.

Unfavorable aberrations (11q-, 17p-) occur more frequently in VH unmutated cases, and favorable aberrations (13q-, 13q- single) more frequently in the VH mutated subgroup. On the other hand, about two-thirds of the VH-unmutated CLL cases show no unfavorable genomic aberrations indicating a differential influence of these factors.

In multivariate analysis of the survival time, the VH mutation status, 17p deletion, 11q deletion, age, leukocyte count and LDH were identified as independent prognostic factors. The clinical stage of disease according to the staging systems of Rai or Binet was not identified as an independent prognostic factor indicating that with the knowledge of genetic parameters, the clinical stage of the disease may lose its independent prognostic value (Figure 1). Similar
results, demonstrating a strong prognostic and independent impact of the VH mutation status and genomic aberrations were found in other series.\textsuperscript{12}

VH mutation status and genomic aberrations enable insight into the biological bases of the clinical heterogeneity of CLL and may lead to future risk-adapted treatment strategies for individual patients. Table 1 gives a summary of the incidences of genomic aberrations and the VH mutation status observed in a single center cohort of patients distributed over all stage groups and from several multicenter trials of the GCLLSG. It should be noted that there are significant differences in the occurrence of high-risk and low risk markers in the different studies indicating the different biological background of the cohorts of patients.

**Surrogate markers for the VH mutation status: CD38, ZAP-70, LPL, etc**

In order to make the estimation of prognosis based on genetic markers accessible to the routine hematology laboratory, surrogate markers for the VH status have been identified. Originally, a correlation was observed between the VH mutation status and CD38 expression of the CLL cells pointing to CD38 expression as a prognostic marker.\textsuperscript{8} Based on genome-wide gene expression studies other surrogate markers such as ZAP-70 expression were identified and validated.\textsuperscript{7,13,20} ZAP-70 expression appears to correlate strongly with VH mutation status and was, therefore, a strong prognostic marker for disease progression in a pivotal study.\textsuperscript{8} However, subsequent studies have yielded controversial results with regard to validity of both CD38 and ZAP-70 as surrogate markers for VH and prognostic indicator. The facts that i) divergent results have been obtained in different laboratories (CD38 and ZAP-70), ii) the expression level may change over time (CD38), iii) a careful separation of T cells is necessary (ZAP-70), iv) different cut-off values to distinguish positive from negative cases were defined (CD38 and ZAP-70), and v) approximately 10-30% of cases show discordant status for CD38 or ZAP-70 as compared to VH in all series described, indicate that these markers may not be as useful as initially thought for routine diagnostics.

The discordance of ZAP-70 and VH mutation status in some patients may in part be explained by the presence of additional genetic high-risk features such as 11q or 17p deletion and V3-21 gene usage: in a recent study, discordant cases with V3-21 usage were almost exclusively ZAP-70 positive and VH mutated, whereas all but one of the discordant cases with high-risk aberrations were ZAP-70 negative and VH unmutated.\textsuperscript{14} Therefore, the search for additional markers appears mandatory to allow for a refined risk assessment. This has already been demonstrated for markers such as LPL and ADAM29\textsuperscript{21,22} and risk estimation may be improved based on global or targeted gene expression analyses.

**Validation of prognostic factors in clinical trials: risk of progression in early stage CLL**

In the CLL1 trial of the German CLL Study Group CLL patients with Binet A disease are stratified into the high risk arm if they have a lymphocyte doubling time < 12 months and/or a diffuse bone marrow infiltration pattern and a thymidine kinase level > 7 U/L and/or β2-microglobulin level > 3.5 mg/L. Based on this stratification patients in the high risk group are randomized to either immediate treatment with fludarabine or a watch-and-wait strategy while the low risk group is followed up. In addition, genomic aber-
rations and VH mutation status are analyzed at enrollment. Table 1 gives a summary of the genetic results obtained so far. These results show that high risk aberrations (11q-, or 17p-: 14%) and unmutated VH (41%) occur in a significant number of asymptomatic early stage patients. When comparing the results from CLL1 with our single center study involving patients diagnosed in all stages and the CLL4 study (fludarabine vs. fludarabine and cyclophosphamide for untreated Binet B / C patients) it is interesting to note that the incidence of low risk markers (mutated VH, 13q- single) is higher and the percentage of high risk markers (unmutated VH, 11q, 17p-) is lower.

In the CLL1 study preliminary correlations of genetic parameters with progression-free survival among untreated patients showed that unmutated VH as well as +12q, 11q- and 17p- are associated with more rapid disease progression (Figure 2). Moreover, the genetic parameters and the other parameters used for risk stratification appear to be correlated. Unmutated VH and high risk aberrations (17p-, 11q-, +12q) were significantly associated with the trial-defined high risk group and with the individual parameters defining this group. In multivariate analysis thymidine kinase, lymphocyte doubling time, unfavorable genomic aberrations (11q-, 17p-, +12q) as well as unmutated VH status were identified as independent variables. Therefore, the current CLL7 trial includes the parameters thymidine kinase, lymphocyte doubling time, genomic aberrations and VH mutation status for initial risk stratification among Binet A CLL patients.

**Predictors for response to treatment and survival in advanced stage CLL**

The observation that overall survival was inferior among patients with untreated VH, 11q-, or 17p- indicated that response to therapy may be different in genetic subgroups. In particular, the deletion 17p and/or abnormalities of the p53 gene involved in this aberration have been associated with failure after treatment with alkylating agents, purine analogs and rituximab.\(^{24-26}\) In a chromosome banding study in an alkylating agent trial, 17p aberrations were the only chromosomal aberration of prognostic relevance.\(^{21}\)

An interphase-FISH study also showed that patients whose CLL cells showed a 17p- / p53 deletion had significantly shorter survival times than had patients without this aberration and a relationship was found between the deletion and the response to treatment.\(^{26}\)

In the prospective CLL4 trial of the GCLLSG (comparing first line treatment with fludarabine versus fludarabine plus cyclophosphamide), clinical outcome was evaluated in subgroups defined by genomic aberrations and VH status for both treatment arms combined. In univariate analyses, significant associations were found for the following parameters: the overall response rate was significantly lower in the subgroup with 17p- (53.8% vs. 89.6%, \(p=0.001\)), the median progression-free survival was significantly shorter in the subgroups with 11q- (17.4 vs. 26.8 m, \(p=0.044\)), and 17p- (11.0 vs. 24.1 m, \(p=0.002\)), and the median overall survival was significantly shorter in the subgroup with 17p- (15.9 m vs. not reached, 75% survival at 43.8 m, \(p<0.001\), Figure 3). Multivariate analysis was performed including the treatment arms, specific genomic aberrations, and the VH mutation status as possible prognostic factors: the parameters with significant adverse impact were fludarabine monotherapy (HR 1.70, 95%CI 1.12-2.58, \(p=0.013\)) and 17p- (HR 3.67, 95%CI 1.66-8.14, \(p=0.001\)) regarding progression-free survival, but only 17p- (HR 7.32, 95%CI 2.44-21.90, \(p<0.001\)) regarding overall survival.\(^{27}\) Similar data on a strong prognostic role of genetic parameters on the outcome after first-line treatment have been reported from preliminary analyses of the UK CLL4 and the US ECOG 2997 studies.\(^{28, 29}\)

In contrast to the treatment failure after fludarabine-based therapy, there is anecdotal evidence that durable remission can be achieved in CLL with 17p-
p53 mutation using the monoclonal anti-CD52 antibody alemtuzumab.30 This observation has been expanded in a retrospectively evaluated series of CLL cases mostly refractory to fludarabine therapy.31 In the CLL2H study of the GCLLSG (alemtuzumab for fludarabine refractory CLL), a high incidence (27%) of 17p- aberrations was observed underscoring the association of this abnormality with fludarabine-resistant disease (Table 1). An interim analysis of this ongoing prospective trial has shown a response (complete or partial) in 10 of 21 VH unmutated, five of ten 11q-, and six of ten 17p- cases, providing evidence from a controlled trial that alemtuzumab may be effective in CLL with 17p- / p53 mutation.

Risk evaluation for autologous and allogeneic stem cell transplantation in CLL

Autologous and allogeneic stem cell transplantation (SCT) are increasingly considered in the management of medically fit patients with active CLL. There is a need to identify the role of prognostic factors which may be helpful to decide whether a patient is a good candidate for SCT or not and whether an allogeneic or autologous SCT should be considered.

Data from prospective autologous SCT trials demonstrating safety, improved remission after transplantation and long survival times are emerging. In the MRC series the early transplant-related mortality was 1.5% and the 5-year overall and disease-free survival rates after transplantation were 77.5% and 51.5%, respectively.32 In the multicenter prospective autologous SCT study of the GCLLSG (CLL3) the treatment-related mortality was 5% with a 2-year overall survival rate of 88% among 105 patients.33 This result appears promising considering the high-risk features present in the majority of patients (see also Table 1 for the CLL3 trial: 68% unmutated VH, 25% with 11q- or 17p-). However, the continuing occurrence of clinical and molecular relapses observed in all series on autologous SCT in CLL is evidence against the curative potential of this procedure in the majority of patients.

Furthermore, both the time to clinical relapse and the time to disease recurrence, as assessed by consensus primer CDR3 PCR, were significantly shorter among patients with unmutated VH genes.34 Nevertheless, the median treatment-free interval of 49 months in the VH unmutated cohort suggested a beneficial effect of autologous SCT for this high-risk population.

As compared to autologous SCT the primary therapeutic mechanism of allogeneic SCT after dose-reduced conditioning is the graft-versus-leukemia effect which may offer long-term disease control and eventually cure. Indeed, a recent comparative study of minimal residual disease detected by CDR3 PCR provided evidence that the graft versus leukemia effect is operational in CLL with unmutated VH.35 In this study, only a modest decrease in minimal residual disease levels was observed immediately after allogeneic SCT, but minimal residual disease became undetectable in seven of nine (78%) CLL patients with unmutated VH after tapering immunosuppression, chronic graft-versus-host disease or donor lymphocyte infusions. Therefore, allogeneic SCT after dose-reduced conditioning appears to combine the favorable features of low treatment-related mortality with the activity of the graft-versus-leukemia effect making this procedure a promising option when aiming for a cure for high risk CLL.

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References


Our understanding of chronic lymphocytic leukemia (CLL) has dramatically changed over the last 5 years as has the variety of available therapies. This is leading to a revolution in the approach to treating the disease. We have now moved from the purely palliative approach of the 1990s to attempts to produce profound and durable remissions at the present time. It is not unreasonable to believe that in the near future we might be considering a cure for more than just a small minority of patients. The current and future management of patients with CLL will require treatment stratification by individual patient risk, the application of techniques to detect minimal residual disease and the intelligent use of the variety of novel therapies that are currently being developed.

### Treatment by Risk Stratification

CLL can now be stratified according to a variety of biological characteristics. The two most extensively studied and robust are the presence or absence of somatic mutations in the immunoglobulin gene of the CLL cell\(^1,2\) and chromosomal abnormalities detected by fluorescent in situ hybridization (FISH).\(^3\) Mutational status and FISH can be used to predict the course of a patient’s disease and are now being evaluated to define whether patients with poor risk disease should have therapy initiated before disease progression as is currently recommended or whether different therapeutic approaches should be applied to different risk groups. Patients whose CLL cells that have no or few (>98% homology with germ-line sequence) somatic mutations in the immunoglobulin disease (VH unmutated) appear to have a similar response to therapy as those with mutated CLL but remain in remission for a shorter period and have a poorer overall survival.\(^4\) It appears that when unmutated CLL relapses after initial therapy there are frequently abnormalities of the p53 pathway (either deletion of 17p or 11q) leading to a poorer response to conventional therapy and subsequently a poorer survival. In contrast, when mutated CLL progresses after therapy these poor risk chromosomal abnormalities appear to occur rarely. It is therefore logical to consider therapies that are either more intensive or targeted in order to achieve more profound remissions for unmutated CLL but to de-escalate therapy to reduce toxicity for patients with mutated disease. When patients either present with or develop a dysfunctional p53 pathway (either 17p- or 11q deleted) they are inherently resistant to therapies that damage DNA or interfere with its repair, such as alkylating agents or purine analogs.\(^5\) These patients have a very low response rate to conventional therapy and this may explain the resistance in most, if not all, patients who fail to respond to fludarabine-based combination therapies. In these cases it is logical to use treatments that do not depend upon an intact p53 pathway for their activity, such as high dose steroids or monoclonal antibodies.\(^6\)

For example, data from the recent LRF CLL4 trial in the United Kingdom suggests that patients with CLL can be divided into three different risk groups by the molecular characteristics of their leukemia cells: (a) **good risk** CLL is defined by the presence of somatic mutations in the immunoglobulin gene of the CLL cell, excluding those cases utilizing the VH segment VH3-21, and comprises approximately 30% of patients requiring therapy; (b) **standard risk** patients are those cases with unmutated immunoglobulin genes or utilizing VH3-21 and comprise approximately 65% of patients; and (c) **poor risk** patients are defined as those patients with greater than 20% CLL cells which have loss of the short arm of chromosome 17 (17p-), as determined by FISH, and comprise about 5% of patients.\(^7,8\) These risk groups will be used to stratify therapy in the forthcoming CLL6 trial in the United Kingdom.
Minimal residual disease

The currently used response criteria in CLL were published in 1996 prior to the advent of purine analogs, monoclonal antibodies and stem cell transplantation as conventional therapies in CLL. A patient with complete remission was defined as one in whom the clinical examination was normal with an essentially normal blood count and a morphologically normal bone marrow (Table 1). These criteria have proven to be extremely useful to allow comparisons between the results of trials from the various collaborative groups. However, it is now clear that there can be as many as 2% CLL cells in the marrow of a patient who is in an NCI complete remission. This has driven the development of techniques that can detect extremely low levels of CLL. The two most frequently used sensitive approaches are molecular techniques, such as allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) directed against the immunoglobulin gene of the CLL clone, and multi-parameter, four-colour flow cytometry (MRD Flow). Both of these techniques will detect a single CLL cell in 10,000 leukocytes or more. ASO-PCR is slightly more sensitive than MRD Flow but has several disadvantages (Table 2) which make flow cytometry more likely to become the standard approach. In all the series that have been reported, whether treated with combination chemotherapy, immunochemotherapy, monoclonal antibody-based therapy or stem cell transplantation, patients who achieve a negative MRD status have better progression-free and overall survival. However in all of these series the aim of therapy was to try to eradicate MRD and therefore they do not prove beyond doubt that MRD is critical (the patients achieving MRD negativity may have had a biologically better risk and, therefore, could have had a better survival, regardless of therapy, than their more resistant counterparts). Therefore the next series of clinical trials will address, in a randomized fashion, whether attempting to eradicate MRD is an important end-point of therapy.

Combination chemotherapy

Purine analogs, and in particular fludarabine, are the most active chemotherapeutic agents in CLL and form the base for most of the effective combination therapies. Three large trials have recently been reported that overall randomized more than 900 patients between fludarabine and fludarabine plus cyclophosphamide. All three trials showed a significant improvement in complete response rate for the fludarabine plus cyclophosphamide combination, being over double that for fludarabine monotherapy. In addition, fludarabine plus cyclophosphamide doubled the progression-free survival rate compared to that achieved with fludarabine monotherapy; this was the primary endpoint of the trials and the difference was statistically significant. In the LRF CLL4 trial there was no upper age limit and 30% of the patients recruited were over 70 years of age. It was somewhat surprising that even in patients over 70 years old there was no significant increase in treatment-related toxicity or mortality. In addition, the benefit in response rates for the combined regimen of fludarabine plus cyclophosphamide was seen in all age groups. There was, however, no improvement in overall survival, is probably due to a cross-over from fludarabine to fludarabine plus cyclophosphamide at progression. However in the LRF CLL4 trial there was extensive quality of life assessment which showed that patients who achieved a complete or nodular partial remission had a significantly better quality of life over the next two years. It is expect that patients treated with fludarabine plus cyclophosphamide will also experienced improved quality of life.

Therefore the combination of fludarabine plus cyclophosphamide can now be considered the gold standard for the initial treatment of CLL, also for elderly patients without co-existing co-morbidities. Recently Bosch et al. reported on the results of adding mitoxantrone to fludarabine and cyclophosph-

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Table 1. Criteria for NCI complete remission (Cheson et al., 1999).

| B-symptoms | Absent* |
| Lymph nodes | Not palpable* |
| Peripheral blood lymphocytes | ≤4000/µL |
| Peripheral blood neutrophils | ≥1500/µL |
| Haemoglobin | >11.0 g/dL |
| Bone marrow aspirate | <30% lymphocytes** |
| Bone marrow trephine | No nodules*** |

*, no requirement for imaging; **, no requirement for immunophenotyping; ***; this can equate to up to 2% CLL cells.

| Applicable patients | >95% |
| Sensitivity limit | 0.01% |
| Quantitative range | 0.01% |
| Cost & complexity | Moderate |
| Pre-treatment material required | Essential |
| Turn-round time | Hours |

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Table 2. Comparison of methods of residual disease monitoring in CLL.

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<th>MRD flow cytometry</th>
<th>Allele-specific oligonucleotide PCR</th>
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<td>Applicable patients</td>
<td>&gt;95%</td>
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<td>Sensitivity limit</td>
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remissions. The most important predictor of relapsed, refractory CLL range between 33% and 80% with a reasonable toxicity profile. This led to the CAM307 trial in which 297 previously untreated patients with CLL were randomized to receive either chlorambucil or intravenous alemtuzumab. Somewhat surprisingly the toxicity to alemtuzumab was not significantly greater than that in the chlorambucil arm of the trial. In fact there was little difference in treatment-related toxicity between the two arms. The higher overall and complete response rate for alemtuzumab compared were significantly higher than those in response to chlorambucil. Alemtuzumab monotherapy might find its most useful role in the consolidation of response to chemotherapy in an attempt to eradicate minimal residual disease (see below).

**Monoclonal antibody therapy in CLL**

The only monoclonal antibody that is approved for use in CLL is alemtuzumab (Campath or MabCampath). It is approved for fludarabine-refractory CLL. Rituximab (Rituxan or MabThera) has also been used, both alone and in combination, in large numbers of patients with CLL. Rituximab, used as a single agent at the conventional dose of 375 mg/m2/weekly for 4 weeks, has little efficacy in relapsed or refractory CLL since only partial remissions occur in a minority of patients and these remissions only persist for a few months. The partial remission rate increases as the dose of rituximab increases, but again complete remissions are not achieved and the doses used are extremely high (up to 2250 mg/m2). Conventional doses of rituximab have also been reported to give higher response rates in untreated CLL (up to 50% of patients) but still very few complete remissions and these responses are not durable. Therefore rituximab has no proven role as a single agent but will probably find its role in combination with chemotherapy (see below).

In contrast, alemtuzumab is effective as a single agent in refractory and untreated CLL. The response rates to alemtuzumab, used as a single agent, in relapsed, refractory CLL range between 33% and 50% with up to 25% of patients achieving complete remissions. The most important predictor of response to alemtuzumab is the presence or absence of significant lymphadenopathy. Patients with massive lymphadenopathy have a very low response rate and in these patients a more effective strategy is to try to control the lymphadenopathy prior to alemtuzumab therapy. Two recently reported phase II trials of subcutaneous alemtuzumab in fludarabine-refractory CLL suggest that the drug has a similar efficacy when given subcutaneously, but has a much improved toxicity profile when administered by this route. A phase II trial of subcutaneous alemtuzumab in previously untreated CLL was reported by Lundin _et al._ in 2002. The response rates were in excess of 80% with a reasonable toxicity profile. This led to the logical approach is to combine rituximab with the most effective front-line therapy for CLL, namely fludarabine and cyclophosphamide. This combination (FCR) was recently reported to produce extremely high response rates in a group of 300 previously untreated patients, with an impressive overall response rate of 95% and 72% of patients achieving a complete remission by NCI response criteria. In addition, this series of patients in whom detectable disease was eradicated according to a sensitive PCR-based assay, had a very small chance (<10%) of progression at 5 years follow-up. The combination of FCR is the subject of at least two large international studies to address whether or not it is more effective than fludarabine and cyclophosphamide: these studies should complete recruitment within the next year and their results are eagerly awaited.

Kennedy _et al._ demonstrated for the first time in 2002 that patients who were refractory to both fludarabine and alemtuzumab as single agents could respond to the two agents combined. In fact in a subsequent update of their data 8 of 11 refractory patients responded better to the combination than to either fludarabine or alemtuzumab alone with detectable minimal residual disease being eradicated in two patients. Elter _et al._ have reported an alternative combination of alemtuzumab and fludarabine, again with high response rates. This combination is now being studied in a randomized phase III trial for relapsed CLL.

**Alemtuzumab consolidation therapy**

The fact that patients who achieve an NCI complete remission will inevitably relapse and that
patients who achieve an MRD negative remission appear to have a prolongation of their disease-free period suggests that consolidating patients into deeper remissions after conventional therapy might be an effective strategy. In addition, the fact that alemtuzumab is relatively ineffective in the presence of bulky lymph node disease and that the pharmacokinetics of the antibody indicate that it is likely to be more effective with low bulk disease suggests that the setting in which alemtuzumab is likely to be most effective is consolidation. There has been one randomized trial of alemtuzumab in the consolidation setting following fludarabine-based initial therapy. This was the GMLCLLSG CLL4b trial which showed a significant prolongation in progression-free survival for patients given alemtuzumab consolidation. However this trial was stopped prematurely as there was a high incidence of infections during alemtuzumab therapy. This was probably because alemtuzumab was given a median of 2 months after the-fludarabine, which was probably not long enough to allow recovery from the initial therapy. Three other studies of alemtuzumab as consolidation therapy have been reported and all had a longer interval between completing the induction chemotherapy and alemtuzumab therapy. Each of these studies has shown a degree of activity with an acceptable toxicity profile. Therefore it appears that the strategy of using alemtuzumab to consolidate remissions following conventional therapy is very likely to be effective if an appropriate schedule and dose can be identified.

**Allogeneic stem cell transplantation**

There is convincing evidence of graft-versus-CLL effect in the setting of both conventional myeloablative and reduced intensity conditioning stem cell transplantation. It is apparent that patients with MRD detectable post-transplant can frequently become MRD negative during follow-up or after donor lymphocyte infusions. This reversion to MRD negativity has not been reported after the completion of other therapies, strongly supporting the importance of the graft-versus-CLL effect as a biological phenomenon.

**Novel therapies**

There are a number of novel agents that are now being developed in CLL. These include flavopiridol which, despite disappointing results following the initial clinical studies, appears to be potentially very effective with a modified dosing schedule. Recently both thalidomide and lenalidomide have been reported to have activity in CLL and are being developed for use in the disease. Bcl-2 is upregulated in the vast majority of patients with CLL and this has led to the use of Bcl-2 antisense in initial studies in CLL. A variety of new monoclonal antibodies to pre-existing and novel targets are now being studied in CLL.

**References**


It is well recognized that multiple myeloma (MM) is a B-cell malignancy with a greatly variable clinical outcome: median survival times are approximately 3 years with standard-dose therapy and about 4 to 5 years with intensive treatment programs, but survival may range between only a few months and more than 10 years. Therefore, it has been important to identify prognostic indicators in order to estimate the individual patient’s outcome. Knowledge of such factors is critical not only for an improved understanding of disease outcome, but also for the development of strategies to optimize treatment, particularly with the aim of using risk-adapted therapies. The latter aspect has gained substantial importance due to the availability of novel agents for MM therapy.

