HODGKIN'S LYMPHOMA

Treatment options in early stages of Hodgkin’s Lymphoma, high cure rate with lower short and long-term toxicity

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Abstract
The definition of early stages in HL varied among cooperative groups and clinical trials. Most of them considered early stages; stage I, II, and IIIA without bulky disease. Bulky disease has been defined at the Costwolds Meeting as those tumors with more than 10 cm or a mediastinal involvement of more than one-third of the chest wall diameter. Other factors that have been considered unfavorable within the early stages are old age, high ESR, mixed cellularity or lymphocyte depleted histology, B symptoms or multiple sites of disease.

Keywords: Hodgkin’s lymphoma, early stage, treatment

Brief history of the therapy of HL from 1960s to 1980s
The standard treatment in pathological stage I–II HL in 1960s and 1970s was total nodal irradiation (mantle and inverted Y) at 35 Gy to 40 Gy. The combination of chemotherapy MOPP and MOPP like regimens (COPP, CVPP, LOPP, etc.) were used in the 1970s only in advanced stages or in patients relapsing to radiotherapy. ABVD emerged as a second-line treatment in patients who relapsed or were refractory to MOPP. In the 1980s MOPP was used with ABVD as sequential, alternated or hybrid form in order to increase response rate and DFS.

Best combination chemotherapy
Randomized studies done by European and later by USA groups demonstrated better results with ABVD than MOPP, and similar results with ABVD versus alternated or hybrid MOPP/ABVD with less myelosupression, gonadal toxicity and second malignancies.

The German Hodgkin’s Disease Study Group (GHSG) performed the HD11 trial in 1047 intermediate stage patients. It showed that there was no difference for the comparison between four courses of ABVD and four courses of standard BEACOPP, or for the comparison of 20 Gy IFRT with 30 Gy IFRT, with a 90% DFS rate and 97% OS rate at 2 years [1].

The EORTC and GELA randomized 808 patients in the H9-U trial in stage I–II with unfavorable clinical features to 6 vs. 4 cycles of ABVD vs. 4 cycles of BEACOPP baseline followed by 30 Gy IFRT in all the arms. The 4 years DFS were 94%, 89%, and 91% in the 3 arms respectively (P = 0.23). The 4 years OS rates were 96%, 89%, and 93% respectively (P = 0.89) [2].

Number of cycles of chemotherapy
The GATLA performed a randomized study in stage I–II without bulky disease of CVPP for 3 vs. 6 cycles without radiotherapy. The DFS at 5 and 10 years were 85% and 85% for these treated with 3 cycles; 93 and 73% for 6 cycles of CVPP. The OS at 10 years were 89% in both arms concluding that 3 cycles of CVPP without radiotherapy were equally effective than six cycles [3].

The GHSG in a trial HD 10 compared in stage I–II without risk factors 4 vs. 2 cycles of ABVD and 30 Gy vs 20 Gy of IFRT. The DFS and OS at 2 years in 847 evaluable patients was 97% and 98% respectively,
with no statistical difference between ABVD 2 vs. 4, and IFRT 30 Gy vs. 20 Gy [4].

**Extended field vs. involved field radiotherapy as consolidation after chemotherapy**

The GHSG randomized 1064 patients with early stage unfavorable prognosis after 4 cycles of alternate COPP and ABVD to extended-field radiotherapy or IFRT 30 Gy plus 10 Gy to bulky disease. The CR, DFS and OS were 98%, 83% and 91% at 5 years without difference between type of RT arms [5]. Bonadonna et al. [6] showed that in 136 patients with early stage of HL after 4 cycles of ABVD, 36 Gy of subtotal nodal plus spleen irradiation were equivalent to IFRT. The overall 12 years DFS and OS were 93% and 95% respectively.

**Chemotherapy vs. combined modality therapy**

The Canada Clinical Trial Group (CCTG) and the Eastern Cooperative Oncology Group (ECOG) reported the results of a randomized trial comparing ABVD 4–6 cycles alone vs. ABVD plus STNRT 35 Gy in limited-stage HL. No difference was observed in DFS (88% vs. 86%), or OS (94% vs. 96%) between both treatment modalities [7]. Strauss et al. [8] randomized 152 untreated stage I–IIIA nonbulky HL to 6 cycles of ABVD alone versus ABVD followed by 36 Gy of radiation. The 5 years DFS and OS for ABVD and ABVD + RT was 81% vs. 86% and 90% vs. 97% being the differences not significant.

In the current trial of the Argentine Group for Treatment of Acute Leukemia (GATLA), patients with stage I–IIIA without bulky disease and who achieved CR after the third cycle of ABVD received IFRT 25 Gy. A total of 172 patients were evaluable, the DFS and OS at 60 months were 92% and 98% respectively [9].

The GHSG compared in a trial, patients who received eight cycles of BEACOPP, 30 Gy IFRT or no radiation to bulk or residual disease. CR was achieved in 93%, the DFS for the total group was 89% and OS was 95%. There was no difference for DFS or OS in an attempt to treat analysis between the RT and no RT arm [10].

The GATLA has compared 6 cycles of CVPP vs. 6 cycles of CVPP plus IFRT 30 Gy in early stages of HL without bulky disease. Also, the comparison of the DFS and OS at 5 and 10 years showed no difference (3). The EORTC and GELA (2) in a H9-F trial compared in 783 patients with early and favorable disease who achieved CR after 6 cycles of EBVP, 36 Gy vs. 20 Gy of IFRT vs. no radiotherapy. The 4 years DFS was 87%, 84% and 70% respectively (P < 0.001) being superior in the two arms with radiotherapy.

**Second malignancies**

The combination of regimens that included alkylating agents as MOPP, BEACOPP, has shown to produce higher rate of AML/MDS, mainly during the first 5 years since treatment started. The incidence of second solid tumor continued to increase compared to the normal population more than 30 years later. However most of the long-term reports were in patients treated two or three decades ago with MOPP like regimens and wider fields and higher doses of radiation, that is not the current practice today.

**Conclusion**

The current treatment of choice for localized stages without “bulky” disease is 2 or 4 cycles of combined treatment with ABVD and low doses of radiotherapy (20 to 25 Gy) to involved areas in partial responders or bulky disease at diagnosis. The new imaging techniques as positron emission tomography (PET) will help more sensitive discriminate between early responders, late responders, and nonresponders with ABVD chemotherapy and tailored consolidation radiation therapy only for these late responders, and nonresponders that probably will be less than 20% of the early stages of HL.