**Standard clinical and laboratory factors**

In 1975, Durie and Salmon proposed a staging system based upon readily available clinical parameters (serum hemoglobin, size of the paraprotein, serum calcium, and number of osteolytic bone lesions by skeletal radiography). The Durie and Salmon staging system, which correlated with tumor burden and survival, was widely used despite its limitations, in particular with respect to the definition of bone lesions. However, the search for more accurate prognostic factors continued, and several studies identified serum β2-microglobulin as a powerful prognostic indicator for survival.

However, cut-off levels as well as additional parameters that could be combined with β2-microglobulin remained a matter of controversy. As summarized in Table 1, factors related to demographics, features of the tumor itself, and laboratory abnormalities were associated with poor outcome in patients with MM at presentation.

Combinations of parameters were proposed for staging and prognosis, but none of the models turned out to be superior to the Durie and Salmon staging system.

**International Staging System for MM**

This background provided the basis for an international co-operative project aimed at identifying a simple and reliable staging system for MM. Clinical and laboratory parameters from 10,750 previously untreated, symptomatic patients with MM were collected (69.1% from clinical trial data). The most powerful classification system was obtained by a combination of serum β2-microglobulin and serum albumin (Table 2). This International Staging System was validated in various MM patient populations. It was found to be effective in MM patients independently of age (less or more than 65 years of age), type of therapy (standard dose or autologous transplantation) and geographic region (North America, Europe, and Asia). It is now suggested that the International Staging System is used, particularly in the setting of clinical trials. An improved definition of patients at risk is expected in the future by incorporation of genetic and proteomic data.

**Genetics and prognosis in MM**

**Ploidy**

Cytogenetic and molecular genetic investigations of MM cells have provided evidence that virtually all cases of MM have chromosomal abnormalities. Karyotypes from MM cells are usually very complex, but careful analyses of large series have demonstrated that MM can be subdivided into two cytogenetic categories: the hypodiploid/pseudodiploid category (which also includes the near-tetraploid karyotypes) and the hyperdiploid category. This observation was extended by recent data obtained using fluorescence in situ hybridization (FISH), which indicated the presence of hyperdiploid and non-hyperdiploid MM variants. The hyperdiploid subtype is defined by presence of multiple trisomic chromosomes (most commonly chromosomes 3, 5, 7, 9, 11, 15, 19, and 21), but a low frequency of IgH translocations. In contrast,
non-hyperdiploid MM is characterized by a high frequency of IgH-translocations and frequent loss of chromosomes, especially chromosomes 13, 14, 16, and 8. Recognition of hypodiploid MM is of clinical significance, since patients with hypodiploid MM have a particularly unfavorable prognosis.

IgH translocations

One of the most frequent structural abnormalities observed in MM karyotypes involves the Ig heavy-chain (IgH) gene locus on 14q32, which is usually part of a translocation. Heterogeneous translocation partners have been described, with 11q13, 4p16.3, 16q23, 20q11 and 6p21 being recurrently involved in 14q32 translocations from primary MM tumor specimens. These five types of primary IgH translocations, which are mutually exclusive, comprise about 60% of all IgH translocations, and are mediated primarily by errors during IgH switch recombination. Relevant correlations have emerged with respect to biology and prognosis: the t(11;14)(q13;q32) resulting in upregulation of cyclin-D1 was originally thought to characterize a group of patients, with a favorable prognosis, in particular when treated with intensive therapy. However, more recent results suggest that t(11;14) does not affect either event-free or overall survival, whereas the presence of t(4;14)(p16;q32) or t(14;16)(q32;q23) identifies a subset of MM patients with short survival, even in the context of autologous transplantation. Translocations t(4;14) and t(14;16) are strongly correlated with a deletion of chromosome 13q.

Deletion of chromosome 13q

Using metaphase cytogenetics, a chromosome 13q abnormality can be found in about 15% of MM patients at diagnosis, whereas interphase FISH studies have shown a higher frequency (39-54%) of 13q deletions in newly diagnosed cases of MM. Several studies have reported a strong association between deletion of 13q and an unfavorable prognosis of MM patients. Recognition of hypodiploid MM is of clinical significance, since patients with hypodiploid MM have a particularly unfavorable prognosis.

Deletions of 17p13 at the TP53 locus, were reported to be clinically important, with similar observations being made in patients receiving standard-dose and high-dose therapy. Comprehensive analyses of cytogenetic abnormalities in MM identified patients with a t(4;14) and/or 17p-deletion as the group of patients with the worst prognosis suggesting that novel approaches are required for the treatment of such high-risk patients with these high-risk indicators.

Studies done by the Arkansas group identified a region on chromosome 1, which was linked with an aggressive clinical course in MM. Global gene expression profiling on plasma cells from newly diagnosed patients treated with autologous transplantation revealed a significant over-representation of chromosome 1 genes in a group of about 70 genes whose expression was associated with poor outcome. Further analyses showed that overexpression of CKS1B was strongly correlated with a gain of DNA copy numbers at chromosomal region 1q21, and that this abnormality conferred a poor prognosis. As a possible mechanism, reduced levels of p27Kip1 protein were observed in cases with 1q21 amplification, suggesting dysregulated cell cycle control in these cases.

Additional chromosomal aberrations

Genome-wide gene expression profiling based on DNA microarrays currently represents one of the most powerful tools in the area of genomics. This
technique has become feasible and broadly accessible, and in MM it is a valuable tool to identify all myeloma-specific genetic abnormalities on a single platform.24 When this technique was used to identify genes associated with therapeutic outcome in 221 patients with previously untreated MM, unsupervised clustering led to the identification of four distinct MM subgroups.24 Further studies indicated that three genes in this analysis can be used to predict event-free survival. Furthermore, gene expression profiling provided the basis for a novel molecular classification of MM because overexpression of one of the cyclin-D genes was found to be a universal molecular feature of MM.25 The so-called TC-classification combines the cytogenetic information about the 14q-translocations with cyclin-D gene expression, as summarized in Table 3. Patients in the TC4 and TC5 categories have shortened survival suggesting that they should be considered for clinical studies exploring investigational therapies.

Impact of novel agents on prognosis

Prognostic factors conferring a poor outcome in MM have been defined according to the experience with chemotherapy, with no apparent differences being found between patients treated with standard-dose or high-dose therapy (compare all studies referenced above). Recent studies have addressed the question of whether or not treatment for high-risk patients may be improved by the use of novel agents.

Thalidomide

Prognostic information in patient populations treated with thalidomide is mainly available in the relapsed/refractory setting. Among 75 patients treated with single agent thalidomide, advanced age (> 65 years), elevated serum lactate dehydrogenase (LDH), and elevated serum creatinine were predictive of inferior outcomes.26 In a similar analysis of relapsed MM patients treated with thalidomide-based regimens, elevated serum LDH, advanced International Staging System stage, and reduced performance status were independent predictive factors for survival.27 Based on these three variables, a scoring system was developed with survival times of 58.1, 28.8, and 5.8 months for patients with scores 0, 1, and 2, respectively. The authors concluded that the addition of LDH and performance status to the prognostic information provided by the International Staging System may help select patients who will likely derive benefit from treatment with thalidomide-based regimens.

According to the experience of the Arkansas-Group (phase 2 trial of single agent thalidomide in 169 patients with pretreated MM), favorable survival rates were observed in patients with normal metaphase cytogenetics, low proliferative activity (plasma cell labeling index < 0.5%) and serum β2-microglobulin below 3 mg/L.28 Overall, these results suggested that prognostic factors for treatment with thalidomide are similar to those observed in patients treated with chemotherapy.

Bortezomib

A potential association between baseline characteristics and outcome was explored in patients enrolled in the SUMMIT trial.29 By multivariate analysis, two parameters emerged as being significantly associated with lower response: age > 65 years and plasma cell infiltration > 50%. Parameters predicting for shortened overall survival were low serum albumin, bone marrow plasma cell infiltration > 50%, and thrombocytopenia. Of particular note, elevated serum β2-microglobulin and presence of a chromosome 13q deletion (tested in a subset of study patients) were not predictive of poor outcome bortezomib-treated patients in this clinical trial.

Among patients treated in the APEX trial, a matched-pair analysis was performed between 21 patients with a deletion 13q (metaphase analysis) and 41 patients without this deletion.30 Patients were

| Table 3. TC molecular classification of MM as proposed by Bergsagel and Kuehl.25 |
|---|---|---|---|---|
| Group | Translocation | Gen(s) | CyclinD | Ploidy* | % |
| TC1 | t(11;14)(q13;q32) | cyclinD1 | D1 | NH | 15 |
| TC2 | None | None | D1 | H | 37 |
| TC3 | None | None | D2 | H > NH | 22 |
| TC4 | t(4;14)(p16;q32) | fgr-3/mnsset | D2 | NH > H | 16 |
| TC5 | t(14;16)(q32;q23) | c-maf | D2 | NH | 5 |
| | t(14;20)(q11) | mafB | D2 | NH | 2 |

*NH, non-hyperdiploid; H, hyperdiploid.
balanced for other adverse prognostic factors including age, lines of prior therapy, β2-microglobulin, and albumin. Presence of a chromosome 13q-deletion was associated with a markedly decreased survival in the dexamethasone arm; in contrast, in the bortezomib arm, deletion 13q was not associated with a difference in survival or response rate.

In our own analysis of 51 patients with relapsed/refractory MM, treatment with bortezomib as a single agent resulted in similar response rates and durations of response in patients with and without a chromosome 13q-deletion. Serum β2-microglobulin did not emerge as a relevant parameter associated with treatment outcome after bortezomib (lack of prognostic information for response rate, time to treatment failure, and overall survival). Low serum albumin levels correlated with a short time to treatment failure and poor overall survival, and also identified those patients with a deletion 13q who did not benefit from treatment with bortezomib.

Thus, although additional data from prospective clinical trials are needed, existing data indicate that prognostic factors established from chemotherapy trials cannot be uniformly applied to patients treated with bortezomib.

Conclusions and future directions

During the past decade, considerable progress has been made in our understanding of the molecular basis and biology of MM. Molecular genetic analyses and gene expression profiling have contributed to the recognition of distinct subtypes of MM with different prognoses. Both cytogenetic and molecular findings are correlated with laboratory and clinical characteristics, and we are beginning to use this information as diagnostic and prognostic indicators for the selection of treatment options. Clinical trials are under way to examine the therapeutic efficacy of agents targeting specific molecular defects in myelomatous plasma cells. It is hoped that novel molecular structures will continue to be discovered for specific therapeutic interventions. Standardization of techniques such as gene expression profiling will become helpful in predicting response to therapy and eventually tailoring therapy to specific molecular MM entities. These advances should result in further improvements of our therapeutic strategies for patients with MM.

References


Treatment of elderly myeloma patients

High-dose chemotherapy is regarded as the treatment of choice for young myeloma patients, whereas conventional chemotherapy is considered more suitable for the elderly. Even though the upper age limit remains to be determined, the recent introduction of new drugs as front line therapy is changing the scenario.

Superiority of high-dose chemotherapy over conventional chemotherapy

High-dose chemotherapy was the only real improvement for the treatment of multiple myeloma in the 1990s that allowed a significant increase in complete remission rates. In a recent large study by Child et al., 407 patients were randomized to receive standard chemotherapy or high-dose chemotherapy followed by autologous transplantation. The median overall survival was significantly prolonged, by almost one year, in the high dose arm: 54 versus 42 months. To date, this superiority has clearly been demonstrated in younger patients with a median age of 50 years. However, the median age of newly diagnosed myeloma patients is approximately 65 years old. Thus, high-dose chemotherapy has progressively been employed in older patients with a higher toxicity. It is currently assumed that high dose treatments should be employed up to the age of 65 years, but only few data are available on the subgroup of patients above this age.

In a non-randomized study, our group showed that a conditioning regimen with melphalan at the dose of 100 mg/m² (MEL100) was less toxic than the standard dose of 200 mg/m² (MEL200). MEL100 was inferior to MEL200 in terms of event-free survival but not in terms of overall survival. Halving the melphalan dose did not significantly affect the response rate suggesting the lack of a clear dose/response relationship.

MEL100 appeared a suitable treatment for elderly patients. A randomized study by our group in untreated patients, aged 50-70, demonstrated the superiority of MEL100 over standard melphalan/prednisone (MP). The near-complete remission rate was 6% after MP and 25% after MEL100 ($p=0.0002$). At 3 years, MEL100 increased event-free survival from 16% to 37% and overall survival from 62% to 77% ($p<0.001$) (Table 1). The superiority of high-dose was also confirmed in patients aged 65-70 (Table 2).

Upper age limit for high-dose chemotherapy

The Nordic Myeloma Study group showed the impact of age on survival after high-dose chemotherapy in a population-based study involving 414 patients (261 younger than 60, 98 between 60 and 65 years old). The overall survival rate, compared with that of a historic control group treated with conventional chemotherapy, was higher the younger group: 67% vs 44% ($p<0.0001$) at 4 years. The survival advantage persisted after corrections for prognostic factors between groups. Survival was also prolonged in the older group treated with high-dose chemotherapy, but with weaker statistical significance, which disappeared after corrections for prognostic factors between groups. In conclusion, this study clearly showed the impact of age on outcome, but was unable to determine an upper age limit.

Another study reported on the Mayo Clinic experience with high-dose chemotherapy in 35 patients over 70 years old, compared to 70 patients matched for several clinical and biological characteristics. The two groups showed similar toxicities, response rates and 2-year overall survival rates. The authors concluded that high-dose chemotherapy is feasible in selected elderly patients.

A third report by the Intergroup Francophone du Myelome (IFM) group compared high-dose chemotherapy (MEL100) versus standard oral MP in patients aged 65-75 years. In a third arm, patients were
treated with MP + thalidomide (see new drugs). The median progression-free survival was 19.0 and 17.1 months in the MP and MEL100 groups, respectively. Thus, in this preliminary analysis high dose chemotherapy does not appear superior to conventional chemotherapy in a patient population older than that in our study (65-75 versus 65-70).6

Overall, these three studies addressed the issue of age in the treatment of myeloma patients and showed that in older patients the toxicity related to high-dose chemotherapy generally offsets its advantage over conventional chemotherapy. However, selected medically fit patients can still benefit from high-dose programs and should not be excluded a priori solely because of chronological age.

An age cut-off of 65 years old appears reasonable, but this boundary may be crossed in both directions according to the patient’s clinical conditions (Figure 1).

New chemotherapy regimens for patients ineligible for high-dose chemotherapy

Thalidomide is a new drug in the therapeutic armamentarium for myeloma. Thalidomide, as a single agent or in combination is regarded as standard treatment for relapsed/refractory myeloma and its efficacy is now being explored in newly diagnosed patients. It appears suitable for combination regimens with standard chemotherapy in elderly patients ineligible for high dose chemotherapy.

The Italian Multiple Myeloma Network, GIMEMA, randomized 255 patients to receive 6 cycles of MP or MP plus thalidomide (MPT) at the daily dose of 100 mg during MP treatment and then as maintenance until relapse. Near complete response or complete response was achieved in 36/129 patients (27.9%) in the MPT arm as compared with 9/126 patients (7.2%, p<0.001) in the MP arm. The 2-year event-free survival was 54% in MPT patients and 27% in MP patients. (Table 1). This finding suggests a synergistic effect of thalidomide with conventional chemotherapy.7 Toxicity, in particular deep vein thrombosis, infections, and neuropathy, was greater in the MPT arm (Table 3).

The IFM group randomized 436 patients to receive 12 cycles of MP or MPT at the daily dose of 400 mg at the maximum tolerated doses during MP treatment with no maintenance treatment. Moreover, a third arm consisted of MEL100. After a median follow up of 32.2 months, median progression-free survival was 17.1 and 27.6 months for the MP and MPT groups, respectively (p<0.0001). The overall survival was 30.3 and 38.6 months, respectively (p<0.0009). No significant differences were noted between MP and MEL100.6

Bortezomib represents a new class of anti-neoplastic drugs. Bortezomib is a proteasome inhibitor with multiple effects on myeloma cell lines and primary myeloma cells. Based on results from phase II trials, bortezomib was approved by the FDA and the EMEA for patients with relapsed/refractory myeloma treated with at least one prior line of therapy.
Synergy with cytotoxic agents such as melphalan has been described. Thus, bortezomib represents an ideal candidate to combine with MP in elderly patients ineligible for high-dose chemotherapy.

The Spanish group GEM carried out a phase I-II multi-center study to determine the optimal dose of bortezomib in combination with MP (V-MP) in elderly untreated myeloma patients. Moreover, toxicity and efficacy were evaluated on a larger cohort. Sixty patients with a median age of 74 years entered the trial. The phase I part of the study determined the recommended dose of 1.3 mg/m² for the phase II part. Overall, V-MP showed acceptable toxicity and a high response rate (28% immunofixation negative complete response).

Lenalidomide (CC5013) is a thalidomide analog with immunomodulatory properties. A large phase III study showed that the combination of lenalidomide plus dexamethasone was a well-tolerated and active oral regimen for relapsed/refractory myeloma. With a study duration of 18 months, the median time to progression for patients treated with this combination was 13.3 months compared to 5.1 months for patients treated with dexamethasone and placebo ($p<0.000001$). The overall response rate was higher in patients who received lenalidomide plus dexamethasone than in patients who were given dexamethasone alone (58% vs. 22%; $p<0.001$). Lenalidomide appears another promising agent to use in combination with conventional chemotherapy.

Our group carried out a phase I dose finding study to combine lenalidomide with MP and a phase II study to evaluate the toxicity and response rate. The best dose combination was found at melphalan 18 mg/Kg with lenalidomide at 10 mg/day. Preliminary data showed a 70% response rate including 10% immunofixation negative complete responses (Table 1).

Overall, these studies showed that there is an additive or synergistic effect between the so-called new drugs and conventional chemotherapy, namely MP. Complete response rates higher than 5-8% have never been reported with conventional MP. Similar

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<table>
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<tr>
<th>Adverse event</th>
<th>MEL100 (N=95) No (%)</th>
<th>MP (N=126) No (%)</th>
<th>MPT (N=129) No (%)</th>
<th>MPR (N=24) No (%)</th>
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<tr>
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<td>1 (1)</td>
<td>0 (0)</td>
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<td>32 (25)</td>
<td>62 (48)</td>
<td>9 (37)</td>
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results have been observed using thalidomide as first line treatment. However, MP plus thalidomide increased the complete response rate up to 27% showing a synergistic effect. Response rates have also been significantly increased using both bortezomib and lenalidomide in combination with MP (up to 30% immunofixation negative complete responses).

It has been demonstrated that a significant increase in complete response rates after high dose chemotherapy correlates with a prolonged survival. Preliminary data have confirmed that this is also the rule for combination regimens with conventional chemotherapy and new drugs. For instance, in the French and Italian MPT studies, the increase in complete and near complete response rates were associated with significantly prolonged event-free and overall survival.

When complete response is considered a surrogate marker of efficacy of a specific treatment, it is clear that these old/new drug combinations produce response rates similar to those so far obtained with the high-dose regimens. Our MEL100 protocol achieved near complete responses in 27% of patients, but similar results can now be obtained with an oral and easy to administer chemotherapy (Table 1).

The new/old drug combinations are bound to become reference treatments for elderly myeloma patients. However, the following considerations should be taken into account before drawing definitive conclusions:
- longer follow up is needed to confirm preliminary data. Only studies employing thalidomide have now reached an observation period longer than 2 years. Long-term toxicity (Table 2) and remission duration also remain to be determined;
- combinations of conventional chemotherapy with new drugs have provided encouraging results. No data are currently available on combination strategies of high-dose chemotherapy with new drugs. Several questions, such as whether high-dose treatments may be improved with the addition of new drugs, which are the best drug combinations and what is the optimal timing (during induction, maintenance) will have to be answered in the near future.

References

Multiple myeloma (MM) remains an incurable disease; therefore new treatment approaches are needed in order to improve the outcome of patients with this disease. The increased knowledge about MM biology is already contributing to more specific drug design, and we have recently learned that the interaction of malignant cells with the microenvironment is as important as the malignant cells themselves, in the pathogenesis of MM.

MM requires a multistep transformation process that implies the sequential generation of primary immunoglobulin translocations, chromosomal instability (including mutations such as: RAS-, and deletions such as: RB-), and secondary translocations. Most primary immunoglobulin gene translocations occur early in the pathogenesis of MM. These translocations, which are mediated by errors in immunoglobulin heavy-chain switch recombination, result in the juxtaposition of an immunoglobulin enhancer and oncogene. On the basis of IgH translocations, MM patients can be divided into five subgroups: 1) those with D-type cyclins: cyclin D1 on 11q23, cyclin D3 on 6p21 and cyclin D2 on 12p13 (25% of cases); 2) those with MMSET/FGFR3 proteins (4p16.3) (15%); 3) those with B-zip transcription factors: c-maf on 16q23 and mafB on 20q11 (15%); 4) those with other IgH translocations (20%); and 5) those with no IgH translocations (25%). Secondary oncogenic events may involve genes other than the Ig locus, as well as the 14q32 region, as occurs in c-myc translocations: t(8;14), t(2;8), t(8;22), t(14;20).

Some of these molecular events represent potential therapeutic targets. Thus the t(4;14) translocation generates constitutive activation of the oncogenic receptor tyrosine kinase FGFR3 with subsequent phosphorylation of the anti-apoptotic STAT3 signaling pathway. Therefore, inhibitors of the FGFR3 tyrosine kinase as well as inhibitors of cyclin-dependent kinases could be attractive therapeutic targets. Similarly C-maf, which is over expressed in MM patients with t(14;16) and in some MM cases lacking the translocation, also represents a potential target.

The second area of MM pathogenesis that has important implications for treatment intervention is the interaction between the malignant cell and the bone marrow (BM) microenvironment. MM cells adhere to the extracellular matrix proteins and BM stromal cells through a series of adhesion molecules, such as the β1-integrin family (VLA-4, VLA-5 and VLA-6) as well as ICAM-1 and VCAM-1. Adhesion of myeloma cells to the bone marrow microenvironment induces cell-adhesion-mediated drug resistance. Interrupting, by downregulation, the interaction between the tumor cell and its microenvironment can potentially halt MM cell growth and proliferation, and benefit patients with MM. The binding of MM cell to the bone marrow microenvironment also induces the transcription and secretion of cytokines (TNF-α, IL-6, IGF-1, SDF1α, VEGF), by both the plasma cells and bone marrow stromal cells, triggering signaling pathways (such as the RAF/MEK/MAPK, PI3K/AKT, and JAK/STAT pathways) that promote cell proliferation and prevent apoptosis. These pathways are also potential targets for therapeutic intervention.

**Thalidomide**

Thalidomide was initially used in MM because of its anti-angiogenic activity given that increased angiogenesis occurs in the bone marrow of MM patients. Nevertheless, it was soon discovered that thalidomide has additional mechanisms of action: it inhibits the production of TNF-α, stimulates T cell proliferation, induces the secretion of IFN-γ and IL-2, augments NK cytotoxicity, induces apoptosis, and regulates the expression of adhesion molecules.