**References**


NON-HODGKIN’S LYMPHOMA

Treatment of indolent lymphomas from watch and wait to high dose therapy

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Indolent B-cell non-Hodgkin’s lymphomas (IBC-NHL) represent a heterogenous group of chronic diseases [1]. Follicular lymphomas grades 1 and 2 (FL1/2) are by far the most common, accounting for approximately 20% of all NHL worldwide. Indolent IBC-NHL also include small lymphocytic lymphoma (SLL), which is the tissue counterpart of B-chronic lymphocytic leukemia (B-CLL); [2], lymphoplasmacytic lymphoma (LPL; 1%), mantle cell lymphoma (MCL; 6%), and marginal zone lymphomas, either splenic (SMZL; 1%), nodal (NMZL, 2%) or extranodal MALT (8%).

Various treatment strategies ranging from “watch and wait” policies or oral alkylating agent monotherapy to more aggressive combination chemotherapy (CT), chemoimmunotherapy or even CT followed by high dose therapy and autologous stem cell transplantation (ASCT) have been used in previously untreated patients with IBC-NHL. However there is no clear evidence for the superiority of any particular approach in terms of overall survival (OS), because: (i) The natural history of IBC-NHL is generally prolonged and many of these approaches are ultimately integrated in the overall treatment strategy, thus minimizing OS differences, despite significant differences in progression free survival (PFS); (ii) With the exception of FL, the rarity of these disorders raises further difficulties in the design of randomized trials.

We will review here current treatment approaches for FL1/2, MCL, SLL, LPL including Waldenstrom’s macroglobulinemia and NMZL, SMZL and extra-nodal MZ lymphomas of MALT type (EMZL), focusing mainly on first-line therapies.

Follicular lymphomas, grades 1 and 2

Ann Arbor stages I and II

This is the only subgroup of FL1/2, which is considered curable, accounting for approximately 1/4 of the total patient population. Involved field radiotherapy (IF-RT) may cure approximately half of stage I and one quarter of stage II patients. However based on recent developments some questions may be raised. Thus, it is not known whether IF-RT could be curative in patients with conventional stage I or II but with subclinical lesions in PET imaging. Furthermore, many of these patients have molecular evidence of disease dissemination as detected by the presence of BCL-2 or immunoglobulin heavy chain gene rearrangements in the blood or bone marrow DNA. It is not known whether such patients may be cured by and which is the place of rituximab in the potential eradication of residual disease.

Ann Arbor stages III and IV

Given that advanced FL1/2 are clearly incurable with conventional CT, a “watch and wait” policy is applied in patients with asymptomatic, non-bulky disease. This approach is supported by randomized trials [3]. Chemotherapy based treatment should be instituted when the patient develops constitutional symp-
Watch and wait to high dose therapy for indolent lymphomas

toms or symptoms related to tumor burden. There is a plethora of treatment options (Table I), which differ with respect to response rates, PFS, and toxicity, but OS differences are not demonstrable so far.

Elderly patients can be safely treated with oral alkylating agents, such as intermittent chlorambucil, alkylator-based combination chemotherapy (CVP) or monotherapy with the anti-CD20 monoclonal antibody rituximab. Younger patients can also be treated with such approaches, but many centers prefer to administer anthracycline-based CT (CHOP or similar regimens, MCP: mitoxantrone, chlorambucil and prednisone, etc) or CT based on both anthracyclines and purine analogues (FND, FCM etc).

Several randomized trials have now convincingly demonstrated that the addition of rituximab to conventional CT produces superior PFS rates, but follow-up is still short to reveal potential differences in OS [4–7]. The efficacy of rituximab in the setting of relapsed/refractory FL [48% response rate (RR) with 13 mo median PFS] may in part obviate OS benefits of chemoimmunotherapy over CT alone. For the time being, it seems reasonable to add rituximab to the first-line CT regimen, irrespectively of the intensity of the latter.

A recent meta-analysis suggested that interferon-alpha (IFN-α) incorporated in the initial CT regimen and/or given as maintenance to responding patients may result in superior PFS rates and a modest increase in 10-year OS, in the order of 6–8% [8]. OS benefits were apparent only in the subgroups of patients receiving more intensive CT or higher doses of IFN-α. However the individual randomized trials included in this analysis were performed prior to the introduction of rituximab. Thus the benefit of IFN-α in the era of rituximab remains uncertain.

The value of autologous stem cell transplantation (ASCT) incorporate in the first-line approach of FL has been tested in 3 randomized trials [9–11]. Two of them demonstrated significant prolongation of PFS, while the third [11] revealed an OS benefit despite similar PFS! Firm conclusions on OS cannot be derived yet, but notably two trials demonstrated a significant increase of MDS/ANLL in ASCT-treated patients. Furthermore, rituximab, which prolongs PFS compared with conventional CT alone, was not used in anyone of these trials. Thus, ASCT is still experimental in the first-line treatment of FL. In contrast, ASCT is clearly indicated in relapsed/refractory disease.

Rituximab, when given as first-line monotherapy, produces RR of approximately 70% [12]. Maintenance with 4 bimonthly infusions prolongs the PFS over rituximab induction alone (median 36 vs. 19 months) [12]. It is however uncertain whether rituximab maintenance is superior to induction followed by retreatment upon progression. Given the favorable toxicity profile of rituximab and the potential of durable responses, it can be considered as frontline therapy at least in patients not eligible for aggressive CT.

Recently, radioimmunotherapy with 131I-Tositumomab (Bexxar) was shown to be very effective, not only for relapsed/refractory disease, but as first-line therapy as well. A single one week course of therapy produced a RR of 95% with 75% CRs in 76 previously untreated stage III/IV FL patients. Among CRs, 80% were extended at the molecular level as well, while the 5-year PFS was 59% and 5-year OS 89% [13]. In the setting of relapsed/refractory disease radioimmunotherapy with Bexxar or Y 90 Ibritumomab tiuxetan (Zevalin), which can be administered in an outpatient basis, produces RR of 70–80% with approximately 30% CR. Radioimmunotherapy even at myeloablative doses with stem cell support has also produced very promising results in relapsed/refractory disease.

### Mantle cell lymphoma

MCL resembles to the IBC-NHL in that there is a continuous pattern of relapse and no plateau in survival curves. However MCL can not be strictly considered as an IBC-NHL, because median survival is short usually in the range of 2 to 4 years with <10% surviving at ten years after diagnosis. In contrast to FL, a “watch and wait” policy is not advisable in MCL except perhaps a minority of elderly patients with poor performance status. However approaches with very diverse toxicity profiles have been applied. The preferred treatment approach is highly depended on patient’s age.