The therapeutic efficacy of thalidomide
has been confirmed in numerous trials on refractory/relapsed MM patients. The pivotal study, published in 1999 in the New England Journal of Medicine by the Arkansas group and updated two years later in Blood, included 169 refractory/relapsed MM patients. The scheduled treatment consisted of escalating doses of thalidomide (200 to 800 mg/day) and the overall response rate (>25% reduction in M-component) was 37% (2% complete response [CR], 12% near complete response [nCR]), with a 2-year event free survival and overall survival of 20% and 48%, respectively. These results have been confirmed by many other groups, including data on over 1200 patients with overall response rates ranging from 25 to 66% (median 42%) and around 30% partial responses (PR).\textsuperscript{5}

The non-myelosuppressive profile of thalidomide has favored its combination with other agents summarized in Table 1 (selected references for thalidomide, immunomodulatory drugs and bortezomib are included in the corresponding tables). Moreover, thalidomide has been reported to restore the sensitivity of myeloma cells to other drugs and to enhance the anti-myeloma activity of dexamethasone. Between 35\% and 55\% (mean 47\%) of refractory MM patients treated with thalidomide plus dexamethasone achieve at least partial responses. Even higher response rate (55-76\%) have been reported upon adding cyclophosphamide or melphalan or etoposide. In fact, the oral combination of thalidomide plus cyclophosphamide and dexamethasone is widely used in this setting, and in our experience it yields durable responses (57% event-free survival at 2 years).\textsuperscript{5} More recently, thalidomide has been combined with other novel agents, such as proteasome inhibitors (bortezomib). Eighty-five refractory MM patients received these two drugs, together with dexamethasone if the response was suboptimal after three cycles. The response rate (\(\geq PR\)) was 71\% (16\% CR/nCR), without increased toxicity regarding neuropathy and myelosuppression. Combinations of thalidomide and bortezomib with other agents, such as adriamycin and dexamethasone or pegylated liposomal doxorubicin are also being explored, with a

\textsuperscript{5} Anagnostopoulos (Br J Haematol 2003), Dimopoulos (Ann Oncol 2001) and Palumbo (Haematologica 2001); \textsuperscript{a} Kropff (Br J Haematol 2003), Garcia-Sanz (Leukemia 2004) and Dimopoulos (Haematol J 2004.)

Table 1. Thalidomide combinations in relapsed/refractory MM patients.

<table>
<thead>
<tr>
<th>Author</th>
<th>Treatment schedule</th>
<th>Patients</th>
<th>Response rate ((\geq PR)) (%)</th>
<th>Reference</th>
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<tr>
<td>Several\textsuperscript{1}</td>
<td>Thal + Dex</td>
<td>&gt;400</td>
<td>47</td>
<td>*1</td>
</tr>
<tr>
<td>Several\textsuperscript{2}</td>
<td>Thal + Cy + Dex</td>
<td>200</td>
<td>64</td>
<td>*2</td>
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<tr>
<td>Hussein</td>
<td>Thal-PegLD-Vincristine</td>
<td>45</td>
<td>76</td>
<td>Oncology 2004; 18: 1233-5</td>
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<tr>
<td>Zangari</td>
<td>Thal+Bortezomib+/-Dex</td>
<td>85</td>
<td>55 (16 CR+ nCR)</td>
<td>Blood 2005; 106: 2552</td>
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<tr>
<td>Chanan-Khan</td>
<td>Thal+Bortezomib+PegLD</td>
<td>13</td>
<td>54</td>
<td>Leuk&amp;Lymphoma 2005; 46:1103-4</td>
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<tr>
<td>Hollmig</td>
<td>Thal+Bortezomib+Adriamycin+Dex</td>
<td>20</td>
<td>55 (12CR+12nCR)</td>
<td>Blood 2004; 10: 2399a</td>
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</table>

Table 2. Thalidomide combinations in untreated MM patients.

<table>
<thead>
<tr>
<th>Author</th>
<th>Treatment schedule</th>
<th>Patients</th>
<th>Response Rate ((\geq PR)) (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rajkumar</td>
<td>Thal-Dex vs Dex</td>
<td>103 vs 104</td>
<td>63 vs 41</td>
<td>J Clin Oncol 2006;24:431-6</td>
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<tr>
<td>Cavo</td>
<td>Thal-Dex vs VAD</td>
<td>100 vs 100</td>
<td>76 vs 52</td>
<td>Blood 2005;106: 35-39</td>
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<td>Ludwig</td>
<td>Thal-Dex vs MP</td>
<td>83 vs 85</td>
<td>57 vs 50 (CR/nCR: 24 vs 13)</td>
<td>Blood 2005; 106: 782</td>
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<td>Goldschmidt</td>
<td>Thal-Adriamycin-Dex vs VAD</td>
<td>406</td>
<td>80 vs 63 (CR: 7 vs 3)</td>
<td>Blood 2005; 106: 424</td>
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<tr>
<td>Palumbo</td>
<td>Thal-MP vs MP</td>
<td>129 vs 126</td>
<td>768 vs 47 (CR: 28 vs 5)</td>
<td>Lancet 2006; 367:825-31</td>
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<tr>
<td>Facon</td>
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<td>191 vs 190</td>
<td>81 (15 CR)</td>
<td>Blood 2005; 106: 780</td>
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<td></td>
<td>MP vs Mel 100</td>
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<td>40 (2CR)</td>
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<td>38</td>
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</table>
response rate (≥PR) of 55% and 54%, respectively, with a manageable toxicity (Table 1).

The obvious next step was to investigate the efficacy of thalidomide in the up-front setting (Table 2). Several pilot studies have been conducted with thalidomide plus dexamethasone achieving 68-79% PR or better, including 5-16% CR. The ECOG has carried out a phase III randomized trial of thalidomide plus dexamethasone versus dexamethasone alone, showing a higher response rate (≥PR) for the combination arm (63% vs 41%, \(p=0.0017\)). The Bologna group conducted a retrospective matched-case-control analysis and reported that thalidomide plus dexamethasone was superior to VAD (≥PR: 76% vs 52%) as primary therapy in preparation for autologous stem cell transplantation (ASCT). The Hovon/GMMG group compared three cycles of VAD vs thalidomide, adriamycin and dexamethasone (TAD) as induction treatment before ASCT. Although TAD induced a higher response rate (≥PR: 80% vs 65%, with 7% vs 3% CR) \(p=0.01\) (Table 2), this benefit disappeared after ASCT (≥PR: 91% vs 88%, with 19% and 13% CR). Two very interesting randomized trials (Italian and French) compared the combination of thalidomide with melphalan-prednisone (MP) versus MP alone in elderly patients. In both studies thalidomide plus MP (MPT) was clearly superior in terms of response (87-96%, with 15%-28% CR) and event-free survival (29 months in both trials, which represents an approximately 1 year advantage over conventional MP) (Table 2). Moreover, in the French study the overall survival had not been reached at month 56 in the MPT arm vs 30 months in the MP arm \(p=0.008\). Some pilot studies are currently exploring the combination of thalidomide and bortezomib (i.e. VTD), reporting a very high PR (≥PR 78%) with the responses being achieved rapidly. As mentioned earlier, thalidomide is also being combined with adriamycin and dexamethasone (TAD) (Table 2). However, the final value of these regimens requires longer follow-up. In addition, thalidomide is being investigated as maintenance therapy. The IMF group reported a higher progression-free survival rate at 4 years for the group of patients receiving thalidomide versus no maintenance therapy or pamidronate (50% vs 39% vs 37%).

Due to the previous history of thalidomide, a major concern was the toxicity profile of this drug. The side effects are dose-related, and the most common are constipation, weakness, drowsiness and neuropathy. The use of combination therapy has also raised concern about an increased risk of deep vein thrombosis. Apparently the major risk of deep vein thrombosis occurs when tumor load is high and thalidomide is combined with chemotherapy, especially adriamycin (30% incidence vs 4% when used alone).

**Immunomodulatory drugs**

Immunomodulatory drugs (IMID) were developed as thalidomide analogs and so far include two drugs: lenalidomide (Revlimid, CC-5013) and actimid (CC-4047). In vitro, lenalidomide is a 200-50,000 more potent immunomodulator than thalidomide (in terms of TNF inhibition, cytokine modulation, increased response rate (≥PR) of 55% and 54%, respectively, with a manageable toxicity (Table 1).

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stimulation of T-cell proliferation and IL-2 and IFN-γ production), but it has less anti-angiogenic activity. It has fewer side effects and it is not teratogenic. The most relevant side effects of Lenalidomide are neutropenia (grade 3 in 17-30%), and deep vein thrombosis (8-15%).

In a recent phase II study with lenalidomide, given at a dose of 30 mg/day x 3 weeks q28 in 222 relapse/refractory MM patients, the PR + CR rate was 25%, with a median time to progression of approximately 6 months. These positive results prompted the activation of two phase III trials (one in the USA and the other in Europe) comparing lenalidomide plus dexamethasone vs dexamethasone alone in relapsed/refractory MM. In both studies the lenalidomide arm was associated with a significantly higher response rate (≥PR, mean 60% vs 22%) and longer time to progression (median 14 vs 5 months). These positive results prompted the activation of two phase III trials (one in the USA and the other in Europe) comparing lenalidomide plus dexamethasone vs dexamethasone alone in relapsed/refractory MM. In both studies the lenalidomide arm was associated with a significantly higher response rate (≥PR, mean 60% vs 22%) and longer time to progression (median 14 vs 5 months). Lenalidomide is also being evaluated in relapsed/refractory MM patients in combination with other agents, such as liposomal doxorubicin, vincristine, and dexamethasone (DVd-R) with a preliminary response rate (≥PR) of 60%; it is also being combined with bortezomib in a phase 1/2 trial in which 59% of patients reached CR +PR. Ongoing studies are evaluating lenalidomide combinations in first line therapy.

Rajkumar et al. reported that, in 34 newly diagnosed MM patients who received lenalidomide plus dexamethasone, 91% had at least a PR, including 6% with a CR and similar results were reported with the same combination plus clarithromycin in another pilot study (Table 3).

The experience with actimid is significantly shorter. Schey et al. (JCO 2004) reported that the response rate in 44 refractory patients was 71% (17% CR, 37% PR). This stimulating activity is counterbalanced by the toxicity profile: neutropenia (57%), and deep vein thrombosis (16%), and the fact that this drug is also teratogenic.

**Proteasome inhibitors: bortezomib**

We have recently learned that the ubiquitin-proteasome pathway is an attractive therapeutic target in cancer. Proteasomes represent a large complex of proteolytic enzymes responsible for the intracellular degradation of ubiquitinated proteins, including proteins that govern important cellular functions such as cell cycle, cell growth and differentiation. NF-κB is involved in a pivotal route that is also controlled by proteasomes. Blockade of proteosomal degradation pathways results in the accumulation of ubiquitinatised proteins, followed by significant cell stress and cell

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death. Cancer cells seem to be more sensitive than normal cells to the pro-apoptotic effects of proteasome inhibitors, perhaps due to their loss of check point mechanisms for DNA repair.

Bortezomib (Velcade®, formerly PS-341) is a novel dipeptide boronic acid which induces reversible inhibition of the 26S proteasome. In addition to its antiproliferative and proapoptotic effects (via NF-κB blockade), it downregulates the expression of adhesion molecules, inhibits angiogenesis, inhibits effectors involved in DNA repair, and blocks the unfolded protein response resulting in accumulation of improperly folded proteins and subsequent endothelium reticulum stress and cell death. Although other proteasome inhibitors, including oral formulations, are under investigation, bortezomib is the only one that has been introduced at a clinical level.2

Based on the preclinical studies and a promising phase 1 trial, two pivotal phase 2 trials, SUMMIT and CREST, were developed in relapsed/refractory MM patients. Patients were treated with bortezomib 1.3 mg/m2 on days 1, 4, 8 and 11 every 3 weeks and dexamethasone was allowed in patients with suboptimal responses to bortezomib alone. The response rate was 35%, including 10% CR/nCR with an overall survival of 17 months. A subsequent randomized phase 3 trial (APEX) including 669 patients with relapsed MM, showed that bortezomib is more effective than high-dose dexamethasone, as demonstrated by a significant improvement in response rate (43% vs 18%), median time to progression (6.2 vs 3.4 months) and 1-year survival rate (80% vs 67%, respectively)(updated at ASH 2005). Although these results are encouraging, a substantial proportion of patients do not respond to bortezomib, and acquired resistance has already been observed. These facts together with the well documented in vitro synergy of bortezomib with other agents, clearly justify combination therapy. Two pilot studies have shown that bortezomib in combination with melphalan or pegylated liposomal doxorubicin produces a response rate of 50% and 73%, respectively in refractory MM, including a substantial number of CR (Table 4). As mentioned above, over two-thirds of patients responded to the combination of bortezomib and thalidomide (Table 2). These responses rates are clearly superior to those obtained with bortezomib alone, and confirm the synergistic effect found in in vitro studies.

Bortezomib has also shown high activity as first-line treatment in untreated MM. Pilot studies reported at ASH 2005 using bortezomib as a single agent showed discrepant response rate (from 30% to 70%) due perhaps to the difference in the number of cycles administered (median two versus five). The addition of dexamethasone was associated with a higher overall response rate (≥PR 80-90%, with 18% CR+nCR). Similar results (90% response rate, with 15-20% CR/nCR) have been obtained with the PAD regimen (bortezomib, adriamycin, and dexamethasone) and the VTD scheme (bortezomib, thalidomide and dexamethasone). Moreover, peripheral blood stem cells could be successfully mobilized. Finally, bortezomib as first-line treatment was explored in elderly patients in combination with MP, with very promising initial results (86% response rate, with 43% CR/nCR) (Table 4).

An important aspect of all these studies is the toxicity profile of bortezomib, particularly in the combinations. The most frequent grade 3 toxicities reported in these trials included fatigue, gastro-intestinal symptoms, cyclic thrombocytopenia, peripheral neuropathy that resolved or improved in two-thirds of the patients after completion or discontinuation of therapy.

Other promising drugs

Arsenic trioxide (ATO)

The rationale for using ATO in MM is based on its multifaceted effects on MM cell lines and fresh myeloma cells. A phase 2 trial in heavily pre-treated patients with relapsed/refractory MM, showed minor responses or stabilization of M-component. Glutathione has been implicated as an inhibitor of ATO-induced cell death. Since ascorbic acid is able to decrease glutathione concentrations, their combination with ATO would seem appropriate. Likewise, combinations with dexamethasone could be tested because it uses a different pro-apoptotic pathway. Unfortunately, for unknown reasons most trials with ATO have been cancelled.

Farnesyltransferase inhibitors (FTI)

N-RAS and K-RAS mutations are frequent in advanced MM, and they are associated with an adverse prognosis. The activation of the RAS pathway requires the membrane localization of RAS, which implies a prenyl lipid modification mediated by both farnesyltransferase and geranylgeranyltransferase. Therefore, the use of drugs that inhibit RAS farnesylation in MM patients is attractive, particularly in those with RAS mutations. The results of a phase 2 trial with the FTI inhibitor tipifarnib (Zarnestra) are not very encouraging since only disease stabilizations have been achieved. Studies in combination with other agents are ongoing.

Imatinib mesylate (STI 571)

We have shown that STI571 is able to block cell cycle progression in MM cells but in contrast to its action on leukemic cells, it is unable to induce apoptosis of MM cells. In our experience as well as in that
of the Mayo Clinic, it proved of little value in MM patients.

**Aplidin**

Aplidin is an antitumor agent derived from Tunicate Aplidium Albicans. It induces cell cycle arrest, apoptosis (via JNK and p38) and is synergistic with several anti-MM agents. Preliminary data from one ongoing study in heavily pretreated MM patients shows PR in 7% and stable disease in 36%.

**Fibroblast growth factor receptor 3 (FGFR3)**

The t(4;14) translocation is present in 15% of MM patients and results in constitutive activation of the anti-apoptotic STAT-3 signaling pathway. Recent evidence using different molecules such as the small molecule inhibitor PD173074 (Pfizer, Ann Arbor, MI, USA) or CHIR-258 (an inhibitor of class III, IV and V receptor tyrosine kinases), has demonstrated the possibility of inhibiting FGFR3 autophosphorylation, resulting in tumor cell growth arrest and apoptosis. A clinical trial with CHIR-258 is ongoing.

**Mammalian target of rapamycin (mTOR) inhibitor**

mTOR is a downstream target of PI3K/AKT and mediates between the phosphorylation of proteins responsible for translation and expression of D-type cyclins and c-myc. mTOR inhibitors such as rapamycin and its analog CCI-779 are currently under investigation in MM.

**Histone deacetylase (HDAC) inhibitors**

HDAC inhibition results in accumulation of acetylated nucleosomal histones, leading to differentiation and/or apoptosis of MM cells. Several HDAC inhibitors (e.g. SAHA and LBH589) are available. We have shown that LBH589 has potent anti-MM activity in vitro and potentiates the activity of dexamethasone, bortezomib and melphalan. Clinical trials with the Hsp inhibitor geldanamycin (17-AGG) are ongoing.

**Insulin growth factor (IGF-1) receptor inhibitors**

IGF-1 stimulates proliferation of MM cells and protects them from dexamethasone or TRAIL-induced apoptosis. Both monoclonal antibody and specific IGF-1R tyrosine kinase inhibitors are being investigated for the treatment of MM.

**Statins**

Some studies have recently shown the anti-myeloma activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins). These agents inhibit the geranyl-geranylation of target proteins (such as RAS). Statins induce activation of the intrinsic pathway of apoptosis, cell cycle arrest, inactivation of the survival cascade of MAP kinases, and overcome cell adhesion-mediated drug resistance.

**Perifosine**

Perifosine is an oral bioactive novel alkylphospholipid that inhibits Akt and induces apoptosis via FADD and caspase 8. Interestingly, normal cells are resistant to its apoptotic effect, while malignant cells are sensitive to TRAIL. Therefore, its use, alone or in combination, would appear to be attractive.

**Vascular endothelial growth factor (VEGF)**

The presence of increased angiogenic activity and micro-vessel density is a well known feature of the bone marrow of MM patients. VEGF triggers the phosphorylation of its high affinity receptor (VEGFR), which leads to the downstream activation of the MEK/ERK (increasing proliferation) and p13K/AKT (increasing migration) pathways. There are already available several tyrosine kinase inhibitors that target the three VEGFR. In addition, a humanized monoclonal antibodies against VEGF (bevacizumab, Avastin) is already in phase 1/2 trials.

**Heat shock protein (Hsp) inhibitors**

Hsp are responsible for folding proteins into a functional conformation. Hsp27, Hsp70 and Hsp90 are upregulated in MM. The use of Hsp inhibitors will lead to cytotoxic intracellular accumulation of misfolded proteins and disruption of critical signal pathways with subsequent cell death. In vitro studies have shown a synergistic effect with bortezomib and clinical trials with the Hsp inhibitor geldanamycin (17-AGG) are ongoing.

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Acknowledgements

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References

The antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease defined by the presence of antiphospholipid antibodies (aPL) in the plasma of patients with venous and/or arterial thrombosis and/or recurrent complications of pregnancy. In APS thrombotic complications can occur in almost every vessel of the body, in arteries and veins, and in large vessels and in the microcirculation. The pregnancy morbidity includes unexplained death of a morphologically normal fetus at or beyond week 10, a premature birth of a normal fetus before week 34 of gestation and three or more unexplained spontaneous abortions before week 10 of gestation. Other clinical features such as heart valve abnormalities, thrombocytopenia, chorea and livedo reticularis are also frequently observed in patients with APS, although these manifestations are not included in the definition of the syndrome. APL are a heterogeneous family of immunoglobulins with different specificity, which complicates an unequivocal definition of APS. Interestingly, the pathological autoantibodies are not directed against phospholipids at all. It has been shown that aPL are often directed to proteins bound to anionic phospholipids and a large number of possible antigens have been described. Besides anti-β2-glycoprotein I antibodies (a2βGPI) and antiprothrombin antibodies, most of the other antibodies are relatively rare and poorly correlated with clinical features. It is generally accepted that the most relevant antigen for aPL is β2-glycoprotein I (2βGPI). The pathological aPL antibodies are autoantibodies directed against 2βGPI.

2β-glycoprotein I is a glycoprotein present in plasma at concentrations ranging from 50 to 500 µg/mL (0.25-5.0 µM). The major source of synthesis is the liver. 2βGPI is synthesized as a single polypeptide chain 326 amino acids long with a calculated molecular mass of 36.3 kDa. It is built up out of five successive complement binding repeat domains. The fifth domain contains a large positively charged patch, the phospholipid binding site. 2βGPI contains four potential glycation sites and the glycans account for approximately 20% of the total molecular weight. Already in...
Not all these questions can be answered easily. LAC measures the potential of \( \alpha_2 \)GPI and antiprothrombin antibodies to inhibit coagulation, aCL ELISA measures the presence of antibodies directed against cardiolipin and \( \beta \)GPI bound to cardiolipin, while an \( \alpha_2 \)GPI ELISA measures the presence of antibodies that recognise \( \beta \)GPI coated on a plastic surface. LAC thus measures the functional activity of antibodies against \( \beta \)GPI, while the other assays only measure their presence. The results of both LAC and aCL are also determined by the presence of anti-prothrombin antibodies and antibodies directed against cardiolipin directly, respectively. Moreover the results of the assays are also influenced by the conformation that \( \beta \)GPI adopts when it binds to the surfaces exposed in the assays.\(^5\) Clearly, the assays do not measure identical antibody populations. In a meta-analysis of the predictive value of the different types of aPL antibodies, Galli \textit{et al.} showed that the antibodies that induce LAC activity correlate best with a history of thrombo-embolic complications.\(^6\) Whether LAC is also the assay of choice for pregnancy morbidity and all the other additional clinical manifestations is unknown.

In an attempt to improve the correlation between the clinical features and the measurement of the antibodies, we have developed new assays for the detection of \( \alpha_2 \)GPI. \( \alpha_2 \)GPI are a heterogeneous population of antibodies, which is illustrated by the observation that antibodies directed against all five domains of \( \beta \)GPI have been described. We and others have found strong evidence that in particular antibodies directed against domain I correlate with thrombosis.\(^7,8\) Further analyses have shown that specifically antibodies that recognize the positively charged cluster around amino acids 40-43 are the pathological antibodies. The use of a newly developed assay that measures LAC caused by \( \alpha_2 \)GPI only, and an ELISA that specifically detects the autoantibodies directed against domain I of \( \beta \)GPI could improve detection of patients at risk of thrombo-embolic complications. A direct improvement in the specificity for identifying thrombotic risk can be obtained by combining a LAC assay with an \( \alpha_2 \)GPI ELISA, selecting only those patients who are positive in both assays.

A number of recent studies have shown that there is no independent correlation between elevated levels of anticardiolipin antibodies and the risk of venous thrombosis, neither in the general population nor in patients with an autoimmune disease.\(^9,10\) Does that mean that an ELISA for anticardiolipin antibodies is of no value? At present we cannot confirm this statement because we do not have enough information on its relation with arterial thrombosis and pregnancy morbidity. Reliable population-based information is not available for a correlation between arterial thrombosis or pregnancy morbidity and aCL.

Inadequate data are available to estimate the value of detecting antiprothrombin antibodies for the diagnosis of APS and therefore inclusion of these autoantibodies for diagnostic purposes is not advised.

**New concepts for treatment**

A retrospective study in 1995 reported that anticoagulant therapy targeted at an international ratio (INR) of 3.0 or higher protects better against recurrence than does less intense anticoagulant therapy.\(^11\) The results of this study were the lead for treatment for a long period. However, this study had several limitations. The endpoint of this retrospective study was thrombosis, without discrimination between venous and arterial thrombosis, and patients, according to the physician’s judgment, could deviate
from their target INR. In a recent study Crowther et al. reported the results of a prospective, randomized, controlled study in a population of aCL-positive patients with thrombosis in whom maintenance of a high INR (3.1-4.0) was compared with a moderately intensive level of anticoagulation (INR 2.0-3.0). In this study, the lower dose of warfarin was as effective as the higher dose and adverse effects were similar in both groups. These results challenged the claim for general use of high doses of oral anticoagulants.

Is it necessary to treat patients with APS indefinitely with warfarin? The supposedly high recurrence rate for thrombosis has led to the expert opinion of prolonged (lifelong) continuation of treatment. No controlled studies have been performed to support this. Does anticoagulant treatment prevent or treat the additional clinical manifestations such as heart valve disease, thrombocytopenia and neurological dysfunction? No controlled studies have been performed but clinical experience in single cases suggests that it does not.