CHOP or similar regimens are preferred by most centers, while others also use fludarabine-based regimens. The addition of rituximab to CHOP moderately prolonged PFS but had no effect on OS in a recent randomized trial [14]. The MD Anderson group has reported impressive preliminary results

**Table I. Treatment Options for Follicular Lymphomas-Grades 1 and 2**

<table>
<thead>
<tr>
<th>Watch and Wait</th>
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<tr>
<td>Oral alkylating agents (intermittent chlorambucil, etc)</td>
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<tr>
<td>Alkylator – based combination chemotherapy (CVP)*§</td>
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<tr>
<td>Anthracyclin – based combination chemotherapy (CHOP and similar regimens, MCP)*§</td>
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<tr>
<td>Fludarabin – based combination chemotherapy (FND, FCM, etc)*§</td>
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<tr>
<td>Rituximab monotherapy</td>
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<td>Chemotherapy followed by high dose therapy and autologous stem cell transplantation§</td>
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<td>Radioimmunotherapy</td>
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*Interferon possibly added during and/or after chemotherapy, §Rituximab may be added in combination chemotherapy regimens, CVP: cyclophosphamide, vincristine, prednisone, CHOP: cyclophosphamide, vincristine, doxorubicin, prednisone, MCP: mitoxantrone, chlorambucil, prednisone, FND: Fludarabine, mitoxantrone, desamethasone, FCM: Fludarabine, cyclophosphamide, mitoxantrone.
with the combination of rituximab and hyperCVAD regimen [15]. In the absence of randomized trials, this approach is considered experimental given that there is no plateau in PFS curves, the high toxicity of the regimen. Studies evaluating the first-line use of ASCT are ongoing, but no definitive OS data have been published so far. Allogeneic SCT – either myeloablative or based on reduced intensity conditioning regimens are the only potentially curable approaches and deserve further evaluation.

In contrast to these high-intensity approaches, some patients with MCL may achieve relatively durable remissions with chlorambucil monotherapy [16]. Thus, elderly asymptomatic patients without features of histologic aggressiveness (non-blastoid MCL) may be treated in this way. Rituximab monotherapy may produce RR of 25–30% in both untreated and relapsed/refractory MCL patients, but CRs are very rare (~2%) and the median PFS is in the range of 6–12 months. Maintenance rituximab does not appear to improve these results [17]. In the rare "splenic form" of MCL, splenectomy may be a reasonable first-line approach, delaying the administration of CT [18]. Novel agents, as the proteasome inhibitor bortezomib and temsirolimus a rapamycin kinase inhibitor that regulates cyclin-D1 translation are effective in relapsed/refractory patients and require further evaluation [19,20].

**Splenic marginal zone lymphoma**

SMZL is another entity for which the "watch and wait" policy was the preferred approach for patients having asymptomatic splenomegaly or non significant cytopenias. When treatment is needed, splenectomy is still a reasonable option. Various CT regimens, including CHOP or fludarabine-based ones as well as oral alkylating agents have been used, mainly in splenectomy failures or in patients not eligible for splenectomy [21]. The selection of the CT regimen is present arbitrary. The goal of treatment is to achieve a good response, but not necessarily a CR. Interesting preliminary results have been reported with rituximab monotherapy, which may delay splenectomy [22]. The subgroup of patients with SMZL and hepatitis C virus infection achieve long-lasting partial remissions of excellent quality with interferon-a and/or ribavirine without CT [23].

**SLL, LPL/MW, NMZL**

Specific data for these subtypes of IBC-NHL, particularly for NMZL, are lacking. We favor treatment of these patients, who present usually with advanced age, with monthly intermittent chlorambucil for 1–2 years. High-dose chlorambucil produced a RR of 72% with 30% CRs in a recent randomized trial, with 5-year PFS rates of 20–30%. The addition of epirubicine or the administration of IFN-a maintenance did not improve these results [24]. Rituximab produces responses similar to that observed in FL and can be considered as monotherapy in these patients [25]. As many as 70% of previously untreated patients respond to induction plus maintenance rituximab, with a median PFS of approximately 2.5 years.

Combination CT (CVP, CHOP) is usually deserved for relapsed disease while purine analogues may also have a role in the treatment of these patients, although clear data are not yet available.

**EMZL**

MALT lymphomas commonly arise in the stomach but also can be seen in other extranodal sites such as the skin, salivary glands, lung, ocular adnexa and thyroid. Gastric lymphomas respond well to antibiotic therapy in conjunction with proton pump inhibitor therapy. A considerable percentage of patients achieve CR [26]. Radiation therapy or alkylating agent therapy alone or in combination with Rituximab is reserved for non-responders. For non-gastric MALT lymphomas the optimal management is not well defined. Single alkylating agent or CVP regimen, radiotherapy, immunotherapy, surgery alone or in combination have been successfully used [27]. Anthracycline based regimens do not seem to improve the response rate [28].

**Conclusion**

IBC-NHL may be treated with various strategies, without clear superiority of anyone of them. Evidence-based approaches are likely to emerge for FL (and probably MCL), but are difficult to be obtained for SLL, LPL and marginal zone lymphomas. The introduction of rituximab has revolutionized the treatment of IBC-NHL. ASCT and newer approaches including bortezomib and temsirolimus may improve their eventual outcome. For the time being, only ASCT can cure a fraction of relapsed/refractory patients at the expense of a high rate of early mortality.

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**References**


NON-HODGKIN’S LYMPHOMA

Update on lymphoma management: Diffuse large B-Cell NHL

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Keywords: Diffuse large cell, lymphoma, treatment

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most commonly occurring subtype of non-Hodgkin's lymphoma (NHL) in the Western Hemisphere, comprising about one-third of all adult lymphomas [1]. The natural history of this subtype is aggressive, with a median survival of less than 1 year in untreated patients. Over the past decade, remarkable progress has been made in understanding the biological heterogeneity of DLBCL. There is clear evidence that, in many cases, the clinical behavior of certain DLBCLs can be profiled by the expression of molecular markers [2,3]. These markers have not only contributed to the development of novel prognostic models, allowing clinicians to refine their ability to identify patients at high risk but they have also been integral in the identification of new therapeutic targets.

The clinical management of DLBCL has changed dramatically over the past five 5 years. Routine incorporation of monoclonal antibody therapy in induction treatment regimens has improved OS in most subgroups of patients with DLBCL. In addition, studies evaluating high-dose chemotherapy and autologous stem cell transplantation (SCT) as consolidation treatment during first remission have shown promise. Perhaps most exciting is the multitude of promising new agents now under development.

Despite many recent advances, most patients with advanced-stage DLBCL are not cured with conventional therapy. Given this reality, treating physicians must recognize the inadequacy of current therapies and urge their eligible patients to participate in well-designed clinical trials. The development of novel therapies may result in improved outcomes for patients diagnosed with these common NHL subtypes.

Diffuse large B-Cell lymphoma—clinical risk stratification

Clinical risk stratification is necessary to define optimal therapy for patients with “early stage” DLBCL. “Early stage” NHL usually refers to disease limited to a single side of the diaphragm, including, at most, stage 1 contiguous extranodal site. It has been well documented that patients with “bulky” stage 2 disease (i.e., a mediastinal mass >10 cm or >1/3 of the maximum diameter of the chest) have a prognosis indistinguishable from that of patients with advanced-stage disease; thus, these patients should be treated differently from other patients with early-stage disease.

Randomized clinical trials have demonstrated that a combined-modality approach incorporating a brief duration of chemotherapy followed by involved-field radiation remains a reasonable standard of care for most patients with early stage DLBCL. A SWOG study randomized 401 patients with aggressive non-bulky stage 1 or 2 NHL (mainly DLBCL) to 3 cycles of CHOP followed by involved-field radiation (40-50 Gy) or to 8 cycles of CHOP alone [4]. At 5 years, PFS and OS rates were significantly higher in the combined-modality arm than in the chemotherapy-alone arm (77% vs. 64% and 82% vs. 72%, respectively, with less life-threatening toxicity in the combined modality arm (P=0.06).
More recently, the Eastern Cooperative Oncology Group (ECOG) enrolled 210 patients with either diffuse, aggressive stage 1 lymphoma with mediastinal or retroperitoneal masses greater than 10 cm in diameter (bulky disease), or stage 1E, 2, or 2E disease. The 172 patients who had attained CR after 8 cycles of CHOP were randomized to receive no further therapy or involved-field radiation [5]. Disease-free survival at 6 years was superior in the combined treatment arm (73% vs. 56%; 2-sided \( P = 0.05 \)). However, there was no difference in overall survival. Therefore, the benefit of radiation therapy following a full course of chemotherapy appears to be limited to enhanced local control.

When any risk factor (age >60 years, high lactate dehydrogenase (LDH) level, stage 2 disease, and performance status \( \geq 2 \)) by the stage-modified (“Miller Modification”) International Prognostic Index (IPI) is present, outcome is inferior to that of patients with no risk factors [6]. For example, in the SWOG study, 5-year overall survival was 94%, 71% and 50%, respectively, for those with 0 or 1, 2, or 3 risk factors; and 5-year failure free survival estimates were 82% for patients with 0 or 1 risk factor, 71% for patients with 2 risk factors, and 48% for patients with 3 risk factors [4].

These findings have been confirmed by Canadian researchers who evaluated combined modality therapy in a similarly defined group of early stage patients [7].

The overall survival rates at 5 years were 97% for patients with no risk factors, 77% for patients with 1–2 risk factors, 58% for patients with 3 risk factors, and 48% for patients with 4 risk factors, with similar decrements in PFS reported for increasing numbers of risk factors [7]. Similarly, in the ECOG study, the following factors were significantly associated with prolonged survival among patients receiving induction CR: age less than 60 years \( (P < 0.001) \), and fewer than 3 disease sites \( (P = 0.01) \). As in the Canadian trial, these factors were also associated with prolonged survival among complete responders receiving induction therapy [5].

The Groupe d’Étude des Lymphomes de l’Adulte (GELA) has reported results from a randomized trial of previously untreated patients younger than 61 years with localized, aggressive stage 1 or 2 lymphoma and no IPI risk factors. The study compared 3 cycles of CHOP plus involved-field radiotherapy \( (n = 329) \) or chemotherapy alone with dose-intensified doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone (ACVB) plus sequential consolidation with high-dose methotrexate, etoposide, ifosfamide, and cytosine arabinoside \( (n = 318) \) [8]. Notably, patients with bulky stage 2 disease were not included in this trial. The 5-year event-free survival estimates were 82% for patients receiving ACVBP chemotherapy alone (followed by intensive consolidation) and 74% for those receiving standard CHOP with radiation therapy. The respective 5-year OS estimates of OS were 90% and 81%, respectively. Thus, ACBVP conferred only a modest survival benefit among patients without bulky disease.

The GELA group presented data from an early analysis of elderly patients with localized aggressive NHL and an age-adjusted IPI risk of 0 [9]. The report suggested that the adding involved-field radiotherapy to 4 courses of CHOP did not improve CR rates, 5-year event-free survival, or 5-year OS [23]. Finally, another preliminary report from a SWOG pilot trial that treated patients with limited stage disease to 3 cycles of R-CHOP, followed by radiation therapy [10] calculated 2-year progression-free survival and OS at 94% and 95%, respectively, superior to historical results with CHOP chemotherapy alone. Of note, this trial required patients to have at least 1 risk factor in the Miller IPI modification. However, 10-year follow-up of SWOG’s original randomized trial [11] suggested an increase in late recurrences \( (>3 \text{ years after completion of therapy}) \) in patients treated with combined radiochemotherapy, including a fixed mortality rate over the first 10 years, with no evidence of a plateau in the survival curve. Therefore, long-term follow-up is clearly required before it can be said that one regimen is superior to another, particularly when that regimen is compared with historical controls.

Despite the preliminary nature of these follow-up findings, the SWOG investigators currently recommend 3 cycles of CHOP plus rituximab in addition to involved-field radiation for most patients with stage 1 and nonbulky stage 2 disease, on the basis of increased survival through the first 9 years and less associated toxicity. Select elderly patients lacking other risk factors may not require radiation therapy, and patients with bulky disease clearly require more chemotherapy and may benefit from intensified regimens. By using new approaches such as radioimmuno-therapy and 18F-fluorodeoxyglucose positron emission tomogram imaging, current clinical trials in patients with early stage DLBCL and at least 1 Miller IPI modification risk factor [12] will be useful in defining which patients may not require external-beam radiation.

In patients with advanced-stage DLBCL, rituximab appears to improve survival when administered in combination with standard chemotherapy, but no additional benefit is observed with the addition of maintenance rituximab.

In a 2002 publication, GELA reported that rituximab added to standard CHOP conferred a higher OS rate for older patients \( (>60 \text{ years}) \) with advanced-stage DLBCL [13]. These results truly changed clinical practice throughout much of the world. Eight cycles of CHOP alone (control arm) or CHOP with rituximab (treatment arm) produced CR rates of 63% and 76%, respectively \( (P = 0.005) \) and a 2-year OS of 57% and 70% \( (P = 0.007) \). A recent update of this
trial demonstrated that the survival benefit was maintained, and actually continued to improve through 5 years of follow-up [14]. A subgroup analysis of this large study has revealed that 2 groups of patients appear to derive particular benefit from rituximab: (1) those with an age-adjusted low IPI risk and (2) those with DLBCL positive for Bcl-2 overexpression, historically a poor prognostic factor. This finding suggests that one of the ways in which rituximab works is to overcome Bcl-2-associated chemotherapy resistance [15].