We still lack evidence-based rules for the treatment of APS, supported by a clear insight in the pathophysiology of the syndrome. This failure is due to the clinical complexity of the syndrome, the absence of an understanding of how the presence of the antibodies causes the disease and the lack of well-designed prospective studies. What should be done for patients who develop recurrent thromboses despite anticoagulant therapy? Should we stop treatment for patients with a history of recurrent thrombosis in whom aPL disappear in time? There is a need for better treatment. In the future, the treatment of patients with APS may not be anticoagulant therapy alone. The perspective of therapies focused on B-cell depletion may be promising but other innovative treatments such as the use of statins, complement inhibitors, vaccination and immunoabsorbent procedures could be considered and tested in prospective randomized trials.

**New concepts concerning pathophysiology**

A unique feature of APS is that it is associated with both arterial and venous thrombosis. Most other risk factors for thrombosis are associated with either venous or arterial thrombosis. One of the intriguing questions is whether a single pathological mechanism is responsible for the different types of thrombosis and for pregnancy morbidity or whether there are different mechanisms. Recurrences of thrombotic complications often take place in the same vessel type but this is not a general rule. As long as we do not have a unifying hypothesis of how aPL cause thrombotic complications, we cannot exclude that venous thromboses, arterial thromboses and pregnancy morbidity are caused by different mechanisms. However, based on observations that the clinical manifestations in patients are frequently quite similar, it is logical to assume that there is one, unique mechanism by which anti-2βGPI antibodies cause thrombotic complications.

We now accept that a2βGPI are gain-of-function antibodies. After antibody binding, 2βGPI forms dimers, which strongly increases their affinity for negatively charged phospholipids. This increase is sufficiently strong to induce competition with the binding of clotting factors to anionic phospholipids. This is illustrated by the most relevant assay for the detection of these antibodies in patients' plasma samples, the LAC assay. There is now convincing evidence that the same complex can also interfere with cellular functions. 2βGPI, dimerized after binding of antibodies, has an increased affinity for a large number of cells, such as platelets, endothelial cells, T cells, dendritic cells, neuronal cells and monocytes. The increased affinity of the 2βGPI-a2βGPI antibody complexes results in binding to and subsequently activation of these cells. Agonists normally activate cells via interaction with a specific cellular receptor. At least nine different receptors have been described that bind 2βGPI: toll-like receptors 2 and 4, annexin A2, apoER2, VLDL-receptor, megalin, LRP, LDL-receptor and glycoprotein Ib. Activation of endothelial cells a2βGPI results in a switch of an anticoagulant phenotype to a more procoagulant phenotype, envisioned among others by the expression of tissue factor, via a signaling pathway identical to the activation pathway of endotoxin. It has been suggested that toll-like receptor 4 has an important role. Interaction of 2βGPI-a2βGPI antibody complexes with apoER2 on the surface of platelets resulted in increased platelet deposition onto collagen in an *in vitro* flow model. Inhibitors of the ApoER2-receptor prevent the increased platelet deposition. Apparently there are many different receptors that have the ability to signal after interaction with 2βGPI-a2βGPI antibody complexes. It seems unlikely that all the different receptors identified *in vitro* as 2βGPI-a2βGPI antibody complex binders are of pathological relevance. Further research should clarify the receptor puzzle.

2βGPI binds with higher affinity to activated cells that express anionic phospholipids than to quiescent cells. Preactivation may be necessary to bind 2βGPI in sufficient amounts to start the complete activation process. The 2βGPI-a2βGPI antibody complexes do not necessarily bind to exposed negatively charged phospholipids, as they can also bind to heparin-sulphate containing structures. These latter structures are exposed on non-activated cells, while negatively charged phospholipids are only exposed on apoptot-
ic cells or activated trophoblasts and activated platelets. We do not know whether the binding of 2βGPI-a2βGPI antibody complexes to cellular phospholipids or heparin sulphates precedes the interaction with the above mentioned receptors. Binding to anionic structures on the membrane can lead to better positioning of 2βGPI for interaction with the receptor through a mass action effect. There is also an entropy factor that must be considered in any binding. 2βGPI-antibody complexes bound to a surface have less mobility than 2βGPI-a2βGPI antibody complexes in solution. The loss of freedom is entropy unfavorable and must be overcome by a favorable energetic effect due to the interaction with the receptor. Moreover, it is possible that the interaction with anionic surfaces induces a conformational change in 2βGPI that allows a subsequent interaction with the receptor.

2βGPI-a2βGPI antibody complexes usually do not fully activate cells, but they render them more susceptible to suboptimal concentrations of other activators. A second hit such as a small vascular injury or an infection is probably necessary for full cellular activation. This could explain the fact that, despite the constant presence of antibodies in plasma of patients, the patients do not suffer from thromboembolic complications continuously. Only the risk of developing thrombosis or obstetric complications is increased.

Conclusions

Important progress has been made with one of the major problems in defining the syndrome, i.e. reliable diagnostic tests for the detection of the pathological aPL. The characterization of patients with definite APS is essential in order to facilitate pathological studies and clinical trials. Progress has also been made in our understanding of the pathophysiology of the syndrome. We have now attractive hypotheses that can be validated in clinical studies. If we can increase our insights into the cause of the syndrome, alternative treatments will be possible, e.g. focused on the prevention of the interaction between the antibodies and 2βGPI, the prevention of the interaction of 2βGPI and cells, or specific interference with cellular activation.

References

Diagnosis and treatment of pulmonary embolism

Pulmonary embolism (PE) occurs in most patients as a complication of a deep-vein thrombosis of the lower extremities. Less often thrombi originate from the pelvic region and in rare cases from the axillary, subclavian or other arm veins. Clots may also form in the right atrium or in the right ventricle. Indeed, as hypothesized by von Virchow in 1846, clots originate in the part of circulation that precedes the lung, and more precisely veins and right heart. They are then dislodged to the pulmonary artery by blood flow.

This vascular disorder most often complicates the course of severely ill, hospitalized patients but may also affect ambulatory and otherwise healthy persons. Even though the importance of PE as a major cause of morbidity and mortality has been gaining attention in the past three decades, its true incidence in the general population remains difficult to determine.

Diagnosis

Although clinicians dispose of new objective tests, the diagnosis of PE is still a difficult clinical problem and is still made in most cases only at autopsy. Unfortunately, the signs and symptoms of PE are similar to those of many other cardiovascular and pulmonary diseases. Moreover, some clinical situations such as chronic obstructive pulmonary disease or congestive heart failure may mask PE but unexpected symptom deterioration or lack of improvement following an appropriate treatment strategy should provoke the clinician’s suspicion. Clearly the first diagnostic procedure to prescribe for a patient with suspected embolism and signs or symptoms of acute deep vein thrombosis is a duplex ultrasonography of the legs given its availability, sensitivity, specificity and cost.

When obstruction of the pulmonary vascular tree is rapid and complete or the basal clinical situation is compromised, there is an emergency situation with syncope or cardiogenic shock or prolonged hypotension. The physical examination may reveal tachycardia, tachypnea, or cyanosis. Signs of acute right ventricular dysfunction such as distended neck veins, a parasternal heave, an accentuated P2, and a tricuspid regurgitation murmur may be present. The ECG is occasionally normal but more often it will have some abnormality such as a sinus tachycardia, an S1Q3T3 pattern, T-wave inversions in V1 to V4, or a pseudoinfarction pattern (Qr) in V1-4. A chest X-ray may show cardiac and/or pulmonary-artery enlargement and/or oligemia of the embolised lung.

In the absence of clear signs of PE, a differential diagnosis of myocardial infarction, cardiac tamponade, severe bleeding, pneumothorax, aortic dissection and septicemia should be considered. The best tool to exclude other clinical situations mimicking PE in the emergency room is transthoracic echocardiography (TTE) but it should be underlined that TTE is diagnostic for PE only in those cases in which there is a direct visualization of thrombi in the right atrium, the right ventricle or the pulmonary artery. In other cases TTE can demonstrate the effect of pulmonary hypertension on the right ventricle but it is not helpful for making a diagnosis. Some authors, however, have found a high specificity with low sensitivity for TTE when the combination of dilated right ventricle and an intensity of tricuspid regurgitation of more than 2.7 m/sec are utilized.1 If low quality of images are obtained by TTE or in case of cardiopulmonary resuscitation, a trans-esophageal echocardiography may be considered.2 When other possible causes have been excluded, the diagnosis can generally be made on the basis of computed tomography (CT) scan or pulmonary angiography. The latter may be preferred if the use of interventional catheter devices are being hypothesized or surgical thrombectomy is being considered.

When the obstruction of pulmonary
Table 1. Pre-test probability of PE.3

<table>
<thead>
<tr>
<th>Signs or symptoms of DVT</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Alternative diagnosis is less likely than PE</td>
<td>3</td>
</tr>
<tr>
<td>Heart rate &gt; 100/min</td>
<td>1.5</td>
</tr>
<tr>
<td>Immobilization or surgery in the previous 4 weeks</td>
<td>1.5</td>
</tr>
<tr>
<td>Previous DVT/PE</td>
<td>1.5</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>1</td>
</tr>
<tr>
<td>Active cancer</td>
<td>1</td>
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<tr>
<td>Probability</td>
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<td>High</td>
<td>&gt;4</td>
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<td>Low</td>
<td>≤4</td>
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vessels is only partial in non-compromised patients, dyspnea, chest pain or cough are frequent initial complaints although the only symptom is sometimes malaise or agitation. In this case, a differential diagnosis from pulmonary edema, pneumonia, airways obstruction, or atelectasia should be made. A physical examination, ECG, chest X-ray and blood gases may or may not be useful for reinforcing the hypothesis of PE. In contrast, the clinical scenario is crucial in assessing the likelihood of PE, as recent surgery or trauma, cancer, prolonged bed rest, pregnancy or puerperium, elderly age as well as a positive family history of venous thromboembolism, thrombophilia, and the use of oral contraceptive may substantiate the diagnostic hypothesis. Wells and colleagues3 have developed a rapid seven-feature bedside assessment. A score of 4 or less indicates PE as unlikely and only about 5% of patients in this low-risk group were subsequently found to have PE. Table 1 reports the seven features of the assessment: clinical signs and symptoms of DVT (3 points); an alternative diagnosis is less likely than PE (3 points); heart rate above 100 bpm (1.5 points); immobilization or surgery in the previous 4 weeks (1.5 points); previous thromboembolism (1.5 points); hemoptysis (1 point); active cancer (1 point). In case of clinical suspicion and a score of more than 4, the above tests must be made so a diagnosis may be made. The exception to this rule are patients with a low a priori probability and a negative D-dimer level as this combination excludes PE.5,6 It should be mentioned here that the measurement of the degradation products of cross-linked fibrin (D-dimer) is a highly sensitive but non-specific screening test for suspected venous thromboembolism. Elevated levels are present in nearly all patients with embolism but are also associated with many other clinical situations, including advanced age, pregnancy, trauma, postoperative periods, inflammatory states, and cancer. The role of D-dimer testing is, therefore, limited to ruling out embolism.

Ventilation/perfusion lung scanning has been used for the past 30 years as the imaging procedure of choice for the evaluation of patients with suspected PE. A negative perfusion lung scan excludes PE while large perfusion defect(s) with normal ventilation lung scans are diagnostic.6,7 As most lung scans do not provide conclusive results, attempts are being made to identify alternative or complementary diagnostic procedures that can minimize the need for pulmonary angiography. These include clinical models, D-dimer assays, compression ultrasound (CUS), spiral CT imaging and nuclear magnetic resonance (NMR). Modern spiral CT scan, a particularly promising procedure, is rapid and able to exclude PE when associated with negative D-dimer and negative CUS results.5,9 As spiral CT scan has a low sensitivity for small subsegmental emboli, other diagnostic tools should be considered in patients with a negative CT scan, a high pre-test probability and/or D-dimer positivity.5,9,11

The choice of diagnostic tests may vary in certain clinical circumstances. Patients with previous PE represent a particular diagnostic challenge as it is not possible to distinguish between an unresolved previous thromboembolism and a new one. In high risk patients a perfusion lung scan after 3 to 6 months of treatment would be useful to judge a following recurrence. Pulmonary angiography may help to distinguish acute from chronic thromboembolism when the pulmonary artery pressure is higher than 50 mmHg. Ventilation-perfusion scanning is of limited usefulness, given the high probability of non-diagnostic results in patients with chronic obstructive pulmonary disease. A spiral CT scan is a better approach as it provides information on lung parenchyma and other structures.

The use of contrast medium required for CT scanning poses the risk of tubular necrosis in patients with renal insufficiency. Ventilation perfusion scanning and duplex ultrasonography are preferable tests in these patients. Pulmonary embolism is the first cause of death during pregnancy. The first diagnostic approach during pregnancy should be duplex ultrasonography. In case of negativity and high clinical probability, a ventilation-perfusion lung scan should be made using the lowest possible amount of radioactive material. In the case that the above mentioned diagnostic procedures fail, phlebography or CT may be used to prove or exclude the diagnosis.

Treatment

Treatment options in critically ill patients comprise thrombolytic therapy, interventional catheter devices for emboli removal, extracorporeal membrane oxygenation (ECMO) and surgical thrombectomy.12 Which of these options should be used is decided taking into account that the in-hospital mortality of these patients depends on the degree of hemodynamic imbalance. The rate of in-hospital mortality of
patients with hypotension, cardiogenic shock and cardiopulmonary resuscitation is 15.2%, 24.2% and 64.8%, respectively.13 Thrombolytic therapy can be administered as reported in Table 2. Administration of a bolus (10 mg) of recombinant tissue plasminogen activator (r-tPA) is the best procedure in critically ill patients. In fact, it is the authors’ experience that this approach can quickly reverse the clinical picture. In this case, a further infusion of 90 mg of r-tPA over 2 hours is recommended. Other options should be considered if patients do not show improvement after 5 days.

The use of thrombolytic therapy is controversial in patients with stable hemodynamic equilibrium. Apart from the general contraindications including recent surgery or neurological accidents, these drugs may not be effective when symptoms have been present for more than 5 days and may not be safe in elderly patients (those 75 years of age or older) who are more exposed to severe bleeding complications. The problem is that hemodynamically stable patients may present signs of right ventricle overload at echocardiography, a finding that increases the risk of death within 2 months’ time.14 When these patients were randomized to receive standard heparin or heparin plus alteplase (100 mg over 2 hours), there was no difference in the mortality or in bleeding complications but the short term clinical course was more severe in patients treated with heparin only.15 It thus appears premature to treat all these patients indiscriminately with thrombolytic therapy and it is urgent to identify with greater precision patients with a worse prognosis and those who may develop chronic thromboembolic pulmonary hypertension (CTPH). In this respect, stable patients with higher troponin levels, similar to those found in myocardial infarction at presentation, may be those with a poor short term prognosis.16,17 Likewise, high concentrations of brain natriuretic peptide may predict an adverse outcome.18 Moreover, a recent cohort study by our group showed that patients at risk of CTPH are young subjects with severe idiopathic PE and previous episodes of PE.19

Heparin is the treatment of choice for patients in a stable condition and should be initiated when there is only a clinical suspicion and before the diagnosis is made.20 A bolus of 5,000-10,000 U of unfractionated heparin is usually administered in these patients, followed by continuous infusion of 1,000-1,500 U per hour to prolong the activated prothrombin time (aPTT) to 1.5 to 2.5 times the normal value. The potential problem with unfractionated heparin infusion is the failure to achieve adequate, rapid anticoagulation using empirical dose adjustments. Utilizing weight-based nomograms helps patients attain a therapeutic degree of anticoagulation more quickly.21 Heparin treatment should be prolonged for at least 5 days and longer in severe cases. Low molecular weight heparin (LMWH) is equally effective and safe in stable patients with PE.22 Its major adverse effect is hemorrhage but heparin-induced thrombocytopenia (HIT) with thrombosis is potentially more serious and this occurs more frequently when unfractionated heparin is used. The platelet count should be measured when heparin treatment is begun, repeated 3-5 days later and once again if heparin treatment is prolonged. In the case of HIT, heparin should be withdrawn and treatment with a direct thrombin inhibitor, generally lepirudin, initiated.

For patients with contraindications to anticoagulants and for those with recurrent venous thromboembolism despite adequate anticoagulation, a filter in the inferior vena cava should be inserted.23 Although filters prevent most recurrent PE, patients with filters are more likely than those without to develop deep vein thrombosis during the next few years. Anticoagulation should therefore be resumed as soon as possible after insertion. Another kind of filter now available, called optional, can be removed 10 days to 12 months later or, if necessary, be left in situ, as a permanent filter.

Oral anticoagulants should be initiated during the first or second day of heparin treatment and discontinued when the International Normalized Ratio is more than 2 for two consecutive days. While antivitamin K drugs are generally recommended for variable intervals of time following the initial treatment period, the long-term use of low-molecular-weight heparins is likely to be more effective than oral anticoagulants for the secondary prevention of PE in cancer patients. New categories of drugs are emerging, which may replace conventional anticoagulants in the near future. These include anti-Xa inhibitors.

### Table 2. Recommended scheme when using thrombolytic drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bolus (Ul/v)</th>
<th>Maintenance dose (Ul/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptokinase</td>
<td>250,000</td>
<td>100,000 (24 hours)*</td>
</tr>
<tr>
<td>Streptokinase</td>
<td>100,000</td>
<td>100,000 (12 hours)</td>
</tr>
<tr>
<td>Streptokinase</td>
<td>1,500,000</td>
<td>1,500,000 (2 hours)</td>
</tr>
<tr>
<td>Urokinase</td>
<td>4,400</td>
<td>4,400 (12 hours)*</td>
</tr>
<tr>
<td>Urokinase</td>
<td>3,000,000</td>
<td>3,000,000 (2 hours)</td>
</tr>
<tr>
<td>Urokinase</td>
<td>2,000</td>
<td>2,000 (2 hours)</td>
</tr>
<tr>
<td>Alteplase</td>
<td>100 mg</td>
<td>100 mg (2 hours)**</td>
</tr>
</tbody>
</table>

*FDA approved; \( * \) or 10 mg i.v. bolus and 90 mg i.v. (2 hours).
such as pentasaccharide, and anti-thrombin inhibitors, such as ximelagatran.

References


Low molecular weight heparins as antitumor agents

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ABSTRACT

LMWH clearly have an important role in the treatment of thrombotic complications in patients with cancer. However, the accumulating evidence from both basic and clinical studies now challenges clinicians to look beyond the antithrombotic effects of these agents and consider their potential as antineoplastic agents. Given the wide variety of biological activities that have been demonstrated in vitro and in vivo, it is not surprising that it has been difficult to identify the exact mechanisms by which LMWH or heparin may interrupt the neoplastic process. Laboratory evidence to date suggests that LMWH are capable of targeting multiple pathways to suppress tumor growth and metastasis and that different tumor types may be susceptible to such activities. Data from clinical trials are encouraging but definitive conclusions about a true antineoplastic effect cannot be drawn given the methodologic limitations of the trials. The optimal dose, regimen and agent remain uncertain. However, because LMWH are relatively less toxic than many of the available chemotherapeutic agents, it is certainly worthwhile pursuing this novel approach to the management of patients with cancer.

Low molecular weight heparins (LMWH) have improved and simplified the treatment of venous thromboembolism (VTE). Since their introduction two decades ago, they have replaced unfractionated heparin as the standard initial treatment for deep vein thrombosis (DVT) and pulmonary embolism (PE). Recent data have also established that monotherapy with LMWH is superior to vitamin K antagonists in preventing recurrent VTE in patients with cancer. These are significant medical advances that have improved quality of care and quality of life.

More recently, however, basic and clinical research activities have focused on the potential antineoplastic effects of LMWH. Although the concept that anticoagulants may have an impact on tumor growth or progression is not new or unique to LMWH, previous studies that evaluated the efficacy of warfarin and unfractionated heparin for the treatment of cancer were inconclusive. Interest in the potential role of antithrombotic agents as anticancer agents was renewed when retrospective analyses of clinical trials in patients with thrombosis showed that LMWH provided a survival advantage in the subgroup of patients with cancer. This unexpected observation is now supported by results of small clinical trials that were specifically designed to examine the effect of LMWH on survival in patients with cancer. Although statistically significant improvements in overall survival were reported in patients who were randomly assigned to receive a LMWH in these trials, whether the survival benefit is derived from an anticoagulant effect-secondary to a reduction in fatal pulmonary embolism-or a true antineoplastic effect remains unanswered. Multiple anticancer mechanisms, including inhibition of tumor angiogenesis, interference with tumor cell adhesion, and suppression of tumor cell invasion, have been demonstrated in experimental models, but none have been definitively confirmed in vivo in human cancers. This review will briefly outline some of these mechanisms and summarize the clinical data from published randomized studies.

Potential antineoplastic mechanisms

Inhibition of angiogenesis

Tissue factor (TF) is a transmembrane protein that is the primary activator of normal coagulation by binding with activated factor VII (FVIIa). Normally expressed on subendothelial stromal cells such as fibroblasts, TF is also found constitutively on malignant cells and its expression can be induced on endothelial cells and monocytes under pathological conditions.

Studies using TF-knockout mice and
Inhibition of cellular adhesion

P- and L-selectin are cell adhesion/signaling receptors that normally mediate the interactions of leukocytes and activated platelets with one another, as well as with endothelial cells lining blood vessels. Since heparin is able to inhibit P- and L-selectin recognition of mucin-like glycoprotein ligands that are found on normal cells and tumor cells, it has the potential to interfere with the cellular binding and adhesion that is necessary for tumor cell metastasis. Indeed, experiments have shown that while heparin effectively inhibited the metastatic progression of a P-and L-selectin-mediated mouse model of carcinoma, it had limited efficacy in mice deficient in these selectins.

Modulation of integrin-mediated adhesion of cancer cells to vitronectin by heparin may be another pathway by which heparin inhibits tumor cell invasion and metastasis. Modified heparin with low anticoagulant activity has been shown to inhibit the extent of lung metastases following intravenous injection of tumor cells in experimental models.

Inhibition of tumor invasion

Heparanase is an endo-β-D-glucuronidase involved in the degradation and remodeling of the extracellular matrix by cleaving heparan sulfate. Not surprisingly, experimental models have shown that heparanase expression by neoplastic cells contributes to the invasive and metastatic properties of the cells. Many different tumor types show over expression of the heparanase gene and enzyme, including those of the breast, prostate, colon, esophagus, pancreas, as well as hematological malignancies. Consequently, inhibition of heparinase activity by heparins is a promising target for anticancer drug development.

Clinical studies

The initial observation suggesting that LMWH may have an antineoplastic effect was reported in meta-analyses of clinical trials that compared LMWH with unfractioned heparin for the initial treatment of acute VTE. These studies consistently demonstrated a statistically significant reduction in mortality in favor of LMWH, but the effect was observed only in the subgroup of patients with cancer. Furthermore, the difference was not explained by a reduction in fatal PE or bleeding. However, none of these trials was designed with survival as the primary outcome and potential biases or imbalances in prognostic factors in the patients with cancer could not be ruled out. Also, it was difficult to explain how a one-week course of an anticoagulant could exert such a dramatic effect on the natural history of malignancy. Nonetheless, the observation triggered renewed interest in the anticancer effects of anticoagulants.

In order to determine specifically whether LMWH can improve survival of cancer patients, a number of randomized trials have now been completed and several are ongoing. The FAMOUS study was the first randomized, placebo-controlled trial to examine the effect of a low dose of LMWH on survival in patients with cancer. Three hundred and eighty-five patients with advanced solid tumors were randomized to the LMWH dalteparin 5000 IU once daily or placebo for up to one year. Overall, 64% of the patients had stage IV disease and the groups were balanced in other prognostic markers of survival. The primary outcome was overall survival at one year. According to an intention-to-treat analysis, the survival estimates for patients receiving placebo at 1, 2 and 3 years after randomization were 41%, 18%, and 12%, respectively, while the corresponding estimates for patients in the dalteparin group were 46%, 27%, and 21%. The trend for survival benefit, however, was not statistically significant (p=0.19). Nevertheless, in a post-hoc analysis of patients who survived beyond 17 months from randomization, there was a statistically significant improvement in survival in favor of the LMWH. This observation suggested that LMWH might have a great impact on survival in patients with early or limited disease.

Shortly following the publication of these results, two studies came out concurrently that gave further support to the hypothesis. In a post-hoc analysis of a randomized trial comparing long-term administration of dalteparin with coumarin derivatives for pre-

other transgenic techniques have now firmly established that TF is also a critical promoter of angiogenesis via intracellular signaling pathways. Through the up-regulation of angiogenic factors such as vascular endothelial growth factor and interleukin, the down-regulation of the anti-angiogenic factor thrombospondin, TF is capable of tipping the angiogenic balance towards a more pro-angiogenic phenotype. The process may occur via a clot-dependent pathway, in which thrombin generated from TF/FVIIa activation leads to subsequent interaction with protease-activated receptors (PAR) 1, 3, and 4, or via a clot-independent pathway initiated by the interaction of the cytoplasmic domain of TF and PAR-2. Therefore, anticoagulants may exert a negative impact on tumor angiogenesis by interfering with thrombin activity or TF/FVIIa activation. Furthermore, the enhanced release of tissue factor pathway inhibitor induced by LMWH as compared with heparin, as well as the greater attenuation of basic fibroblast growth factor-induced angiogenesis by low molecular weight fractions of heparin, may explain why LMWH may be superior to heparin as anticancer agents.
vent the effect of dalteparin in patients with newly diagnosed small cell lung cancer. A total of 84 patients were randomized to chemotherapy plus dalteparin 5000 IU once daily for 18 weeks or chemotherapy alone. The median progression-free survival was 10.0 months in the combined therapy group versus 6.0 months in the chemotherapy alone group (p=0.01) and the median overall survival was 13.0 months and 8.0 months (p=0.01), respectively. The overall tumor response to treatment was also better in the dalteparin-treated group but the difference was not statistically significant. For the first time, LMWH was used as an adjuvant agent. Although the results are certainly encouraging, clear conclusions about the potential benefits of LMWH and long-term survival remain premature because the study was small.

References


Von Willebrand disease (VWD) is, together with hemophilia A, the most frequent bleeding disorder, with a prevalence of 66 to 100 cases per million in the general population, taking patients referred for clinical manifestations of bleeding as a basis for the estimate.1 Much higher prevalences (1 per 100) are reported in population-based studies, but the clinical relevance of many of these cases is uncertain.1 This year (2006) marks the 80th anniversary of the first description of the disease by the Finnish pediatrician Erik von Willebrand, who used to reach his patients with a rowing boat in the Aland Islands’ archipelago. Over nearly a century, a lot of progress has been made in our understanding of von Willebrand factor (VWF), the protein deficient or defective in VWD, as well as of the molecular basis, natural history and treatment of the disease.

**Structure-function of von Willebrand factor**

VWF is a large circulating glycoprotein synthesized by endothelial cells and megakaryocytes.2 The gene encoding VWF, located on chromosome 12p13.2, is a large gene that spans 178 kilobases of DNA and contains 52 exons. A non-coding, highly homologous pseudogene was identified in chromosome 22, spanning the gene sequence from exon 23 to 34.3 The primary product of the VWF gene is a 2,815 amino acid protein made of a signal peptide of 22 amino acids, an unusually large propeptide of 741 amino acids and a mature subunit of 2,050 amino acids. In keeping with a recently proposed nomenclature,4 numbering of VWF starts from the first amino acid of the signal peptide, so that number 764 is the first amino acid of the mature protein. Different regions, corresponding to four types of repeated protein domains (D1, D2, D3, D4, A1, A2, A3, A4, B, C1, C2), are responsible for the different functions of VWF. Mature VWF is the result of ordered maturation steps as it moves along the secretory pathway of endothelial cells, leading to storage in Weibel-Palade bodies and then to constitutive or regulated secretion of a huge multimeric glycoprotein. Circulating VWF, which is mainly derived from the endothelium has two major functions in hemostasis.5 It is essential for platelet adhesion to the subendothelium, platelet-to-platelet interactions and platelet aggregation in vessels such as small arteries and large stenotic arteries in which rapid blood flow results in high shear stress. Adhesion is promoted by the interaction of a region of the A1 domain with the platelet membrane glycoprotein Ibα (GpIbα).5 High shear stress activates the A1 domain of VWF bound to subendothelial collagen by stretching the largest multimers into filamentous forms. The interaction between GpIbα and VWF can be mimicked in platelet-rich plasma by ristocetin, which promotes binding of VWF to GpIbα. Aggregation of platelets within the growing hemostatic plug is promoted by the interaction of VWF with another platelet receptor, glycoprotein IIb-IIIa (or integrin αIIbβ3), which once activated binds to VWF and fibrinogen to recruit more platelets into a stable plug. Both these binding activities are highly expressed by the largest VWF multimers.5 VWF is also the carrier of factor VIII (FVIII) in plasma. VWF protects FVIII from proteolytic degradation, prolonging its half-life in the circulation and efficiently localizing it at the site of vascular injury.5 Each VWF monomer has one FVIII binding domain located in the first 272 amino acids of the mature subunit (D-domain). Therefore, any change in plasma VWF level is usually associated with a parallel change in FVIII. In this review article we use the recently recommended nomenclature and abbreviations of FVIII/VWF activities.5

**Classification of VWD**

The current classification of VWD identifies two major types, characterized by
quantitative (types 1 and 3) or qualitative (type 2) VWF defects. A partial quantitative defect is the hallmark of type 1, whereas type 3 is characterized by the nearly total absence of VWF in plasma and platelets. Type 1 is easily distinguished from type 3 by milder VWF deficiency (usually in the range of 10–30 U/dL), its autosomal recessive dominant pattern of inheritance (autosomal Table 3 and the presence of a milder bleeding tendency. In the past type 1 was reported to be the most frequent form of VWD, accounting for approximately 70% of cases. A recent study based on the reappraisal of diagnoses of type 1 after 10 years (1994-2004) in 1234 patients followed by 16 Italian Centers established that only 671/1234 (54%) had VWD type 1, because many cases previously diagnosed as type 1 were re-diagnosed as having type 2 due to discrepant VWF measurements (ratio of ristocetin cofactor activity (VWF:RCo) to VWF:Ag <0.7). Four type 2 subtypes have been identified, reflecting different pathophysiological mechanisms. Types 2A and 2B lack high molecular weight VWF multimers in plasma but in type 2B there is also an increased affinity of VWF for GpIba. In subtype 2M there are qualitatively abnormal variants with decreased platelet-dependent function and a normal multimeric structure. Type 2N shows a full array of multimers, the defect being in the N-terminal region of the VWF where the binding domain for FVIII is located. This type is phenotypically distinguishable from mild hemophilia A by the abnormal binding of FVIII to VWF (VWF:FVIIIB). The current classification of VWD, summarized in Table 1, was published in 1994. A working party is preparing an updated classification.

Clinical manifestations
Clinical manifestations are excessive mucocutaneous bleeding and prolonged oozing after surgical procedures. In women menorrhagia may be the only clinical manifestation. Soft tissue and joint bleeding is rare, except in patients with type 3 VWD and severe deficiencies of VWF and FVIII (prevalence approximately 1 per million in the general population). The clinical expression of the disease is usually mild in most patients with type 1, whereas severity increases in type 2 and particularly in type 3. Generally, the severity of bleeding correlates with the degree of reduction of VWF:RCo and FVIII. To date, only few detailed descriptions of symptoms are available. Table 2 shows the relative frequency of bleeding symptoms in three large series of patients diagnosed at specialized centers. Several attempts were recently made to evaluate the sensitivity and specificity of bleeding symptoms as disease predictors, especially in the mild cases with type 1 VWD and VWF:RCo levels >20U/dL. In a multicentre study carried out in obligatory carriers of type 1 VWD, men-

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>VWD Symptoms</th>
<th>Iranians (n=348)</th>
<th>Italians (*)</th>
<th>Scandinavians</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 3 (1=671)</td>
<td>Type 2 (n=497)</td>
<td>Type 3 (n=66)</td>
<td>VWD (n=264)</td>
</tr>
<tr>
<td><strong>Epistaxis</strong></td>
<td>77</td>
<td>61</td>
<td>63</td>
<td>66</td>
</tr>
<tr>
<td><strong>Menorrhagia</strong></td>
<td>69</td>
<td>32</td>
<td>32</td>
<td>56</td>
</tr>
<tr>
<td><strong>Post-extraction bleeding</strong></td>
<td>70</td>
<td>31</td>
<td>39</td>
<td>53</td>
</tr>
<tr>
<td><strong>Hematomas</strong></td>
<td>n. r.</td>
<td>13</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td><strong>Bleeding from minor wounds</strong></td>
<td>n. r.</td>
<td>36</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td><strong>Gum bleeding</strong></td>
<td>n. r.</td>
<td>31</td>
<td>35</td>
<td>56</td>
</tr>
<tr>
<td><strong>Post-surgical bleeding</strong></td>
<td>41</td>
<td>20</td>
<td>23</td>
<td>41</td>
</tr>
<tr>
<td><strong>Post-partum bleeding</strong></td>
<td>15</td>
<td>17</td>
<td>18</td>
<td>26</td>
</tr>
<tr>
<td><strong>Gastrointestinal bleeding</strong></td>
<td>20</td>
<td>5</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td><strong>Joint bleeding</strong></td>
<td>37</td>
<td>3</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td><strong>Hematuria</strong></td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td><strong>CNS bleeding</strong></td>
<td>n. r.</td>
<td>1</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

n. r.: not reported; (*) Bleeding symptoms in Italian patients have been recently recalculated according to the updated results of the Italian Registry of VWD and therefore are different from those previously reported.
orrhagia and epistaxis were poor predictors of the disease while cutaneous bleeding and bleeding after dental extractions were more sensitive symptoms for diagnosis. A bleeding score has been developed (Table 3) and was validated in affected and non-affected members of 154 families enrolled prospectively in a large European study, as well as in 200 normal individuals.15

**Laboratory diagnosis**

The bleeding time, the original hallmark of the disease, is not always prolonged and may be normal in patients with mild forms, such as those with type 1 and normal platelet VWF content.16 Hence, it is not particularly useful for diagnosis. The Platelet Function Analyzer (PFA-100) gives a rapid and simple measure of VWF-dependent platelet function at high shear stress: it can be performed in whole blood and therefore can be employed instead of the bleeding time in children or when the bleeding time is not feasible. The PFA-100 is sensitive and reproducible for VWD screening, but is not specific.

The differential diagnosis of VWD types can be performed following the flow chart shown in Figure 1. Type 3 VWD is diagnosed when VWF:Ag is unmeasurable or less than 1 U/dL. A proportionate reduction of both VWF:Ag and VWF:RCo, with a RCo/Ag ratio > 0.7, suggests type 1 VWD. If the VWF:RCo/Ag ratio is < 0.7, type 2 is diagnosed. Type 2B VWD is diagnosed when ristocetin-induced platelet aggregation (RIPA) is heightened (<0.8 mg/mL), whereas types 2A and 2M are usually associated with low RIPA (>1.2 mg/mL). Multimeric analysis of plasma VWF is necessary to distinguish between type 2A VWD (lack of the largest and intermediate multimers) and type 2M VWD (all the multimers present). Type 2N VWD can be suspected in case of discrepant values for FVIII and VWF:Ag (ratio <1) and diagnosis should be confirmed by the specific test (g) of VWF factor VIII binding capacity (VWF:FVIII-B). In type 1 VWD the ratio between Factor VIII and VWF:Ag is always ≥1 and the severity of type 1 VWD phenotype can usually be evaluated from platelet VWF (f) measurements.11

**Molecular diagnosis**

A registry of mutations identified in types 2A, 2B, 2M and 2N is available in a web site (www.shef.ac.uk/vwf). Most type 2A cases are due to missense mutations in the A1 domain, with R1597W or Q or Y and S1506L accounting for about 60% of them.17 The majority of type 2B cases are due to missense mutations in the A1 domain, about 90% being caused by R1306W, R1308C, V1316M and R1341Q.17 A few heterogeneous mutations, also located within the A1 domain, underlie type 2M. A recurrent mutation in type 2M Vicenza has been identified in families from Europe (R1205H); another mutation (M740I) is seen exclusively in families from the area around Vicenza in north east Italy.18,19 Missense mutations in the FVIII-binding domain at the amino-terminal portion of VWF are responsible for type 2N.10

The genetic causes of type 1 VWD, are still elusive in many cases, especially in those with a mild phenotype. More information on the molecular basis of type 1 will be available when the data of an European project are published in 2006. In the study, recruitment was based on the historical diagnosis of
type 1 VWD as made by 12 expert centers, which included 278 affected cases, 312 non-affected family members and 1166 controls. Three broad groups of patients were identified: 53 had a normal multimeric structure, a VWF:RCo/Ag ratio \( \geq 0.7 \) and mutations in the VWF gene; 55 had VWF gene mutations but abnormal multimers and a ratio <0.7, and 43 had normal multimers, a ratio \( \geq 0.7 \) but no detectable mutation. From these preliminary data it is apparent that with the current criteria only the first group of 53 patients would fit the diagnosis of type 1 VWD, whereas the 55 patients of the second group would be classified as having type 2 and the 43 patients of the third group would not be bona fide cases of inherited VWD. In type 3 VWD, partial or total gene deletions were initially reported. Gene defects in type 3 patients from different populations were subsequently identified, the majority of them being null mutations predicting no VWF production.

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### Table 3. Bleeding score used to evaluate the bleeding history (see reference 15).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Score</th>
<th>-1</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epistaxis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No or trivial (less than 5)</td>
<td>&gt; 5 or more than 10'</td>
<td>Consultation only</td>
<td>Packing or cauterization or antifibrinolytic</td>
<td>Blood transfusion or replacement therapy or desmopressin</td>
<td></td>
</tr>
<tr>
<td>Cutaneous</td>
<td></td>
<td>No or trivial (&lt; 1 cm)</td>
<td>&gt; 1 cm and no trauma</td>
<td>Consultation only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleeding from minor wounds</td>
<td></td>
<td>No or trivial (less than 5)</td>
<td>&gt; 5 or more than 5'</td>
<td>Consultation only</td>
<td>Surgical hemostasis</td>
<td>Blood transfusion or replacement therapy or desmopressin</td>
<td></td>
</tr>
<tr>
<td>Oral cavity</td>
<td></td>
<td>No</td>
<td>Reported at least one</td>
<td>Consultation only or antifibrinolytic</td>
<td>Surgical hemostasis</td>
<td>Blood transfusion or replacement therapy or desmopressin</td>
<td></td>
</tr>
<tr>
<td>GI bleeding</td>
<td></td>
<td>No</td>
<td>Associated with ulcer, portal hypertension, hemorrhoids, angiodysplasia</td>
<td>Spontaneous</td>
<td>Surgical hemostasis, blood transfusion, replacement therapy, desmopressin, antifibrinolytics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tooth extraction</td>
<td></td>
<td>No bleeding in at least 2 extractions</td>
<td>None done or no bleeding in 1 extraction</td>
<td>Referred in &gt;25% of all procedures, no intervention</td>
<td>Resuturing or packing</td>
<td>Blood transfusion or replacement therapy or desmopressin</td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td>No bleeding in at least two surgeries</td>
<td>None done or no bleeding in 1 surgery</td>
<td>Referred in &gt;25% of all procedures, no intervention</td>
<td>Surgical hemostasis or antifibrinolytic</td>
<td>Blood transfusion or replacement therapy or desmopressin</td>
<td></td>
</tr>
<tr>
<td>Menorrhagia</td>
<td></td>
<td>No</td>
<td>Consultation only</td>
<td>Antifibrinolytics, pill use</td>
<td>Dilatation &amp; curettage, iron therapy</td>
<td>Blood transfusion or replacement therapy or desmopressin or hysterectomy</td>
<td></td>
</tr>
<tr>
<td>Post-partum hemorrhage</td>
<td></td>
<td>No bleeding in at least two deliveries</td>
<td>No deliveries or no bleeding in 1 delivery</td>
<td>Consultation only</td>
<td>Dilatation &amp; curettage, iron therapy, antifibrinolytics</td>
<td>Blood transfusion or replacement therapy or desmopressin</td>
<td>Hysterectomy</td>
</tr>
<tr>
<td>Muscle hematomas</td>
<td></td>
<td>Never</td>
<td>Post trauma no therapy</td>
<td>Spontaneous, no therapy</td>
<td>Spontaneous or traumatic, requiring desmopressin or replacement therapy</td>
<td>Spontaneous or traumatic, requiring surgical intervention or blood transfusion</td>
<td></td>
</tr>
<tr>
<td>Hemarthrosis</td>
<td></td>
<td>Never</td>
<td>Post trauma no therapy</td>
<td>Spontaneous, no therapy</td>
<td>Spontaneous or traumatic, requiring desmopressin or replacement therapy</td>
<td>Spontaneous or traumatic, requiring surgical intervention or blood transfusion</td>
<td></td>
</tr>
<tr>
<td>CNS bleeding</td>
<td></td>
<td>Never</td>
<td>-</td>
<td>-</td>
<td>Subdural, any intervention</td>
<td>Intracerebral, any intervention</td>
<td></td>
</tr>
</tbody>
</table>
Treatment

The goal of treatment is to correct the dual defects of hemostasis, i.e., abnormal platelet adhesion due to low or defective FVIII and abnormal intrinsic coagulation due to low FVIII [for review and additional references, see ref. 24]. Two weapons are available: desmopressin which releases endogenous VWF from endothelial cells, and exogenous VWF contained in FVIII/VWF plasma-derived concentrates.

Desmopressin

Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is a synthetic analog of vasopressin that is relatively inexpensive and carries no risk of transmitting blood-borne infectious agents. DDAVP, infused intravenously at a dose of 0.3 µg/Kg diluted in 50 mL saline over 30 minutes, usually increases plasma FVIII/VWF 3 to 5 times above baseline levels within 30-60 minutes and, in general, high FVIII/VWF levels last for 6 to 8 hours. Because the responses in a given patient are consistent on different occasions, a test dose of DDAVP at the time of diagnosis helps to establish the individual response pattern.25 The protocol of the test infusion, with the clinical and laboratory parameters to be measured, are reported in detail by Federici et al.23 DDAVP infusions can be repeated every 12 to 24 hours depending on the type and severity of the bleeding episode. However, most patients repeatedly treated become less responsive to therapy. The drug is also available in concentrated forms for subcutaneous and intranasal administration, which can be convenient for home treatment. Despite the widespread use of DDAVP in the treatment of VWD, there are no prospective clinical studies on efficacy and safety aimed at determining the benefits and limits of this therapeutic weapon.25

Transfusional therapies. FVIII/VWF concentrates are the first choice for treatment in patients with type 3 VWD; it is also used in patients with type 2B, because DDAVP can induce transient thrombocytopenia, and in patients with types 1 and 2 who are unresponsive to DDAVP or have contraindications to its use.25 Among several concentrates containing VWF, only four have been extensively evaluated in pharmacokinetic trials as well as in retrospective or prospective studies.24

Haemate P/Humate P. This intermediate-purity FVIII/VWF concentrate has been widely used and is considered the gold standard for treatment of VWD. The product was introduced into clinical practice in Europe in 1984 (Haemate P) and in the United States in 1999 (Humate-P). Prospective and retrospective data collected in USA, Canada and Italy, showed excellent-good responses in 99% of surgical cases and in 97% of bleeding episodes. The prospective Alphanate Study Group published results on pharmacokinetics and clinical efficacy, showing that 75% of bleeding episodes were controlled with one or two infusions of this FVIII/VWF concentrate, and that 71% of patients who received treatment before surgery or invasive procedures had good clinical responses. In another retrospective study, 22 Italian patients were treated with Fanhdi, a FVIII-VWF concentrate similar to Alphanate. Excellent-good clinical responses were obtained in 92% of bleeding episodes and in 93% of surgical procedures.25 Another plasma-derived VWF concentrate with low FVIII levels was first introduced in France in 1992. An improved version of this concentrate, almost devoid of FVIII and treated with three virus-inactivation methods, was evaluated in two large French and European studies. As expected, there was an approximate 6-hour delay before FVIII increased with this concentrate, so that the additional administration of FVIII is recommended in cases of acute life-threatening bleeds or emergency surgery.26

The aforementioned data derived from pharmacokinetic and clinical studies have contributed to helping the clinical choice of FVIII/VWF concentrates. The accumulation of FVIII that is exogenously infused, together with that endogenously synthesized and stabilized by infused VWF, may cause very high FVIII levels when multiple infusions are given to cover major surgery.24 Sustained high levels of FVIII may increase the risk of deep vein thrombosis: however, this is a rare event reported only in patients with concomitant risk factors.25 When repeated injections of FVIII/VWF concentrates are used to control recurrent bleeding episodes and to prevent excessive bleeding after major surgery, daily monitoring of FVIII levels and adjustment of the concentrate dosage are necessary to keep FVIII levels between 50 and 150 U/dL. The minimal VWF:RCo levels needed to secure hemostasis are not well established. Retrospective data from a large cohort of well characterized Italian patients suggest that levels > 30 U/dL are associated with a low rate of spontaneous mucosal bleedings.7 The treatments recommended according to VWD types are summarized in Table 4.

Treatment of patients with anti-VWF allo-antibodies.

For the rare patients with type 3 VWD who develop anti-VWF allo-antibodies after multiple transfusions, the use of VWF-containing concentrates not only is ineffective, but may also cause severe post-infusion anaphylaxis due to the formation of immune complexes. Recombinant FVIII can be used to avoid this complication, because this product, containing no VWF, does not cause anaphylactic reactions. Due to the very short half-life of FVIII without its VWF car-
rrier, recombinant FVIII must be administered at very large doses by continuous i.v. infusion to keep plasma levels above 50 U/dL.28,29 Another possible therapeutic approach is recombinant activated factor VII, which can be used at the same dosages and with the same regimens as those employed for hemophilia A patients with inhibitors.30

Secondary prophylaxis. Patients with severe forms of VWD may have frequent hemorrhages, especially when FVIII levels are below 5 U/dL, so that some of them develop target joints like patients with severe hemophilia A. Some patients have recurrent gastrointestinal bleeding, often without lesions in the digestive tract and need treatment every day or every other day. Finally, there are children who have epistaxis frequently and severely enough to cause anemia. In these frequent and severe bleeders, the optimal therapy may be regular prophylaxis with FVIII/VWF concentrates rather than on demand treatment on the occasion of bleeding episodes. The largest experience on secondary prophylaxis in VWD has been collected in Sweden in 35 patients with severe forms of VWD.31 Secondary prophylaxis was also implemented in a cohort of Italian patients with VWD.32 Among 89 patients who needed treatment with FVIII/VWF concentrates during the last two years because of one or more bleeding episodes, 11 (12%) were included in a prophylaxis program because of frequent recurrence of bleeding at the same sites.32 Prophylaxis was started because of gastrointestinal bleeds in seven patients with types 3 (n=1), 2A (n=4), 2M (n=1) and type 1 (n=1) and for joint bleeds in four patients with type 3 VWD (n=4). Prophylaxis prevented bleeding completely in eight patients and greatly reduced hospitalization for blood transfusions in the other three. When prophylaxis was compared with previous on demand regimens in all the 11 cases the annual total consumption of concentrate, the number of transfused blood units and days spent in hospital were significantly reduced.32 FVIII levels were always higher than 180 U/dL but no side effects, including thrombosis, were observed. These two retrospective studies suggest that cost-effectiveness of these prophylaxis regimens versus on demand therapy should be further evaluated in larger prospective studies.