Preliminary results from the MabThera International Trial (MInT), currently evaluating CHOP-like chemotherapy regimens plus rituximab in patients younger than 60 years, were recently presented [16]. As in the GELA trial, patients who received rituximab plus chemotherapy had a significantly longer 2-year time to treatment failure (81% vs. 58%) than patients receiving chemotherapy alone. In addition, the 2-year OS rates also significantly favored chemotherapy plus rituximab (95% vs. 85%).

Similar to the GELA trial, the as-yet unpublished larger (N = 632) US Intergroup Study randomized a population of 632 elderly patients to 8 cycles of CHOP or CHOP plus rituximab given every other cycle [17]. Responder patients were then randomized to receive either rituximab “maintenance” (4 doses, every 6 months for 2 years) or no maintenance therapy. A weighted analysis was used to mathematically model the groups that had been treated with CHOP alone or CHOP plus rituximab as induction therapy, controlling for maintenance exposure. The magnitude of the OS benefit of induction therapy with CHOP plus rituximab was similar to that seen in the GELA trial, which essentially confirmed the GELA results. Perhaps the most important contribution of the United States Intergroup Study, however, was that it demonstrated a lack of benefit with “maintenance” rituximab when rituximab was included in the initial chemotherapy regimen. The role of dose-intense regimens which are rituximab-based therapies is unclear. Recently published results of 2 large German trials (NHL-B1 and NHL-B2) suggest that modifications to the CHOP regimen may improve survival. The major limitation of these trials is that they did not include rituximab. These 2 trials randomized patients to 6 cycles of CHOP-21 (every 3 weeks) or CHOP-14 (every 2 weeks) vs. CHOEP-21 (CHOP plus etoposide 100 mg/m² Days 1-3 every 3 weeks) or CHOEP-14 (CHOP plus etoposide 100 mg/m² Days 1-3 every 2 weeks). Patients in these trials also received radiotherapy (36 Gy) to both extranodal and bulky disease sites. One trial (NHL-B2) was limited to patients older than 60 years [18]. Five-year event-free and OS rates were respectively 32.5% and 40.6%, for CHOP-21 and 43.8% and 53.3% for CHOP-14. Toxicity was similar among CHOP-14 and CHOP-21 participants, but CHOEP-21, and, especially, CHOEP-14 were more toxic than either CHOP regimen. In the parallel trial (NHL B1) for patients younger than 61 years, better complete remission rates were obtained with CHOEP than with CHOP (87.6% vs. 79.4%; P = 0.003) as well as improved 5-year event-free survival rates (69.2% vs. 57.6%; P = 0.004, primary end point) [19]. The benefit of interval reduction was less clear in the younger than in the older patients. Although the CHOEP were more myelosuppressive, they were reasonably well tolerated. Only 3 therapy-associated deaths occurred, 1 (0.5%) among the CHOEP-21 and 2 (1.1%) among the CHOEP-14 participants.

The magnitude of benefit seen with these dose-intense regimens is similar to that observed with the addition of relatively nontoxic rituximab therapy reported in other trials. Indeed, a recent retrospective analysis of patients included in the MInT trial suggests that survival differences between different CHOP-like regimens, including CHOEP, disappear when rituximab is added to standard therapy [20]. Since trials incorporating monoclonal antibody therapy into these dose-intensified regimens are ongoing, the routine use of dose-intense regimens outside of a clinical trial is not currently recommended.

Ongoing prospective trials are underway to define the role of autologous stem cell transplantation (ASCT) consolidation for patients with high-risk, advanced-stage DLBCL in first remission. Several phase 3 trials have evaluated ASCT in newly diagnosed patients with DLBCL, either as consolidation therapy after CR or as induction therapy. In most of these trials, however, high-risk disease was identified by criteria other than the IPI, and a variety of schedules incorporating ASCT have been used [21].

The Groupe Ouest-Est des Leucémies et des Autres Maladies du Sang (GOELAMS) trial randomized 197 consecutive patients to receive either 8 courses of standard CHOP chemotherapy, or a complicated regimen of ASCT plus chemotherapy, starting with cyclophosphamide, vindesine, epirubicin, and prednisone (CEEP), followed by high-dose methotrexate and cytarabine, then treated with carmustine, etoposide, cytarabine, and melphalan (BEAM) for stem cell conditioning prior to ASCT [22]. Overall, 78% of the patients completed the assigned treatment. With a median follow-up of 4 years, the estimated event-free 5-year survival rate was significantly higher for patients who received ASCT than for those who received standard CHOP (55% vs. 37%). A retrospectively performed subgroup analysis demonstrated a survival benefit in patients with age-adjusted high-intermediate IPI risk (OS, 74% vs. 44%).

These data are reminiscent of the LNH87-2 trial results previously published by GELA [23]. In this trial, 1043 patients were initially randomized to
treatment with 4 courses of an anthracycline-based regimen. Patients who achieved CR were randomized to receive additional cycles of sequential chemotherapy or ASCT. As in the GOELAMS trial, a retrospective assessment of 451 high-intermediate or high IPI risk patients showed that the 8-year OS rate was higher in the ASCT arm than in the sequential chemotherapy arm (64% vs. 49%). Of course, these retrospective subgroup analyses must be interpreted cautiously because these higher risk patients were not initially identified as the target population for these trials. Most of the current mature phase 3 trials have reported improved disease-free survival (DFS) but not improved OS with ASCT therapy in patients younger than 60 years with high or high-intermediate IPI risk scores [21]. Moreover, none of these trials included rituximab therapy, so it is not known whether the benefit of is abrogated by the addition of rituximab to induction or consolidation therapies. This is a particularly important question in light of the MInT trial analysis, suggesting that rituximab may abrogate the benefit of intensified regimens.

Radioimmunotherapy with iodine-131 (I-131) tositumomab or ibritumomab tiuxetan is quite active in the treatment of indolent B-cell lymphoma and is worthy of further investigation in other lymphoma subtypes. Zelenetz and colleagues analyzed 71 patients whose indolent lymphomas underwent Richter's transformation to more aggressive histologic forms who were treated with I-131 tositumomab in 5 clinical trials [24]. The overall response rate for a single treatment with I-131 tositumomab was 39%, with a median response duration of 20 months. In 24% of these patients, response duration was longer than 1 year. Given the relatively low toxicity profile of the I-131 tositumomab regimen compared with that of ASCT, [25] the radioimmunotherapy approach holds significant promise for patients with transformed disease.

Morschhauser and colleagues have recently completed a prospective, multicenter phase 2 trial to evaluate the efficacy and safety of yttrium-90 ibritumomab tiuxetan in elderly patients with histologically confirmed primary refractory or relapsed DLBCL for whom ASCT is contraindicated [26]. An overall response rate of 44% was observed for the entire study population. The median response duration was 22 months in those patients who had never received rituximab. By contrast, only 19% of patients treated with prior chemoimmunotherapy responded to radioimmunotherapy.

These results are encouraging. Both I-131 tositumomab and yttrium-90 ibritumomab tiuxetan are currently being evaluated for the treatment of DLBCL in multicenter trials. For example, SWOG is currently conducting a trial with I-131 tositumomab as consolidation therapy following standard R-CHOP for patients over 60 with DLBCL.

Despite the many recent advances summarized in this manuscript, most patients with advanced stage DLBCL are not cured with conventional therapy. Hence, each treating physician must recognize the inadequacy of current therapy and urge all eligible patients to participate in well-designed clinical trials. Several of the investigators conducting ongoing clinical trials have emphasized that providing optimal therapy often involves experimenting with both new and old agents in novel ways. Further development of the aforementioned novel therapies should result in improved outcomes for patients suffering from these common subtypes of NHL.

References


CHRONIC MYELOID LEUKEMIA

Allogeneic transplantation for chronic myelogenous leukemia

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Keywords: CML, transplantation

Related donor transplantation

Over the last quarter century investigators have demonstrated that allogeneic hematopoietic cell transplantation (HCT) can cure chronic myelogenous leukemia (CML). Early reports suggested the efficacy of related donor transplant after a myeloablative preparative regimen containing total body irradiation (TBI) [1–3]. Subsequent studies have identified variables which improve outcome such as transplant in early chronic phase, younger recipient age, donor/recipient compatibility at the major HLA loci and male donor gender [4,5]. Myeloablative regimens which do not contain TBI have also proven effective in transplant for CML [6]. The use of peripheral blood progenitor cells as a source of stem cells for transplant mobilized with G-CSF is comparable in most respects to non-mobilized related donor marrow, although long-term studies may uncover differences in the incidence of chronic GVHD and in the risk of relapse [7].

Adult unrelated donors

Alternative sources of stem cells for CML patients without a suitably HLA-matched related donor have been developed. Adult, volunteer unrelated donors (URD) can be obtained through the National Marrow Donor Program (NMDP) and other registries for many, but not all, otherwise eligible patients. Factors predicting success of transplant are comparable in the unrelated and related donor setting [8–10]. Large, retrospective analyses have identified certain donor-recipient disparities such as mismatch at HLA-C as a predictor of poor outcome, while DNA-based methodology has identified certain single and multiple allele mismatches with adverse effects on outcome in URD transplant for CML [11].

Umbilical cord blood

Umbilical cord blood (UCB) has been identified as a source of hematopoietic stem cells for transplantation [12]. Furthermore, UCB may be particularly useful in the unrelated donor transplant setting since HLA-typed, frozen and stored cells are usually available within 1–2 days through international registries, can be used to repopulate hematopoiesis in adults as well as children, and may (arguably) tolerate a greater degree of HLA mismatch with the recipient than hematopoietic stem cells obtained from adult volunteer URD [13,14]. The role of UCB transplant in CML is promising, but not yet fully explored.

Donor Leukocyte Infusions

Although a clinically relevant “graft-versus-leukemia” (GVL) effect was first detected over 20 years ago [15], the importance of this effect in transplant therapy for CML has recently been underscored. Early attempts at ex-vivo T-cell depletion after related donor HCT for CML resulted in a reduced incidence of acute GVHD, but an unexpected, extraordinarily high incidence of relapse. Analyses of a large International Bone Marrow Transplant Registry (IBMTR) data set demonstrated a correlation between development of GVHD and protection from CML relapse [16].
Subsequently, Kolb et al. have demonstrated the efficacy of donor leukocyte infusions (DLI) to suppress persistent or recurrent malignant clones after allogeneic HCT [17–19]. Predictably, such infusions may provoke or exacerbate GVHD.

**Nonmyeloablative preparative regimens**

These clinical observations, underpinned by considerable preclinical data [20], prompted investigators to develop less intensive and often non-myeloablative (NMA) preparative regimens for allogeneic HCT therapy of CML. These NMA regimens are intended to minimize toxicity while exploiting the GVL effect [21–25]. Current results suggest that HCT with a NMA preparative regimen is a feasible treatment option for individuals not eligible for more standard preparative regimens. The incidence of nonrelapse mortality may be lower; however non-engraftment, GVHD, infection and disease persistence or recurrence still complicate — transplants [26].

**Current clinical results**

Analysis of long-term results using a large data set from the Center for International Blood and Marrow Transplant Research (CIBMTR) reveals an approximately 60% incidence of overall survival at 10 years for over 3300 first chronic phase CML patients receiving related donor HCT between 1978 and 1997 and 50% for over 1300 similar patients receiving transplants from alternative donors. The incidence of relapse at 10 years for these two groups is approximately 20%. Of note, the overall survival curve does not plateau. Important late-occurring causes of death include GVHD, infection and relapse [27].

**Late effects and quality of life**

Late effects and quality of life after allogeneic HCT therapy for CML are important issues in a field where effective alternative therapy is developing rapidly [28]. A retrospective analysis of late effects in 248 CML transplant recipients who had survived at least 2 years was very informative [29]. Compared to siblings, survivors had a high prevalence of long-term health-related complications including endocrine, ocular, oral health, gastrointestinal, musculoskeletal, neurosensory and neuromotor impairment. In a non-overlapping study of 46 CML transplant recipients, investigators observed a high incidence of late cognitive deficits and an increase in psychosexual problems compared to the general population [30]. Of interest, in a third study CML patients and physicians reported an improved Quality of Life (QOL) Index score, decreased signs and symptoms of depression and less alcohol consumption at 12 months following transplant compared to the study patients’ immediate pretransplant baseline [31].

**The imatinib era**

The development of the selective Bcr-Abl tyrosine kinase inhibitor imatinib (STI-571, Gleevec) has fundamentally changed therapy of CML. Imatinib, given orally on a daily basis as first-line therapy in newly diagnosed chronic phase CML patients, results in hematologic, cytogenetic and molecular remissions in the majority of cases [32]. Relapses occur and can often be attributed to mutations in the BCR-ABL gene [33]. It is possible that such mutations can be treated successfully with a second generation of tyrosine kinase inhibitors possessing higher binding affinities for the ABL kinases [34–37]. These exciting developments call into question the historical first-line role of allogeneic HCT therapy for CML.