Future treatments

On the whole, treatments currently available for patients with VWD are quite satisfactory, so that there are even fewer incentives than in classic hemophilia to cure the disease through gene replacement therapy. For patients unresponsive to DDAVP, FVIII/VWF concentrates are the only form of available treatment and the fact that they are fractionated from plasma is of concern for some, even if more than one viral inactivation method is used for all concentrates in the manufacturing process. Haemate P/Humate P is the only concentrate that is produced using only one viral inactivation method (pasteurization), but the safety record of this product is impeccable. Notwithstanding this favorable situation, there are advanced plans to develop a therapeutic preparation of recombinant VWF. This product, containing only VWF, will require the concomitant administration of FVIII for the control of acute bleeding episodes and for the prevention of excessive bleeding at the time of emergency surgery.

References

Bridging of anticoagulation during invasive procedures

Bridging of anticoagulant therapy with vitamin K antagonists at the time of invasive procedures is frequently debated. The protocols range from interruption of vitamin K antagonists without bridging to bridging with full (therapeutic) dose of unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH). The guidelines of the American College of Chest Physicians (ACCP) recommend bridging with UFH or LMWH in most situations (Table 1) but all these recommendations are of the weakest grade, i.e. 2C. The reason for this is that no randomized controlled trials have been performed. Until 2001 information could only be obtained from retrospective series of patients. More recently, data have been obtained from registries and prospective cohorts.

In a systematic review of all English-language studies, 31 reports were identified, but the quality was generally considered as poor. These were typically small series of patients, with the timing of anticoagulation and the duration of follow-up rarely mentioned.

General concepts of risk

The risk of thromboembolism needs to be assessed for each patient, as it will have a major impact on the intensity of anticoagulation required during the peri-operative period. Patients with venous thromboembolism more than 3 months previously have a relatively low risk of recurrence when vitamin K antagonists are stopped for a few days, but the surgical trauma and ensuing immobilization increases this risk.

However, a low dose of the anticoagulant agent of choice, corresponding to usual thromboprophylaxis for surgery or somewhat higher (for example dalteparin 5,000-7,500 U or enoxaparin 40-60 mg once daily), is generally considered sufficient. When the thromboembolic event is more recent, in the presence of malignancy, or when there are severe thrombophilic defects such as phospholipid antibodies, deficiency of antithrombin, the homozygous form of factor V Leiden mutation or combined defects, the risk of recurrent VTE increases considerably.

Non-valvular atrial fibrillation without any additional risk factors for stroke is also considered to carry a low risk of thrombosis. With one or more of the following factors the risk increases: age >75 years, hypertension, congestive heart failure, diabetes mellitus and previous stroke or transient ischemic attack. Patients with mechanical prosthetic heart valves have a higher risk of valvular thrombosis or systemic embolism if the valve is in the mitral rather than the aortic position, if it is an older caged-ball or caged disk valve, or if it is combined with atrial fibrillation, left atrial enlargement or low ejection fraction. Patients with bioprosthetic valve replacement are at the low end of the risk range.

The intensity of the bridging anticoagulant regimen must also take into account the risk of bleeding. The main risk factors for bleeding are summarized in Table 2.
Recent studies

Intravenous unfractionated heparin

During the past decade several studies, involving with more homogenous treatment or large numbers of patients, have been published. Nevertheless these studies were flawed by many defects. Registries may have missed important subsets of patients. Treatments were inconsistent and may have been biased. The vitamin K antagonist was stopped 5 days (range 4 to 6) before surgery in most studies. This is the typical time needed to lower the intensity of vitamin K antagonist from the therapeutic range down to the normal range. In cardiac surgery this may not be necessary, since in a controlled study the blood loss was not worse among patients with uninterrupted anticoagulation, presenting with a therapeutic intensity (International Normalized Ratio [INR] - 2.4) on the day of surgery than in patients not receiving vitamin K antagonist.9

It is important to note that the duration of follow-up differed considerably between the studies, ranging from 14 to 90 days.56 Although the shorter follow-up should be sufficient to catch virtually all major bleeding events, this difference may be important for the observed incidence of thromboembolic complications.

One of the first large series, studying 197 patients, was based on a fairly consistent treatment with intravenous UFH.7 The infusion rate was reduced 8 h before surgery to 42 U/h (a very low dose), then increased postoperatively upon return to the ward to 210 U/h (a low prophylactic dose), then progressively to half and full therapeutic dose. In a more recent publication a comparison was made between two cohorts, in which the older one received bridged treatment with intravenous UFH.5 The major outcomes are shown in Table 3. In REGIMEN, which was a prospective registry from many centers in the USA and Canada of patients treated with UFH in hospital, and similarly to the other two studies, the incidence of bleeding was rather high (Table 3).6 Due to the necessity of hospitalizing the patients for several days before and after surgery, this regimen has been largely abandoned. Moreover, the rate of major hemorrhage was relatively high.

Subcutaneous low-molecular-weight heparin

LMWH has several advantages over UFH, being user-friendly for outpatients without the need for monitoring of coagulation parameters and weight-
adjusted doses given subcutaneously have predictable effect. Some studies have used a twice-daily regimen, whereas others gave the full dose in a single daily injection. The bridging therapy with LMWH was usually started 3 days or sometimes 2 days before surgery with the last preoperative dose 12 to 24 h before the procedure. It was then resumed 24 h or sometimes 12 h after surgery and continued until treatment with vitamin K antagonist, usually resumed the evening of surgery, reached the therapeutic range. It appears that the LMWH regimen is associated with a lower risk of major hemorrhage than the treatment with UFH, and without any reduction of the antithrombotic effect (Table 3).

Table 3. Major outcomes in recent studies, according to type of bridging regimen.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>UFH i.v.</th>
<th>LMWH full dose</th>
<th>Dose-reduced VKA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>197</td>
<td>26 164 %</td>
<td>668 174 650 37</td>
</tr>
<tr>
<td>Valvular thrombosis</td>
<td>0 0 1 0.3</td>
<td>0 0 0 0 0 0</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>Stroke</td>
<td>2 1 1 1.0</td>
<td>0 2 1 0 0 0.2</td>
<td>1 1 1 1 1 0</td>
</tr>
<tr>
<td>TIA</td>
<td>0 1 1 0.5</td>
<td>0 2 0 1 0 2.3</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>Systemic embolism</td>
<td>0 0 1 0.3</td>
<td>0 0 0 1 0 1 0</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>DVT</td>
<td>0 1 0 0.4</td>
<td>0 2 0 0 0 0.1</td>
<td>1 1 1 0 0 0</td>
</tr>
<tr>
<td>PE</td>
<td>0 0 0 0</td>
<td>0 0 0 0 1 0.1</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>Major bleed</td>
<td>11 3 9 5.9</td>
<td>6 22 4 6 2 9 2.7</td>
<td>2 2 2 2 2 2</td>
</tr>
<tr>
<td>Death</td>
<td>0 0 2 0.5</td>
<td>0 4 4 2 0 0.5</td>
<td>0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

For patients with only venous thromboembolic disease of older date and possibly also patients with atrial fibrillation without additional risk factors for stroke, a lower dose of LMWH (approximately 5000 U once daily) should be used. As in most of the other studies the patients had a mixture of both arterial and venous thromboses. The treatment with warfarin was reduced to approximately half from 7 days before surgery and the mean INR was 2.1 the day before surgery and 1.8 the day of surgery. It is still premature to conclude that this alternative is as effective and safe as the LMWH regimen (Table 3) and randomized controlled trials are desperately needed.

Concomitant treatment with platelet function inhibitors

Acetylsalicylic acid at a low dose (approximately 80 mg) is nowadays used by a considerable proportion of patients receiving vitamin K antagonists and planned for surgery. Again, we lack randomized controlled trials. A recent review of the literature demonstrated that withdrawal of low-dose aspirin was followed by acute cardiovascular syndromes in up to 10% of cases. The risk of bleeding complications with aspirin was increased by a factor of 1.5, but the severity of bleeding only increased for intracranial surgery and possibly transurethral prostatectomy. Therefore, for the majority of surgical procedures the benefit of maintaining low-dose aspirin exceeds the risks. This is only valid for patients with previous acute coronary syndromes, whereas patients receiving aspirin for other indications may have a net benefit from temporary withdrawal.

Clodipogrel, a P2Y12 antagonist, which blocks part of the ADP-receptor, provides a more profound impairment of the platelet function than low-dose aspirin. Continuation of treatment with clodipogrel beyond 5 days before major surgery is associated with increased blood loss. Obviously, the risks of discontinuation, for example in patients with recent stenting of coronary arteries, must be weighed against the risks of bleeding. Patients planned for cardiac catheterization should definitely continue on clodipogrel, which is crucial in case angioplasty and stenting are performed.
**Specific surgical procedures**

Cohort studies have shown that for dermatological surgery there is no need to discontinue anticoagulant therapy. Likewise, joint and soft tissue aspirations and injections can be performed without disruption of anticoagulant therapy. It is also possible to perform cataract surgery while receiving therapeutic doses of vitamin K antagonists, although mild hyphema or subconjunctival hemorrhage have occasionally been described. In a survey only one quarter of surgeons discontinued anticoagulation before this procedure, although topical anesthesia and clear incision in the cornea should eliminate any risk of bleeding. Several randomized controlled trials have demonstrated the safety of performing tooth extractions with maintained anticoagulation, provided that mouth rinses with the fibrinolytic inhibitor tranexamic acid are used. For all of the above-mentioned procedures it is strongly recommended to avoid supratherapeutic levels of vitamin K antagonists.

**Conclusions**

The studies of bridging of anticoagulant therapy have to a large extent not been designed according to modern research methodology. The exceptions are for some types of minor surgery. Profound controversies prevail regarding the necessity of bridging for major surgery, the anticoagulant agent to be used in case of bridging, timing and magnitude of doses and the management of antiplatelet agents. Differing opinions are found even within a hospital, and little support is provided by the guidelines that suggest a variety of regimens but with the weakest level of evidence. Two multicenter randomized controlled trials are currently being planned, and the objective is to compare regimens with and without LMWH. The rate of major bleeding is likely to be reduced without LMWH. In addition, some of the thromboembolic events in the studies were observed after stopping LMWH due to bleeding and may thus be avoided if no LMWH is given. However, with the low event rates observed, large numbers of patients are required, which imposes a burden on trial organization and finances at the same time as interest from the industry for this type of study has faded.

**References**

**Clinical presentation**

Disseminated intravascular coagulation (DIC) is frequently observed in intensive care unit patients. Typical clinical conditions associated with DIC are sepsis or any other severe infection, organ destruction, such as in severe pancreatitis or trauma, malignancies, especially myeloproliferative and lymphoproliferative tumors, but also adenocarcinomas, obstetric calamities, such as amniotic fluid embolism or abruptio placentae, various vascular abnormalities, such as aortic aneurysms and large hemangiomas, severe hepatic failure, and various toxic and immunologic reactions, such as the catastrophic anti-phospholipid syndrome, transfusion reactions, transplant rejection, heparin-induced thrombocytopenia, and toxic snake bites (Table 1). According to the definition of the DIC Subcommittee of the International Society on Thrombosis and Hemostasis (ISTH), DIC is an acquired syndrome characterized by the intravascular activation of coagulation with loss of localization arising from different causes. DIC can both originate from, and cause damage to, the microvasculature, which if sufficiently severe, can produce organ dysfunction.¹

The clinical presentation of DIC is quite variable²,³ (Table 2). The majority of patients show signs of massive coagulation activation in their laboratory analyses, including high levels of fibrin derivatives such as D-dimer, together with decreased levels of coagulation factors, coagulation inhibitors and platelets. Some patients display very low levels of fibrinogen (defibrination syndrome), whereas in other patients, plasma fibrinogen levels are normal or even elevated, due to an acute phase reaction secondary to underlying diseases such as sepsis.

Bleeding may occur in patients with low, normal, and high fibrinogen levels, but is commonly more extensive in the defibrination syndrome. Thrombotic complications of DIC are frequently observed in sepsis-induced purpura fulminans, which is commonly caused by meningococcal and pneumococcal infections, catastrophic anti-phospholipid syndrome, and heparin-induced thrombocytopenia. All variants of DIC may be associated with microvascular thrombosis, leading to organ dysfunction.

Low levels of coagulation factors and inhibitors in DIC may be the consequence of intravascular consumption by activation of coagulation (consumption coagulopathy). In patients with sepsis, severe trauma, and various other conditions, this may be caused by impaired hepatic synthesis, loss of plasma volume, and hemodilution.⁴,⁵

**DIC score systems**

Score systems for the diagnosis of DIC typically include parameters of hemostatic potential, such as the prothrombin time and fibrinogen level, as well as the platelet count, and an indicator of intravascular fibrin formation, such as D-dimer, fibrin degradation products, or soluble fibrin. In the ISTH DIC-score, this indicator is termed fibrin-related marker (FRM), in order to allow for further developments of specific assay systems.

The DIC Subcommittee of the ISTH originally proposed two score systems: one score for overt DIC (Table 3), and one score for non-overt or evolving DIC (Table 4). The overt DIC score is a static score, using platelet count, prothrombin time, fibrinogen level, and FRM as parameters for the diagnosis of DIC.¹ The non-overt or evolving DIC score also uses platelet count, prothrombin time, and FRM, but adds a kinetic component by comparing the initial values with the results of additional laboratory analyses performed 24 hours later. A trend towards normal levels would lead to a lower score level, whereas a trend towards pathological levels increases the score. Indicators such as antithrombin and protein C levels may also be added, if available.
Table 1. Clinical conditions which may be typically associated with DIC.

<table>
<thead>
<tr>
<th>Clinical conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepsis/severe infection (any microorganism)</td>
</tr>
<tr>
<td>Trauma (polytrauma, neurotrauma, fat embolism, …)</td>
</tr>
<tr>
<td>Organ destruction (e.g. severe pancreatitis)</td>
</tr>
<tr>
<td>Malignancy (solid tumors, myeloproliferative/lymphoproliferative malignancies)</td>
</tr>
<tr>
<td>Obstetricial calamities (amniotic fluid embolism, abruptio placentae)</td>
</tr>
<tr>
<td>Vascular abnormalities (Kasabach-Merrit-syndrome, large vascular aneuvesms)</td>
</tr>
<tr>
<td>Severe hepatic failure</td>
</tr>
<tr>
<td>Severe toxic or immunologic reactions</td>
</tr>
<tr>
<td>(snake bites, recreational drugs, transfusion reactions, transplant rejection)</td>
</tr>
</tbody>
</table>

In the original version of the ISTH DIC score, prothrombin time (seconds) (3–6 seconds prolongation = 1 score point, >6 seconds prolongation = 2 points) is used. This approach is impractical for countries in which either the PT ratio (Quick), or International Normalized Ratio (INR) are used as prothrombin time parameters. For the INR, the numbers used in Japanese scoring systems (INR 1.25±1.67 = 1 score point, INR >1.67 = 2 score points) may be used. For the Quick ratio, various cut-off values have been used, depending on the method employed. Prothrombin time values are influenced by fibrinogen concentration and presence of fibrinogen/fibrin degradation products, resulting in a prolongation of prothrombin time at low plasma concentrations of fibrinogen, and high plasma concentrations of fibrinogen/fibrin degradation products.

There is also no consensus on the cut-off values of FRM. In the USA, fibrinogen/fibrin degradation products are often used as FRM, whereas in Europe, D-dimer is the most prevalent FRM. Since D-dimer assays (similar to fibrin/fibrin degradation product assays) are not standardized, cut-off values need to be determined individually for each assay. Since the majority of intensive care unit patients display fibrinogen/fibrin degradation product, and D-dimer levels above the normal range, the cut-off value for assigning two score points may be in the range of two times the upper limit of the normal range. The FRM cutoff for assigning three score points in published studies was typically in the range of four to six times the upper limit of the normal range.

Fibrinogen levels may be variable, depending on the method used to measure them. Kinetic assays (such as the Clauss assay) are influenced by the presence of fibrinogen/fibrin degradation products, resulting in an underestimation of fibrinogen levels in plasma samples containing high concentrations of fibrinogen/fibrin degradation products. The derived fibrinogen method, being an end-point assay, is not impaired by substances, that delay fibrin formation. On the other hand, soluble fibrin complexes are incorporated into the clot, resulting in an overestimation of functional fibrinogen levels. Immunological assays generally detect both intact fibrinogen, as well as fibrinogen/fibrin degradation products and soluble fibrin, generating the highest apparent fibrinogen concentrations in patients with DIC.

**Evaluation of score systems**

The overt DIC score was evaluated in a number of studies, including retrospective analyses of the data from the PROWESS trial on the use of drotrecogin alfa (activated), recombinant human activated protein C in severe sepsis. According to these results, the ISTH overt DIC score, like other clinical scoring systems such as the APACHE system, identifies a subgroup of patients with severe sepsis with high risk, who may benefit from treatment with coagulation inhibitors such as recombinant activated protein C.

Toh et al. recently showed that the evolving DIC score does not identify patients who later develop overt DIC, but identifies a group of patients with similar mortality as the overt DIC score.

An alternative approach was suggested by Gando et al. on behalf of the Japanese Association for Acute Medicine (JAAM), using a revised version of the DIC score system suggested by the Japanese Ministry of Health and Welfare (JMHW). The original JMHW DIC criteria include fibrinogen/fibrin degradation products, platelet count, fibrinogen, prothrombin time, plus the clinical criteria bleeding tendency and organ failure due to thrombosis. In the revised version, fibrinogen level was dropped, and the clinical criteria were replaced by systemic inflammatory response syndrome criteria. Compared with the original JMHW DIC score, and with the static ISTH overt DIC score, the JAAM DIC score identified a larger proportion of patients and showed good correlation with organ dysfunction parameters. Use of the JAAM DIC-criteria thus had a quite similar effect as combining the ISTH overt DIC score and the evolving DIC score.

The latter approach has now been evaluated in a recent retrospective analysis of date from the Kybersept study. In the Kybersept study, patients...
with severe sepsis received either a high dose of antithrombin concentrate, or placebo. Use of unfractionated heparin or low molecular weight heparin in doses commonly used for the prophylaxis of venous thromboembolism was at the discretion of the treating physicians, resulting in four groups of patients: patients treated with antithrombin with and without heparin, and patients receiving placebo with and without heparin. The initial evaluation of the study data showed a benefit of antithrombin treatment only in one subgroup of patients with high risk according to severity scoring (SAPS II score) and not receiving heparin, whereas there was no benefit in patients with moderate, as well as very high risk, and no benefit in patients receiving concomitant heparin. According to the recent evaluation, antithrombin treatment led to a similar reduction of mortality in sepsis patients with overt DIC and non-overt or evolving DIC.

**Therapeutic consequences**

There is no specific therapy for DIC. Within groups of patients with similar underlying disease, the diagnosis of DIC according to the scoring systems identifies patients with a particularly high risk. These patients typically have a higher risk of dying or of developing organ dysfunction in the course of disease, and may benefit from specific treatment options, such as treatment with recombinant activated protein C, or antithrombin concentrate in DIC associated with severe sepsis. Currently, there are no prospective studies using sepsis-induced DIC as an inclusion criterion.

Patients with sepsis-induced purpura fulminans, which is a typical manifestation of DIC in patients with severe meningococcal or pneumococcal sepsis, benefit from protein C replacement, as well as from treatment with recombinant activated protein C, but these suggestions are based on retrospective subgroup analyses from sepsis trials, and on case reports.

Treatment with coagulation inhibitors may be helpful in some, but detrimental in other conditions associated with DIC. If DIC is associated with severe bleeding, treatment will be mainly focused on the bleeding, and patients are in most cases treated with fresh-frozen plasma, cryoprecipitate, platelets, prothrombin complex concentrates, fibrinogen concentrate, and in especially severe cases of bleeding, with recombinant activated factor VII (epoxapase alfa activated).

In patients with hematological malignancies, especially those with acute promyelocytic leukemia, the diagnosis and treatment of the underlying disease is essential. Treatment of acute promyelocytic leukemia with all-trans-retinoic acid leads to a reduction in tissue factor expression by the malignant cells, and a reduction in systemic coagulation activation.

Patients with disseminated adenocarcinomas, as well as patients with hemangiomias and other vascular malformations, may display a chronic, compensated DIC rarely associated with relevant bleeding complications or organ dysfunction induced by microvascular thrombosis, despite massively elevated levels of fibrin derivatives in the blood.

Defibrination syndromes induced by snake bites may be treated with specific antiserum, if necessary, although defibrination in the absence of other toxic effects (e.g., neurotoxins) is in most cases well tolerated.

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**Table 3. ISTH overt DIC-score.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count</td>
<td></td>
</tr>
<tr>
<td>&gt;100/μL</td>
<td>0</td>
</tr>
<tr>
<td>50-100/μL</td>
<td>1</td>
</tr>
<tr>
<td>&lt;50/μL</td>
<td>2</td>
</tr>
<tr>
<td>PT (INR)</td>
<td></td>
</tr>
<tr>
<td>&lt;1.25</td>
<td>0</td>
</tr>
<tr>
<td>1.25 - 1.67</td>
<td>1</td>
</tr>
<tr>
<td>&gt;1.67</td>
<td>2</td>
</tr>
<tr>
<td>D-Dimer</td>
<td></td>
</tr>
<tr>
<td>&lt;2×ULN</td>
<td>0</td>
</tr>
<tr>
<td>2.5×ULN</td>
<td>2</td>
</tr>
<tr>
<td>&gt;5×ULN</td>
<td>3</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td></td>
</tr>
<tr>
<td>≥1g/L</td>
<td>0</td>
</tr>
<tr>
<td>&lt;1g/L</td>
<td>1</td>
</tr>
</tbody>
</table>

**Evaluation**

≥5 points: compatible with overt DIC
<5 points: use evolving DIC score

---

**Table 4. ISTH evolving (non-overt) DIC score.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underlying disease compatible with DIC</td>
<td>no = 0</td>
</tr>
<tr>
<td></td>
<td>yes = 2</td>
</tr>
<tr>
<td>Platelet count</td>
<td></td>
</tr>
<tr>
<td>Day 1: &gt;100/μL = 0</td>
<td>rising = -1</td>
</tr>
<tr>
<td>&lt;100/μL</td>
<td>stable = 0</td>
</tr>
<tr>
<td></td>
<td>falling = 1</td>
</tr>
<tr>
<td>PT (INR)</td>
<td></td>
</tr>
<tr>
<td>Day 1: ≥1.25 = 0</td>
<td>falling = -1</td>
</tr>
<tr>
<td>&lt;70%</td>
<td>stable = 0</td>
</tr>
<tr>
<td></td>
<td>rising = 1</td>
</tr>
<tr>
<td>D-Dimer</td>
<td></td>
</tr>
<tr>
<td>Day 1: &lt;2×ULN = 0</td>
<td>falling = -1</td>
</tr>
<tr>
<td>≥2×ULN</td>
<td>stable = 0</td>
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<tr>
<td></td>
<td>rising = 1</td>
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<tr>
<td>Antithrombin</td>
<td></td>
</tr>
<tr>
<td>≥70%</td>
<td>-1</td>
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<tr>
<td>&lt;70%</td>
<td>1</td>
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<tr>
<td>Protein C</td>
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<tr>
<td>≥70%</td>
<td>-1</td>
</tr>
<tr>
<td>&lt;70%</td>
<td>1</td>
</tr>
</tbody>
</table>

**Evaluation**

≥5 points: compatible with overt DIC
<5 points: no DIC
The catastrophic antiphospholipid syndrome, and other severe immunological reactions, may require anticoagulant therapy as well as immunomodulating treatment with corticosteroids, immunoglobulins, or other immunosuppressants.