**Transplant in the imatinib era**

Recently, investigators have demonstrated that imatinib can be used effectively to treat CML relapse after allogeneic HCT [38]. By 6 months after imatinib therapy for post-transplant relapse, 9 of 10 patients achieved cytogenetic remission, and the BCR-ABL transcript could not be identified in 7 of these patients. Of note, patients achieving cytogenetic remission also converted to complete donor chimerism. These early results raise the issue of substituting imatinib therapy for DLI in the post-transplant relapse setting.

In small, uncontrolled trials, imatinib has also been administered as prophylaxis during the first 100 days following transplant for poor prognosis CML [39]. Early results suggest that this approach is feasible, but requires reduced doses of imatinib and tacrolimus and resulted in reversible hematologic suppression. The investigators suggest that an “adjuvant” strategy incorporating imatinib in the early post transplant regimen may reduce risk of relapse in high-risk individuals.

Imatinib has also been used to prepare patients for subsequent NMA HCT therapy of CML. In a recently reported study, CML patients receiving pretransplant imatinib had equivalent time to engraftment and transplant related mortality compared to equivalent patients not pre-treated with imatinib [40]. Of note, the incidence of molecular remission in the imatinib pre-treatment group (83%) was significantly higher than that in the group not pre-treated with imatinib (40%) (P = 0.11). Such approaches might be useful to reduce the leukemia load prior to NMA HCT.

Current results suggest that the majority of chronic phase patients receiving imatinib as first-line therapy will achieve a complete cytogenetic remission without
undue toxicity. On the other hand, a recent study suggests that patients achieving a complete cytogenetic remission with imatinib have a lower incidence of molecular remission, less durable molecular remissions and a higher level of residual disease in molecular remission (determined by replicate RT-PCR testing) than comparable patients treated with allogeneic HCT [41]. Furthermore, some CML patients will not respond to imatinib or will develop imatinib resistance, and the role of newer agents with increased binding activity to the ABL-kinase domain in the treatment of imatinib-resistant CML is not yet fully understood. Early transplant in patients unlikely to have a durable response to imatinib may be indicated, and methods to predict those who will benefit from early transplant are needed.

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MULTIPLE MYELOMA

Myeloma bone disease

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Abstract

Bone destruction is a hallmark of multiple myeloma, and recent studies demonstrated a strong interdependence between tumor progression and bone resorption. Increased bone resorption as a major characteristic of multiple myeloma is caused by osteoclast activation and osteoblast inhibition (uncoupling). Myeloma cells alter the local regulation of bone metabolism by increasing the receptor activator of NF-κB ligand (RANKL) and decreasing osteoprotegerin (OPG) expression within the bone marrow microenvironment, thereby stimulating the central pathway for osteoclast formation and activation. In addition, they produce the chemokines MIP-1α, MIP-1β and SDF-1α, which also increase osteoclast activity. Furthermore, myeloma cells suppress osteoblast function by the secretion of osteoblast inhibiting factors, e.g. Dickkopf (DKK)-1. The resulting bone destruction releases several cytokines, which in turn promote myeloma cell growth. Therefore, the inhibition of bone resorption could stop this vicious circle and not only decrease myeloma bone disease, but also the tumor progression. Preclinical studies provided strong evidence that the suppression of the osteoclast activity using bisphosphonates, RANKL blockade or inhibition of MIP-1α or MIP-1β is effective both in reducing myeloma bone disease and tumor growth and therefore may offer an important treatment strategy in multiple myeloma.

Keywords: Myeloma, bone, RANKL

Introduction

Multiple myeloma is a clonal malignancy of terminally differentiated plasma cells. Skeletal complications, including bone pain, osteolytic lesions, pathological fractures and hypercalcemia, are a major cause of morbidity and are found in up to 80% of myeloma patients at presentation [1]. In contrast, these symptoms are rarely seen in other B-cell malignancies. The increased bone turnover has recently been characterized as an important facilitator of proliferation and tumor cell survival in myeloma. Several studies have given growing evidence that blocking the osteolytic process may have an antmyeloma effect. This review will give an overview of novel aspects in myeloma bone disease and show possible developments for novel targets in the therapy of bone destruction in multiple myeloma.

Osteoclast activation

Histomorphometric findings

The main principle of myeloma bone disease is an uncoupled bone remodeling with enhanced osteolytic resorption and decreased bone formation, resulting in prevailing bone destruction. Bone resorption is mediated through osteoclasts, which are derived from the granulocyte-macrophage colony-forming unit and represent differentiated, multinucleated cells. Histomorphometric analysis of bone biopsies from myeloma patients showed osteoclast accumulation only on bone-resorbing surfaces adjacent to myeloma cells, whereas osteoclasts were not increased in bone not invaded by myeloma [2]. Therefore, it has been suggested that osteoclast activity is upregulated by local osteoclast activating factors (OAFs) which are produced by either myeloma cells or cells of the microenvironment [3]. Moreover, ex vivo
receptor activator of NF-

three major groups of factors have been identified as pathogenesis of myeloma bone disease. Recently, several OAFs have been implicated in the turnover support survival and proliferation of myeloma cells. Several OAFs have been identified as major osteoclast inducers in multiple myeloma: the receptor activator of NF-κB ligand (RANKL), the chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β, and stromal derived factor-1α (SDF-1α).

**The RANK/RANKL/OPG system**

The receptor activator of NF-κB (RANK)/RANKL/ osteoprotegerin (OPG) system has been characterized and described as the final common effector system and the central pathway of osteoclast activation. RANKL (synonym: tumor necrosis factor-related activation induced cytokine, TRANCE) is a member of the tumor necrosis factor (TNF) superfamily. It exists as a cell membrane bound isoform, a secondary soluble variant that is cleaved from the cellular form by metalloproteases and TNF-α converting enzyme (TACE), and a primary secreted isoform. Under normal conditions, RANKL is mainly produced by osteoblastic lineage cells and stromal cells. The receptor for RANKL, RANK, is expressed by osteoclast precursors and mature osteoclasts. Activation of RANK by RANKL results in differentiation, formation, fusion and survival of preosteoclasts. In addition, RANKL directly acts on mature osteoclasts, inducing actin ring formation and activating mature osteoclasts to resorb bone [4]. OPG acts as a soluble neutralizing receptor for RANKL and inhibits osteoclast differentiation and activation. It is secreted by stromal cells and other cell types including osteoblast lineage cells. The biologic effects of RANK, RANKL and OPG have been evaluated by several in vitro and in vivo studies. In animal models, unbalanced expression of these cytokines led to extreme skeletal phenotypes, for example severe osteopetrosis in RANKL knockout mice. In contrast, OPG deficient mice develop osteopenia. In humans, an abnormal RANKL/OPG ratio was found both in benign and malignant bone disease.