Patients with heparin-induced thrombocytopenia may display various venous and arterial thrombotic and embolic complications. Therefore it is essential to switch to a full dose of an alternative anticoagulant, such as argatroban, danaparoid, or lepirudin as soon as the diagnosis of heparin-induced thrombocytopenia is suspected, even if no thrombotic complications have occurred at that time.

References

Natural killer cell biology: relevance of missing self-recognition and other aspects of basic natural killer cell biology for clinical approaches

Natural killer (NK) cells represent the third group of lymphocytes. In many respects they resemble the cytotoxic T-cells, but in contrast to them, NK cells do not require the thymus for maturation, and they do not express clonal receptors encoded by somatically rearranged genes. NK cells constitute 5-15% of lymphocytes in the blood, where they can be distinguished by their morphology: slightly larger than resting T- and B-lymphocytes, with more cytoplasm rich in granules. These cells are therefore sometimes referred to as large granular lymphocytes. This reflects that they have organelles that allow them to act immediately after encountering target cells. NK cells are thus constitutively active, and can kill cells or secrete cytokines such as interferon-α within minutes to hours after stimulation by cytokines or cognate interactions with other cells, without need for immunization, clonal expansion or differentiation. This natural activity gave rise to the name natural killer cell.

The interaction between NK cells and other cells is controlled by a set of germ line encoded receptors. Some of these are activating, others are inhibitory, and the final outcome of the NK-target interaction is determined by the balance between positive and negative signals received through these receptors. In animal models, mostly performed with inbred strains of mice, NK cells can contribute to the defence against certain viral, bacterial and parasitic infections. They can mediate resistance to tumor growth and metastasis, as well as rejection of MHC-mismatched bone hematopoetic grafts. There is also evidence that NK cells can regulate innate and adaptive responses by interaction with T-cells, macrophages and dendritic cells. Finally, pregnancy is an emerging field in the studies of NK cell function. NK cells are the dominating lymphocytes in the placenta during the first trimester of pregnancy. They are particularly rich in the maternal-fetal interphase, where they are believed to regulate transformation of spiral arteries, required for optimal interchange of blood and nutrients. Disturbances in this NK-mediated regulation may be one factor contributing to pregnancy complications such as pre-eclampsia.

The possible exploitation of these various NK cell functions in clinical diagnostics, prevention and therapy requires identification of critical receptors and ligands in the interactions between NK cells and other cells, as well as understanding of the biology of NK cell development, education and tolerance.

Cell surface markers and cytokine receptors

There is no cell surface marker that identifies all NK cells and nothing but NK cells. They usually have to be defined or isolated by a combination markers, most commonly as CD3–CD56+ cells. They also express CD16, and adhesion molecules such as LFA-1 and CD2. Two different subsets of NK cells can be distinguished in the blood. The dominant population (80-95%) is CD56(dim)/CD16+. The remaining CD56(bright)/CD16− cells display lower cytolytic activity, but higher cytokine secretion capacity. Recent studies have brought attention to NK cells in lymph nodes, where the evidence suggests that they be predominantly of the efficient cytokine-producing types. In mouse experiments, these NK cells showed the capacity to interact with T cells and dendritic cells, thereby promoting Th1 responses. NK cells themselves have receptors for and respond to several cytokines, such as interleukin (IL)-2, IL-12, IL-15, IL-18 and type I interferons. Cytokine stimulation can lead to enhanced cytotoxicity, secretion of cytokines such as interferon-α, tumor necrosis factor and granulocyte-mono
cyte colony-stimulating factor, or proliferation by the NK cells.
**Missing self-recognition, a function with a critical role for inhibitory receptors**

The interaction with other cells is determined by a set of activating and inhibitory receptors. This was originally proposed in the *missing self-recognition hypothesis*, postulating that NK cells have one set of activating receptors recognizing ligands expressed by most if not all normal cells, and one set of inhibitory receptors specific for host MHC class I molecules. This allows NK cells to interact with and receive a preliminary triggering signal from most normal cells, which is then counteracted by a negative signal delivered by recognition of self MHC class I molecules. If the target cell expresses no, too few, or the wrong (non-self) MHC class I molecules, inhibitory input is insufficient and killing proceeds by default.

This allows NK cells to identify aberrant cells by detecting missing information (for self), as opposed to B- and T-lymphocytes, which recognize the presence of new information (non-self).

Multiple inhibitory receptors have now been identified. Most of these belong to the family of killer cell immunoglobulin-like receptors (KIR), encoded by a locus in the leucocyte receptor complex on chromosome 19. They are membrane anchored monomers, with two or three immunoglobulin (Ig)-domains. KIR do indeed recognize MHC class I molecules in a selective way, although they do not discriminate individual MHC alleles with the same precision as T-cell receptors. Rather, each receptor tends to recognize a subset of MHC class I alleles, e.g. KIR2DL1 (where 2D stands for Ig-like domains and L for long cytoplasmic tail) recognize alleles in the HLA-C group 2 (with a lysine at position 80), while KIR2DL2 and KIR2DL3 recognize alleles in the HLA-C group 1 (with an asparagine at position 80). KIR3DL1 recognize alleles in the HLA-Bw4 group (with an isoleucine at position 80). The inhibitory KIR bind on top of the peptide-binding groove and mediate the negative signal by means of immunoreceptor tyrosine-based inhibitory motifs (V/IxYxxV/L) in the cytoplasmic tail. Upon phosphorylation, these motifs recruit intracellular phosphatases that dephosphorylate substrates in the signal transduction chain elicited by activating receptors. As predicted by the missing self hypothesis, NK cells kill MHC class I-deficient cells more efficiently than MHC class I-expressing cells, and the latter can be killed if inhibitory KIR are blocked by antibodies.

The KIR family also contains activating receptors. These have a similar molecular structure, although they lack a long cytoplasmic tail and ITIM. They instead have a charged amino acid residue that allows them to interact with the ITAM containing signaling adapter molecules such as DAP. The function of the activating KIR is unclear. They do not represent the critical activating receptors for missing self-recognition, since this can be elicited against target cells lacking MHC class I molecules altogether. The evidence indicates that the activating KIR also recognize HLA molecules with the same specificity as the homologous inhibitory KIR, albeit with lower affinity.

Current hypotheses for the function of activating KIR include recognition of microbial ligands, either by themselves or presented by MHC class I molecules, and recognition of enhanced MHC class I expression, which is seen in certain infections.

The KIR genes demonstrate considerable complexity at a population level. Individuals can differ by the number of genes within the locus, as well as by the alleles for each respective gene. The minimal essential KIR locus, usually referred to as the A haplotype contains seven genes, most of which encode inhibitory KIR. B haplotypes have one or several more genes, mostly of the activating type. Ethnic groups vary with respect to KIR, e.g. the A haplotype occurs frequently in Japanese, while B haplotypes dominate among Australian aboriginals. This probably reflects selection of these KIR under a dynamic, still ongoing evolutionary pressure of infections. At present, many investigations are being conducted to investigate possible associations with certain KIR, haplotypes or combinations of KIR and KIR-ligands (i.e. the relevant MHC class I molecules). Interesting associations have been found in studies of human immunodeficiency virus-1, hepatitis C virus, autoimmune conditions such as psoriasis vulgaris, vasculitis associated with arthritis and scleroderma as well as in pre-eclampsia. The mechanistic explanations for these associations require further studies.

There are additional inhibitory receptors containing intracytoplasmic ITIM. NKG2A, a member of the lectin superfamily, is expressed as a heterodimer with CD94. It recognizes HLA-E, a non-classical MHC class I molecule, whose transport to and stable expression at the cell surface is dependent on loading the presentation cleft with leader sequence peptides from other MHC class I molecules. Since the loading of these require the presence of transporters associated with antigen presentation (TAP), the NKG2A represents an alternative and complementary pathway to monitor that cells have an intact MHC class I antigen processing machinery. It can therefore also be interpreted within the concept of *missing self-recognition*. NK cells express additional ITIM-containing receptors in the immunoglobulin superfamily. LIR-1, which is found also on myeloid cells and on subsets of T-cells, recognizes a variety of different MHC class I molecules. For other of these inhibitory receptors, the ligands are not MHC class I molecules or still unknown; one may speculate that these are critical to protect targets that for some reason cannot express MHC class I molecules.
Activating receptors

Although the critical activating receptors in interaction with normal cells have not yet been defined, NK cells express several different receptors recognizing ligands associated with pathological or aberrant cells. NK2G2D, belonging to the lecin superfamily, is a homodimer expressed by all NK cells. It can recognize stress induced molecules, such as MIC-A, MIC-B (both MHC class I resembling molecules) and ULBP1-3 (proteins originally identified by their capacity to bind to certain cytomegalovirus-encoded proteins). The NKG2D ligands are not expressed by normal cells, but they may be induced by events such as infection, DNA damage and malignant transformation. NKG2D ligands are sometimes shed in the serum (e.g. colon cancer) and this can lead to down-modulation of the receptor. NKG2D signals through adapter molecules, mainly DAF-10. Although these activating signals can be counteracted by inhibitory receptors in the KIR family and by NKG2A, they appear quite strong, and in some situations allow NK cells to kill aberrant targets even if they express considerable levels of MHC class I molecules.

There are several activating receptors in the immunoglobulin superfamily. NKp46 is present on all NK cells, and may emerge as the first cell surface marker that is expressed by all NK cells and no other cell. There is evidence that NKp46 recognizes influenza virus hemagglutinin on infected cells, but there are also indications that there may be non-infectious ligands expressed by many tumor cells. NKp80 is an important receptor for NK cell interactions with normal dendritic cells. There is evidence that NK cell can kill immature dendritic cells under certain conditions, while mature cells are spared (by presenting high levels of HLA-E for the NKG2A receptor). NKp44 is expressed only on cytokine-activated NK cells. The ligands for NKp80 and NKp44 remain unclear.

One important line of research in the field is to define which receptors (activating and inhibitory) are active in a given effector-target interaction. This might lead to novel ways to interfere with NK cell function in a more selective way. There are examples of tumor cells whose killing depends almost completely on NKG2D alone or NKp46 alone, while in others it depends on both and in yet others it relies completely on other activating receptors. The MHC class I status of the tumor cell may then further influence the killing via the inhibitory KIR and NKG2A. Finally, it should be noted that cytokine receptors usually act as activating receptors. Stimulation via these may act synergistically with other activating receptors, and it may also change the baseline rules for balance between activating and inhibitory receptors.

NK cells in hematopoietic transplantation-studies in men and in mice

While certain NK receptor are structurally homologous between mice and men (e.g.) NKG2A, NKG2D and NKp46, the two species use entirely different receptor families to recognize polymorphisms of MHC class I molecules. Mice do no express KIR, and the analogous functions are instead performed by Ly49 receptors. These exist in inhibitory and activating variants, and the former contain ITIM, just as the inhibitory KIR. Ly49 receptors belong to the lecin superfamily, and are encoded in the same genetic region (the NK complex) as that for other lecin receptors of NK cells, such as NKG2A and NKG2C. Despite this difference between the two species, there are remarkable similarities between their NK systems, including the functional properties of the MHC class I recognizing receptors. For example, in both species a given receptor: 1) recognizes a subgroup of MHC class I alleles, 2) has a variegated expression pattern, i.e. it is expressed in a significant proportion but not all (15-60%) of the NK cells, 3) can be the only receptor in the family expressed in a given NK cell, or can be co-expressed with one or more family members of either the inhibitory or activating receptor type. It is, therefore, reasonable to use in vivo studies of NK cells in mice for prediction or interpretation of the human NK system.

There is ample evidence from studies in mice that missing self-recognition regulates NK cell attack of normal or malignant cells in the hematopoetic lineage. NK cells of mice promptly reject lymphocytes, bone marrow cells and leukemia cells lacking MHC class I molecules, as measured by short-term in vivo assays tracking the fate of labeled, intravenously inoculated cells. NK cells also reject MHC class I-expressing cells if the host (by outcrossing or transgenesis) possesses one or more inhibitory Ly49 receptor ligand (i.e. MHC class I allele) that is missing in the graft. This situation is referred to as inhibitory receptor ligand mismatch, and the analogous situation in the human is called KIR ligand mismatch. Such incompatibility has been used to analyze the outcome of haploidentical hematopoetic stem cell transplantation (e.g. from a parent to a child, or from a sibling who is matched for only one of the HLA haplotypes). KIR ligand incompatibility in the graft-versus-host direction, i.e. the capacity of NK cells from the transplant to detect missing self on recipient cells, was associated with a reduced risk of relapse (observed for recipients with acute myeloid leukemia, but not those with acute lymphoblastic leukemia) in several studies. This was interpreted as the consequence of a NK-cell-mediated, self-induced graft-versus-leukemia (GvL) effect, as supported by parallel studies in mice based on analogous receptor ligand mismatch. In addition, it
was possible to isolate NK clones with missing self reactivity in the GVL direction for several weeks after the transplantation. Interestingly, there was no increased risk for graft-versus-host (GvH) disease by this mismatch; rather, the clinical as well as mouse experimental study indicated a reduced risk of GvH disease. The proposed explanation was that missing self recognition induced graft NK cells to kill host dendritic cells (required to present host antigens and initiate GvH reactivity by T cells).

While these results suggest ways to exploit NK cells in allogeneic transplantation settings as well as in therapies based on autologous NK cells, it should be noted that KIR ligand mismatch has not been confirmed as a favorable prognostic factor in studies of transplants from unrelated donors (usually matched for HLA-A and HLA-B, but not HLA-C, thus leaving the possibility for a KIR ligand mismatch). This may be due to the different conditioning regimes and stem cell numbers used in the different transplant situations, in which the haploidentical setting might favor NK-cell-mediated effects.

**Future questions from NK cell development and education to cellular therapy**

Three months after haploidentical transplantation as described above, the NK clones isolated from the recipients no longer displayed missing self reactivity towards recipient cells. The NK system may thus develop tolerance to the host, just as mouse NK cells developing in mice with different MHC class I phenotypes acquire tolerance to the autologous cells, while maintaining the capacity to reject cells with one or more missing MHC class I alleles in relation to the host. If KIR ligand mismatch is to be exploited for different therapeutic approaches, it appears important to define the mechanisms whereby NK cells are educated to kill according to missing self rules and remain tolerant to autologous cells. This requires a complete understanding of NK cell development, a process that is only partially clarified so far. Briefly, there is a first phase leading up to NK precursors via initial commitment to the lymphoid lineage and subsequently to the NK cell lineage. The NK precursors in humans as well as in mice express IL-2Rbeta, but lack many of the typical NK cell markers. C-kit ligand and IL-7 can drive NK cells through this phase in vitro. Phase 2, in which IL-15 is critical, leads to immature NK cells; during this phase the NK precursors gradually acquire surface markers (such as CD56) and several functions of NK cells. The MHC receptors are acquired late in a process requiring interaction with stromal cells; those of the NKG2 family precede those of the KIR (human) and Ly49 (mouse) families. Phase 3 involves the export of NK cells to the periphery.

The critical question in this process is how NK cells ending up with no or the wrong inhibitory receptor(s) (i.e. those that do not recognize any self MHC class I ligand) are dealt with. Such potentially autoreactive NK cells have been studied in mice with mosaic MHC class I expression (i.e. with cells of two different MHC class I phenotypes in the same animal, where one represents missing self in relation to the other). The results indicate that potentially autoreactive cells are maintained as anergic, but can be rapidly reactivated to recognize cells of the missing self phenotype if they are separated from these and cultured in IL-2 for at least 24 hours. Recent publications have defined such anergic NK cells by single cell assays for cytokine secretion, and discuss two different models for how they develop (dampening, and licensing). Full understanding of these processes may help us to manipulate NK cell education and tolerance in different settings, such as complete hematopoetic transplantation, or cellular therapy without or different degrees of conditioning. A recent study has shown that in vitro activated NK cells may be reinfused and maintained by cytokine treatment in leukemia patients, and suggested a favourable response if NK cells donors were selected for KIR ligand incompatibility. Other approaches to manipulate NK cell specificity in the autologous situation include attempts to block inhibitory receptors, which requires that one can establish a therapeutic window in which blockade leads to enhanced killing of malignant cells while maintaining tolerance to normal cells. Finally, it may be possible to enhance the effect of transferred or autologous NK cells also by providing specificity by external means: a simple way would be to co-administer leukemia- or tumor-specific antibodies mediating antibody-dependent cell-mediated cytotoxicity. A more complex strategy would involve transfection of NK cells or NK lines with genes for genetically engineered receptors specific for the cells to be targeted. Such approaches may be tried alone or in combination with inhibitory receptor blockade. Further studies of NK cell biology will indicate how the function of these cells can be utilized for the best clinical approaches.
Dendritic cell immunotherapy: promise and challenges

Dendritic cells are antigen-presenting cells specialized to initiate and regulate immunity. The rationale for cellular therapy with dendritic cells is based on the premise that ex vivo loading of antigens to dendritic cells allows optimal targeting of antigens to enhance their immunogenicity. Development of methods to generate dendritic cells ex vivo from progenitors has facilitated the several clinical studies of dendritic cell vaccination. These studies have demonstrated the feasibility of this form of therapy, as well as the ability of injected dendritic cells to boost as well as suppress immunity in vivo in humans. However, attention to several aspects of dendritic cell biology is needed to optimize the immunogenicity of these vaccines and enhance their clinical efficacy.

Lymphocytes and their products such as cytokines and antibodies represent the major work force of the immune system, and mediate protection from pathogens and tumors. Two major classes of lymphocytes exist. Innate lymphocytes such as natural killer (NK) and NK-T cells act quickly but lack immunologic memory. Adaptive lymphocytes such as B and T cells provide antigen-specific immunity and carry immunologic memory. However, lymphocytes do not act by themselves. Dendritic cells (DC) play a critical role in the activation of both innate and adaptive immunity and regulation of the immune response. Both the activation and quality of the immune response depend on the nature of the signals provided by DC. Their central role in the immune system has focused attention on targeting these cells to improve vaccination in humans. Below, I will discuss the impact of newer insights into DC biology on DC-based immunotherapy in humans.

Rationale for DC vaccination

Traditional vaccines have largely relied on random targeting of antigen to DC in vivo. Chemical adjuvants in these vaccines promote the recruitment and perhaps more importantly, activation of antigen-bearing DC at the vaccine site. Development of methods to generate large quantities of DC from progenitors has permitted approaches for ex vivo loading of DC with specific antigens, and their use in immunotherapy. Three major approaches are under study, isolating DC directly from blood, or generating DCs in culture from blood monocytes or CD34+ hematopoietic progenitors. One key advantage of this approach is that the purity, antigen-loading and biological properties of injected DC can be readily monitored and manipulated. The specific culture conditions used to generate DC can have a major impact on their functional properties. For example, the most commonly used approach involves culturing blood monocytes with granulocyte-monocyte colony-stimulating factor and interleukin-4. However, culture in the presence of alternate cytokines such as tumor necrosis factor-α, type I interferons or thymic stromal lymphopoietin (TSLP) leads to distinct subsets of DC with distinct functional properties. In addition to immunogenic DC, DC with tolerogenic properties can also be generated ex vivo. Studies with adoptive transfer of DC in healthy donors have provided the proof of principle that antigen-loaded DC can both enhance as well as suppress immunity in vivo. These cells may provide a useful system for manipulating the human immune system in patients with cancer, infections or autoimmunity.

Some aspects of DC biology of major relevance to clinical immunotherapy

There have been major advances in our understanding of DC biology over the last decade. Many of these insights have been
recently covered in excellent reviews.\textsuperscript{1,2} Here I will focus on some of the recent insights that might have a major impact on improving DC immunotherapy in the near future.\textsuperscript{3}

**DC maturation**

DC normally reside and traffic through tissues in an immature form specialized for antigen capture. Exposure to several stimuli associated with pathogens or inflammation initiates a process termed DC maturation. DC maturation is associated with not only phenotypic remodeling (including increased cell surface expression of MHC and co-stimulatory molecules, as well as other markers such as CD85), but also enhanced capacity for T-cell stimulation. Activation of DC is therefore critical to the development of immunostimulatory DC vaccines. However the specific nature of the maturation stimulus has a major impact on the nature of signaling pathways and functional properties of DC. A combination of inflammatory cytokines that includes interleukins 1 and 6, tumor necrosis factor and prostaglandin E\textsubscript{2} is often used to mature DC for clinical immunotherapy. However, while inflammatory cytokines do lead to phenotypic remodeling, acquisition of potent T stimulatory properties may require additional stimuli such as CD40L or Toll receptor signaling.\textsuperscript{6} Another emerging theme is the cross-talk between various maturation-associated signaling pathways and the potential role of combinatorial signaling via more than one ligand. Optimizing the nature of maturation stimuli delivered to DC (either ex vivo or in vivo) may have a major impact on vaccine efficacy.

**DC subsets**

There are at least four types of human DC, as defined under cytokine driven conditions in vitro. These are conventional or myeloid DC, including blood monocyte-derived DC (Mo-DC), dermal/interstitial DC (IDC), Langerhans’ cells (LC) and plasmacytoid DC. A trace population of myeloid DC also circulates in the peripheral blood. Most studies of human DC therapy to date have been performed with Mo-DCs, largely because they can be obtained in relatively large quantities and at high purity. Cultures of CD34\textsuperscript{+} hematopoietic progenitors lead to a heterogenous population of DC that consists of both IDCs and LC, which are functionally distinct from Mo-DC. Clinical studies with CD34\textsuperscript{+} hematopoietic progenitor derived peptide pulsed DC in advanced melanoma have also yielded promising results.\textsuperscript{3} However, the biological impact of DC heterogeneity on the outcome of DC vaccination remains to be clarified and has not yet been exploited.

**DC migration**

After encounter with antigen in the periphery, DCs migrate to the T cell zone of the lymph nodes where they interact with T cells to initiate immunity.\textsuperscript{3} One of the major regulators of this interaction may be the expression of CCR7 on DCs with CCL19/CCL21 on lymphatics and endothelial venules in the T cell zone. Although only a few studies have monitored migration of DCs after injection, it appears that <1% of injected DCs make it to the lymph node. Therefore improving DC homing may have a major impact on efficacy of DC vaccines. One approach examined in mouse models is to precondition the injection site with inflammatory cytokines. As the current efficiency of nodal migration of injected DCs is so low, it is likely that approaches that enhance this process will have a major impact on the immunogenicity of DC vaccines. A major limitation at present is the paucity of data about real time imaging of injected DCs in patients.

**Antigen uptake, processing and presentation, including cross presentation**

DC use several mechanisms such as phagocytosis, pinocytosis, endocytosis and specific receptors to capture microbial pathogens, dying cells, immune complexes, and other antigens for immune presentation. DC are potent antigen-presenting cells and the captured antigen is efficiently processed and presented on products of major histocompatibility complex (MHC) I and II molecules. Typically, antigens acquired from extracellular environment are presented via MHC II to CD4\textsuperscript{+} T cells, while MHC I molecules bear antigens from the cytosolic compartment. DC are, however, also exceptionally effective at presentation of exogenous antigens on MHC I, a process often termed as cross-presentation. A critical regulator of the ability of DCs to cross present may be signaling via type I interferons.\textsuperscript{3} As tumors themselves are poor antigen-presenting cells, the generation of anti-tumor immunity may critically depend on this property of DC to cross-present antigen. Cross-presentation of tumor antigens by DC has, therefore, been a focus of most approaches to DC vaccination against cancer.

**Control of cross-presentation: role of specific receptors**

The efficiency and outcome of cross-presentation is greatly influenced by the engagement of specific receptors involved in the uptake of the antigenic cargo, as well as receptors that recognize pathogen-associated molecular patterns (e.g. Toll receptors) in the payload. DC express several receptors for specific uptake of apoptotic cells, heat shock proteins, as well as immune complexes. For example, uptake of
antibody-coated myeloma tumor cells via Fcγ receptors greatly enhances the generation of anti-tumor CD4 and CD8+ T-cell responses.\textsuperscript{10} The FcR pathway is further subject to manipulation of the balance between activating and inhibitory Fc receptors.\textsuperscript{11} Targeting tumor antigens to Fc receptors may contribute to anti-tumor effects of monoclonal antibodies in cancer, and provides an example of how targeting of antigens to specific receptors may be exploited towards enhanced immune efficacy. Much of this biology has not yet been fully exploited in the context of DC immunotherapy. For example, most studies of DC vaccination with dying tumor cells have only examined tumor cell lysates.

**Role of Toll-like and C-type lectin receptors in DC biology and in vivo targeting**

DC express several pattern recognition receptors (PRR) that recognize pathogen-associated molecular patterns or molecules from damaged tissues. One such family is Toll like receptors (TLR).\textsuperscript{12} Examples of Toll receptor-ligand interactions include peptido-glycan binding of TLR2, viral dsRNA binding of TLR3, LPS binding of TLR4, viral ssRNA binding of TLR7, and unmethylated bacterial CpG DNA binding to TLR9. Conventional DC express several TLR 1-6 and 8, depending on the state of activation. Another group of PRR are the C-type lectin receptors, which bind carbohydrate moieties of glycoprotein self antigens. Some well studied examples are the mannose receptor, the decalectin DEC-205, and DC-specific intercellular adhesion molecule grabbing non-integrin (DC-SIGN). TLR and C-type lectins likely work in concert to balance immunity to tolerance. For example, DC use C-type lectins to sample and present self and harmless antigens in the steady state to maintain tolerance.\textsuperscript{13} Targeting antigens to C type lectins such as DC-SIGN via monoclonal antibodies has been shown to lead to enhanced T cell immunity.\textsuperscript{14} Activation of TLR signaling leads to immune activation. It is likely that specific targeting of antigens to these receptors will be utilized as means to target DC in vivo for immune therapy in the future.

**Targeting glycolipid antigens and innate lymphocytes**

Although most of the early attention in DC biology was on their ability to process and present protein antigens and stimulate T cells, it is now clear that DC are also efficient at presenting glycolipid antigens and stimulating innate natural killer T (NKT) cells. NKT cells are a trace population of innate lymphocytes that bear an invariant T-cell receptor (Vα24/Vβ11 in humans) and some features of NK cells. NKT cells recognize glycolipid ligands in the context of the CD1d family of molecules. Presentation of self ligands by tissues likely maintains NKT cells in a tolerogenic state in vivo, a situation also present in the tumor-bearing hosts. Presentation of a synthetic or microbially derived glycolipid ligand can lead to NKT activation, with release of cytokines and downstream activation of NK cells and DC, ultimately leading to activation of T-cell immunity. DC appear to be particularly important for activation of NKT cells in vivo,\textsuperscript{15} and in turn are important targets for NKT cells themselves. A similar cross-talk also exists in the lymphoid tissues between DC and NK cells.\textsuperscript{14} For example, DC are efficient at activating of resting NK cells, which otherwise respond to the aggregate of activating and inhibitory signals on target cells.\textsuperscript{16} This ability of DC to activate innate effectors is beginning to occupy an important role in the early response to infections. However, it is also likely that targeting innate effectors via DC will be critical to enhance their ability to boost T cells. In a recent study, we observed that injection of DC loaded with a glycolipid ligand led to marked enhancement of NKT cells in patients with advanced cancer, which was then associated with downstream activation of T cell responses.\textsuperscript{17} Improved understanding of the nature of naturally occurring NKT ligands in cancer patients may allow effective manipulation of these specialized cells in cancer therapy.

**Lessons from initial clinical studies**

Pioneering studies with DC in lymphoma and melanoma were the first to demonstrate the ability of DC to mediate clinical regressions in cancer patients.\textsuperscript{18,19} Many of the early studies were with peptide-pulsed DC, targeting only CD8+ T cells.\textsuperscript{20} Since these initial studies, DC have been a focus of extensive clinical investigation, and several approaches including DC loaded with tumor lysates, viral vectors, RNA and protein antigens have been tried. Several of these studies however did not incorporate emerging concepts in DC biology, for example the need for an effective maturation stimulus. The ability of DC to generate immunity in vivo in humans is now well established. However, the clinical activity observed in these studies has been generally low and variable. In our own studies in melanoma, clinical regressions were also observed, but the small sample size in this and many other studies preclude reliable assessment of clinical activity.\textsuperscript{7} DC vaccination has, however, entered phase III testing in some settings such as melanoma and prostate cancer.\textsuperscript{21} In my view, these studies clearly illustrate the need to better incorporate the emerging knowledge of the biology of DC into the design of future clinical studies to improve outcome (Figure 1). It will be critical not only to monitor the nature of the DC product being injected, but also to track it in vivo,\textsuperscript{22} and carefully
monitor the induction of both effector and regulatory T cells. Issues such as patient selection may also be important, as such immune approaches may be best in the context of minimal residual disease. It is also likely that combining DC vaccination with other approaches such as those aimed at removal of regulatory T cells, or other immune modulating approaches, will be attempted in an effort to improve on these results. The future of this form of cell therapy and its integration into clinical care will depend on the effectiveness of the cross-talk between the bench and the bedside.

References

From tumour-specific T-cells to therapeutic T-cell receptors

Adaptive cellular immunotherapy in allogeneic hematopoietic stem cell transplantation

The most effective form of adoptive, T-cell-based immunotherapy of malignant disease is certainly allogeneic hematopoietic stem cell transplantation (HSCT). This is impressively underscored by both the successful treatment of patients suffering post-transplant from recurrent chronic myeloid leukemia (CML) by donor lymphocyte infusion (DLI) and the development of non-myeloablative HSCT, in which the establishment of mixed donor-recipient chimerism allows selective donor-specific tolerance and, subsequently, donor-derived T cells to respond to leukemic or tumor (stem) cells. In yet another form of allogeneic hematopoietic stem cell grafting, haploidentical HSCT, donor-derived natural killer (NK) cells that miss to recognition by virtue of their highly polymorphic inhibitory killer cell immunoglobulin-like receptors (KIR) certain class I major histocompatibility (MHC) ligands on the surface of recipient cells are no longer silent, but actively target the remaining acute myeloid leukemia (AML) cells in the patient.

The disadvantage of transferring allogeneic T cells into hematopoietic stem cell transplant recipients is that their recognition of leukemic or tumor cells is not, if at all, entirely specific for leukemia- (LAA) or tumor-associated antigens (TAA), but dominated by minor histocompatibility (mH) antigens, peptide epitopes bound by human leukocyte antigens (HLA) and derived from polymorphic proteins that differ by sequence between donor and recipient. As a result, any specific graft-versus-leukemia (GvL) response is largely superimposed by, if not identical to, graft-versus-host disease (GvHD)-mediating T lymphocytes, although the expression of some mH antigens, such as HA-1 and HA-2, is restricted to normal and malignant lymphohematopoietic cells. One possibility way of segregating mH antigen-reactive GvL- from GvHD-inducing T-cell responses is retroviral transduction of suicide genes into the immune effector cells. As soon as severe GvHD develops, the effector cells can be turned off by activation of the suicide mechanism. The prototype suicide molecule that has been used to label adoptively transferred T cells in HSCT has been herpes simplex virus thymidine kinase (HSV-TK), which enables the ablation of transduced alloreactive T lymphocytes by the administration of ganciclovir. However, as HSV-TK itself is immunogenic, alternative suicide mechanisms which are believed to be less immunogenic than HSV-TK have been explored after retroviral expression in human T cells. These include a modified Fas receptor and an inducible caspase 9 molecule that can be switched on to mediate T-cell apoptosis by oligomerization via the bivalent drug AP1903 and by dimerization through a small molecule pharmaceutical, respectively.

The use of retroviral vectors in gene therapy has raised safety concerns for the genotoxic risk associated with their uncontrolled insertion into the human genome. The evaluation of the consequences of retroviral transduction in T cells from leukemic patients treated with HSCT and donor lymphocytes genetically modified with the HSV-TK suicide gene demonstrated that retroviral vectors integrate preferentially within or close to transcribed regions of the genome, with a preference for sequences around promoters and for genes active in T cells at the time of transduction. Quantitative transcript analysis shows that one fifth of these integrations affect the expression of nearby genes. However, transduced T-cell populations maintained remarkably stable gene expression profiles, phenotype, biological functions, and immune repertoire in vivo, with no evidence of clonal selection for up to 9 years after administration. Analysis of integrated proviruses in transduced cells before and after transplantation indicated that integrations interfering with normal
T-cell function were more likely to lead to clonal ablation than expansion in vivo. Despite the potential dangerous interactions with the T-cell genome, retroviral integration is therefore considered to have little consequence on the safety and efficacy of adoptive T-cell transfer.

Polyclonal activation of T lymphocytes ex vivo is a prerequisite for efficient retrovirus-mediated expression of a transgene, such as a suicide molecule. Concerns also arose about whether or not this type of strong preactivation of T cells may lead to their activation-induced cell death and impaired survival and persistence in vivo. We have recently treated a patient suffering from a severe and refractory liver-transplant-associated GvHD with donor lymphocyte-reactive host T cells generated ex vivo and expanded by strong polyclonal stimulation. To control GvHD, activated alloreactive host T cells were repetitively re-transferred into the patient (activated host lymphocyte infusion). The adoptive transfer of these ex vivo polyclonally activated alloreactive host T cells led to the control and eventually to the complete resolution of severe GvHD without inducing allograft rejection, and thus opened a novel therapeutic window for the treatment of fatal solid-organ transplant-associated GvHD while preserving allograft integrity. Interestingly, long-term survival of these preactivated alloreactive T cells was observed in this patient.

The specificity and efficacy of DLI for lymphohematopoietic malignancies can also be improved by taking advantage of donor-patient combinations that differ by expression of the mH antigens HA-1 and HA-2 (HA-1/2 donors and HA-1/2+ patients) and by the transfer of ex vivo selected and expanded leukemia-reactive cytotoxic T lymphocyte (CTL) lines. However, the ex vivo generation of individual HA-1/2- or leukemia-reactive T cells in sufficient numbers for adoptive cellular transfer therapy is laborious and cumbersome. As an alternative, the genes encoding the HA-2- (or HA-1)-specific T-cell antigen receptor (TCR) can be expressed via retroviral gene transfer in donor T cells. To avoid their alloreactive, GvHD-mediating immune potential within the allogeneic HSCT recipient, such TCR gene transfer in allogeneic HSCT is likely to be restricted to human cytomegalovirus (hCMV)- or Epstein-Barr virus (EBV)-specific donor T-cell lines or clones.

In fact, hCMV reactivation and EBV lymphoproliferative disease (EBV-LPD) in immunosuppressed recipients of allogeneic stem cell grafts or organ transplants are highly susceptible to virus-specific adoptive T-cell therapy. The transfer of donor-derived polyclonal hCMV-reactive CTL lines or ex vivo expanded hCMV-specific CD8+ T cells selected from stem cell donors by HLA-peptide tetramers into patients with or without viremia resulted in massive in vivo expansion, protection, and clearance of infection. However, persistence of these cells was observed in only a minority of treated patients. Although dominant hCMV pp65-reactive CD8+ T-cell responses are usually abundant in hCMV infection, it became clear that protection from hCMV disease after (organ) transplantation is actively correlated with hCMV immediate early 1 (IE-1)-specific CD8+ T cells. As with tumor-specific T lymphocyte immunity, it is likely that the simultaneous induction of hCMV-specific CD4+ T helper cells (Th) along with CD8+ CTL responding to a wide array of different hCMV-derived peptide epitopes will improve clinical intervention and immune T-cell persistence. Interestingly, the various immune evasion proteins of hCMV do not seem to prevent the therapeutic impact of a diverse set of anti-hCMV CD8+ CTL responses.

Similar lessons have been learned from the treatment of persistent active EBV infection with autologous EBV-specific CTL and the prophylaxis and therapy of post-transplant EBV-LPD by allogeneic EBV-reactive CTL. The clinical experience with autologous EBV-specific CTL transfer for other EBV-associated malignancies, such as Hodgkin’s disease (HD) and nasopharyngeal carcinoma (NPC) is limited and the results obtained so far indicate that these EBV-specific CTL are less effective than those for EBV-LPD, although even complete tumor responses were observed in a minority of patients. Decreased CTL efficacy is likely to reflect immune evasion strategies by tumor cells, such as downregulation of immunodominant EBV proteins and secretion of inhibitory cytokines, phenomonens that have in fact an important adverse effect on the treatment of solid tumors by adoptive cellular immunotherapy in non-EBV and non-transplant patients as well. Likewise, a number of approaches have been developed to overcome these immune evasion strategies, including targeting CTL to subdominant EBV antigens and genetically modifying CTL to increase their potency.

Adoptive cellular immunotherapy in cancer patients

An alternative to using allogeneic T cells to mediate antitumor responses has been to isolate autologous tumor-reactive T cells, expand the cells ex vivo, and then reinfuse the cells back into the patient. As with the adoptive transfer of T cells in allogeneic HSCT, this strategy required the recent development of methods to extensively manipulate T cells in vitro with retention of specificity and function, such that after infusion the cells survive and migrate to and eliminate tumor cells. The broadest experience with this approach has so far been in patients suffering from malignant melanoma. In a phase I study to evaluate the safety, in vivo persistence, and efficacy of adoptively transferred CD8+ T-cell clones targeting...
melanocyte differentiation antigens such as TAA, MART1/MelanA and gp100 for the treatment of patients with metastatic melanoma, four infusions of autologous T-cell clones were administered with and without low-dose interleukin-2 (IL-2) twice daily. Forty-three infusions of MART1/MelanA- or gp100-specific CD8+ T cell clones were administered to ten patients. No serious toxicity was observed and the adoptively transferred T-cell clones persisted in vivo in response to low-dose IL-2, preferentially localized to tumor sites and mediated an antigen-specific immune response characterized by the elimination of antigen-positive tumor cells, regression of individual metastases, and minor, mixed or stable responses in eight of the ten patients with refractory, metastatic disease for up to 21 months. The use of IL-2 certainly prolongs the persistence and enhances the antitumor activity of the transferred CD8+ T cells. Apart from the additional application of CD4+ Th which likely provide beneficial functions, including cytokine production and activation of professional antigen-presenting cells (APC), alternative cytokines, such as IL-7, IL-15, and IL-21, as well as activation of APC with antibodies to CD40, are currently being evaluated in preclinical studies and early clinical trials. Another means to enhance the activity and survival of transferred cells is to take advantage of endogenous homeostatic mechanisms that restore lymphocyte numbers after an episode of lymphopenia, a phenomenon that does, in fact, appear to be instrumental in HSCT. Intentional lymphodepletion of patients before T-cell transfer can promote extensive proliferation of infused T cells, creating an in vivo repertoire dominated by the desired effector population. Additionally, this promising strategy may help to establish an environment more conducive to mediating an antitumor effect by eliminating suppressive and counterproductive T regulatory lymphocytes (Treg). Accordingly, 35 patients with metastatic melanoma, all but one with disease refractory to treatment with high-dose IL-2 and many with progressive disease after chemotherapy, underwent lymphodepleting conditioning with cyclophosphamide (60 mg/kg) for 2 days followed by 5 days of fludarabine (25 mg/m²). On the day following the final dose of fludarabine, all patients received cell infusion with autologous tumor-reactive, rapidly expanded tumor infiltrating lymphocyte (TIL) cultures and high-dose IL-2 therapy. Eighteen (51%) of 35 treated patients had objective clinical responses including three with ongoing complete responses and 15 with partial responses with a mean duration of 11.5±2.2 months. Sites of regression included metastases to lung, liver, lymph nodes, brain, and cutaneous and subcutaneous tissues. Toxicities of treatment included conditioning-related neutropenia, thrombocytopenia, and lymphopenia, and transient toxicities of high-dose IL-2 therapy. Two patients developed Pneumocystis pneumonia and EBV-LPD was observed in one patient. In some patients, tumor regression was accompanied by a large in vivo expansion of the administered antitumor lymphocytes, which persisted in peripheral blood at >70% of total lymphocytes for many months after transfer. The cells capable of mediating tumor regression consisted of heterogeneous lymphocyte populations with high avidity for tumor antigens that were derived from TIL cultured for limited times in vitro. The success of this treatment likely results from the ability to infuse large numbers of activated antitumor lymphocytes into an appropriate host homeostatic environment depleted of Treg and in favor of a selective in vivo expansion of the transferred effector cells. Although this strategy, including the conditioning of patients appears to be comparably toxic and is based on individual TIL populations, it certainly opens a window of opportunity for more universal adoptive T-cell transfer-based
approaches that rely on genetically modified T lymphocytes.

**TCR gene transfer for adoptive immunotherapy of malignant disease**

Redirection of human T lymphocytes by retroviral expression of TCR genes has been successfully performed in preclinical models. A variety of mostly HLA-A*0201 (A2.1)-restricted TCR specific for numerous human LAA and TAA, mH antigens, and viral epitopes are meanwhile available for clinical application and the first clinical trial of adoptive TCR gene therapy for cancer patients is currently ongoing. There are several advantages to the use of specific TCR in adoptive cellular immunotherapy of malignant disease. High-affinity TCR can be selected by circumventing antigen-specific self-tolerance to target universal human TAA which are not necessarily dispensable for tumor cells as they are derived from proteins which are inherently involved in malignant transformation. Circumvention of self-tolerance requires TCR derived from T cells of allogeneic human donors or HLA transgenic mice.\(^{17,18}\)

Apart from bypassing antigen-specific self-tolerance, there is another important hurdle for a successful CD8\(^+\) CTL-based immunotherapy of cancer. This is its dependency on active CD4\(^+\) Th. Although the majority of malignant targets lack class II MHC, precluding direct attack by CD4\(^+\) Th, it has been demonstrated that antigen-specific Th activity is of pivotal importance for efficient eradication of such malignancies. CD4\(^+\) Th exert their anti-tumor effect independently of CD8\(^+\) CTL by recruitment of innate immune and non-immune effectors, by direct and indirect cross-talk to CTL through cytokine release, and by interaction with professional APC. Direct and indirect interaction of tumor-reactive CD8\(^+\) CTL with CD4\(^+\) Th is also essential for their sustained activity and memory pool formation. We used A2.1 transgenic mice, in which the mouse CD8 molecule cannot efficiently interact with the \(\alpha\)3 domain of A2.1, to generate a high-affinity CD8-independent TCR specific for a commonly expressed, tumor-associated CTL epitope derived from the human p53 tumor suppressor protein. Expression of this CD8-independent p53-reactive TCR in human CD4\(^+\)(CD8–) T lymphocytes led to A2.1-restricted CD4\(^+\) Th that were fully competent to interact with TCR-transduced CD8\(^+\) CTL, professional APC, and directly with class II MHC-negative tumor cells.\(^{19}\) We and others also found that a CD8 co-receptor-dependent TCR is not sufficient to functionally reprogram CD4\(^+\) T lymphocytes.\(^{17,19}\) This result likely explains the failure in previous studies to redirect CD4\(^+\) as opposed to CD8\(^+\) T cells by retroviral TCR gene transfer (Figure 1). However, CD8-independent class I peptide-MHC (pMHC)-specific TCR expressed by CD4\(^+\) or CD8\(^+\) T cells are normally rare events. As an alternative to A2.1 transgenic mice, high-affinity TCR from allogeneic donor T cells or increasing the affinity ceiling of a given TCR by \textit{in vitro} mutagenesis may provide their CD8 coreceptor independence. In contrast to transferring genes encoding a class II pMHC-reactive TCR into CD4\(^+\) T cells, the delivery of a CD8 co-receptor-dependent TCR along with the co-transduction of genes encoding human CD8 or a chimeric molecule that comprises the extracellular domain of CD8 and the cytoplasmic signaling moiety of CD4 may also impart human CD4\(^+\) T lymphocytes with functional class I pMHC-specific Th properties.

The formation of TCR heterodimers consisting of
transgenic as well as of natural TCR chains may create novel and potentially harmful (autoimmune) antigenic specificities. We and others have found that such heterodimeric TCR are in fact generated. The introduction of reciprocal mutations at the transgenic TCR αβ interface substantially impaired their ability to generate heterodimers with natural human TCR αβ chains. An alternative strategy to prevent uncontrolled responses by transgenic-natural TCR heterodimers is the expression of tumor- or MHC antigen-specific TCR in well-defined T cell subsets, such as hCMV- and EBV-specific T lymphocytes or the θ T cell subpopulation, or the use of single-chain TCR molecules (Figure 2).

T-cell and even NK-cell recognition of malignant targets has also been imparted by expressing high-affinity chimeric transmembrane receptors with the external recognition structure of an antibody and the signaling domain of a TCR. The advantage of such T- or NK-bodies is their response to leukemia and tumor antigens in an MHC-independent fashion like antibodies but still employing effector mechanisms inherent to T or NK cells.

Alternative and future genetic strategies for adoptive cellular immunotherapy

An alternative genetic strategy to introducing new structures or functions to T cells is to disrupt effector or signaling molecules and pathways that normally serve to dampen the activation or effector phase of immune responses, such as PD-L1 on tumor cells and PD-1 on tumor-reactive T cells, or Cbl-b, an adapter protein that negatively regulates TCR signal strength. Preliminary studies in which dominant-negative Cbl-b proteins are expressed or Cbl-b expression is reduced through small interfering RNA approaches suggest that increasing signal strength can both reduce the threshold for T-cell activation and restore regulated IL-2 production to effector CD8+ T lymphocytes. Many other targets for these strategies including those that confer resistance to T-cell-mediated tumor cell death have been identified by studies in knockout mice, suggesting the possibility of designing T and NK cells capable of circumventing many of the obstacles posed by malignant disease. The future genetic design of such cellular immune tools does not necessarily rely on retroviral gene transfer, but on potentially safer, transposon-mediated gene delivery systems, such as the so-called sleeping beauty.

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Educational issues related to the pan-European harmonization of specialty training and high quality continuing professional development, are of primary importance for the future of European hematology.

The EHA Education Committee was formed in 2001 to address these issues. The Committee’s current projects include: EHA Scientific Workshops, European Hematology Curriculum Passport and publication of the Education Program of each EHA annual congress.
EHATol is a distance learning tool designed to provide a readily accessible and constantly updated environment for training, education, and CME for scientists and clinicians involved in the field of hematology. It is an Information and Communication Technology (ICT) based platform which currently offers clinical cases, an on-line self-assessment system, links to scientific journals and Pub-Med, a hematological glossary and a preliminary morphology database.

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EHATol is freely available on the EHA website at www.ehaweb.org and at www.esh.org.
The Education Committee coordinated the development of the EHA Hematology Curriculum-Passport, which is a set of recommendations for a harmonized basic curriculum for the specialist training of hematologists throughout the European Union member, associated, and incoming states. These recommendations have been proposed to the national hematology societies in order to determine how they can be applied in their respective countries. This project will contribute to the quality of training and patient care throughout Europe and to the professional status of European hematologists.
Continuing Medical Education (CME) is widely accepted as a means to encourage individual practitioners in Hematology to maintain and develop their professional knowledge and skills. In December 2005, the EHA CME Unit was established to take over the responsibility for this activity from the European Council for Accreditation in Hematology (ECAH).

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