Recent reports suggested that the RANKL/RANK/OPG system is involved in myeloma bone disease. Myeloma cells break the local RANKL/OPG balance [5,6] by several mechanisms, they increase the availability of RANKL within the bone marrow microenvironment. Different studies have demonstrated that myeloma cells induce RANKL by stromal cells through direct cell to cell contact [5,7]. Whether myeloma cells directly express RANKL, has been discussed controversially. Recent data of independent studies performed by several groups showed that human bone marrow myeloma cells express RANKL. In a murine model, Oyajobi et al. demonstrated that interactions between myeloma and stromal cells lead to increased RANKL expression in both cell types. Other authors found RANKL protein and mRNA in murine myeloma cells [8]. The expression of RANKL by human bone marrow myeloma cells was recently demonstrated on protein level by immunocytochemistry [9] and flow cytometry [10]. Expression of RANKL mRNA in plasma cells purified from bone marrow aspirates of myeloma patients could be demonstrated by RT-PCR by several investigators [11–13]. In a study evaluating the clinical impact of RANKL expression, nonmyelomatous plasma cells from controls showed no or only a weak surface expression of RANKL, whereas surface RANKL could be detected on bone marrow plasma cells from all patients with multiple myeloma. According to the bone status determined by conventional radiography, multiple myeloma patients were divided into a group with osteolytic bone lesions and a group without osteolysis. Bone marrow plasma cells derived from patients with osteolytic bone lesions showed a significantly higher level of surface RANKL expression compared to plasma cells from patients without osteolysis (P <0.01) [11]. Moreover, myeloma cells have been demonstrated to enhance RANKL expression by activated T-cells [14] and endothelial cells. Taken together, myeloma cells enhance the local RANKL availability.

In addition to the stimulatory effects on RANKL, myeloma cells decrease the OPG availability within the bone microenvironment. They lead to a down-regulation of OPG mRNA and protein secretion in osteoblasts and stromal cells. Furthermore, myeloma cells produce and shed syndecan-1 (CD 138), a transmembrane proteoglycan, that binds to the heparin-binding domain of OPG and mediates its internalization and lysosomal degradation [15]. Studies showed reduced serum OPG levels in patients with multiple myeloma, compared to normal controls, and an inverse correlation of the OPG levels with the number of radiographic osteolytic lesions [16]. The combination of these effects results in an increased RANKL/OPG ratio that favors the formation and activation of osteoclasts.

**MIP-1α and MIP-1β**

MIP-1α has been identified as another important factor responsible for osteoclastic bone resorption in myeloma. MIP-1α is a low molecular weight chemokine that belongs to the RANTES (regulated on activation normal T-cell expressed and secreted) family of chemokines. It binds to its receptors CCR1, CCR5 and CCR9 and acts as chemoattractant of phagocytes and osteoclasts.
shown to induce late stage of differentiation on human osteoclast progenitors and osteoclast formation in bone marrow cultures. Using an RNAse protection assay, Choi et al. [17] identified MIP-1α as an osteoclastogenic factor in multiple myeloma. In their study, MIP-1α mRNA expression was significantly increased in patients with advanced myeloma as compared to normal controls. Moreover, MIP-1α protein levels were elevated in the bone marrow supernatants of 62% of patients with active myeloma, but only in 17% of patients with stable myeloma and not in healthy individuals. Recombinant human MIP-1α as well as bone marrow plasma from patients with myeloma induced osteoclast formation in human bone marrow cultures. This effect could be inhibited by a neutralizing antibody against MIP-1α or transfection of myeloma cells with an antisense construct to MIP-1α. Abe et al. showed that both MIP-1α and MIP-1β are produced and secreted by myeloma cells, and that the secretion of MIP-1 correlates with the ability of myeloma cells to enhance osteoclastic bone resorption [18]. Antibodies against MIP-1α and MIP-1β or their receptor CCR5 could block these effects. The level of MIP-1α expression or secretion was correlated with the severity of myeloma bone disease by several authors.

There is evidence that the effects of MIP-1α are dependent on the RANKL pathway. Both MIP-1α and MIP-1β enhance RANKL expression in stromal cells. In a murine model of myeloma, injection of recombinant MIP-1α produced a strong increase in osteoclast formation in normal mice, but not in RANKL/OPG animals [19].

Other factors

Before the characterization of RANKL and MIP-1α as osteoclast inducing factors, several cytokines have been implicated in the pathogenesis of myeloma bone disease. These factors, e.g. lymphotoxin, TNFα, IL-1β, IL-6 have been found to be overproduced in some myeloma patients and therefore were discussed as potential OAFs. However, currently they can not be regarded as main inducers of osteoclast activation in multiple myeloma.

A recent study by Zannettino et al. investigated the role of the SDF-1α in the pathogenesis of myeloma bone disease [20]. SDF-1α, a chemokine highly expressed by bone vascular endothelial and marrow stromal cells, increases the recruitment and migration of osteoclast precursors by inducing matrix metalloproteinase-9 (MMP-9) activity. SDF-1α acts through binding to its receptor CXCR4, which is expressed on leukocytes, mature dendritic cells and osteoclast precursors. Zannettino et al. found that myeloma cells produce SDF-1α protein. In their study, myeloma patients exhibited elevated plasma levels of SDF-1α as compared to controls, and the level of SDF-1α positively correlated with the presence of bone lesions on radiology. In an in vitro osteoclast-potentiating culture system, SDF-1α increased osteoclast motility and bone-resorbing activity.

Osteoblast inhibition

In contrast to bone metastases in other malignancies, multiple myeloma causes bone destruction without a propositional osteoblastic reaction, resulting in an uncoupling of the normal bone remodelling sequence. The inability of antiresorptive drugs to repair lytic bone lesions suggests that impairment of osteoblast function is an important factor in myeloma bone disease. Serum concentrations of osteocalcin, which reflect the osteoblastic activity, are reduced in patients with advanced disease. Histomorphometric analysis of bone biopsies from patients with overt myeloma showed a reduced number and activity of osteoblasts on bone surfaces adjacent to myeloma cells [2]. In vitro studies revealed that myeloma cells affect the growth and function of human osteoblast-like cells. Both osteoblast growth and function are inhibited when cultured in medium conditioned by myeloma cells, suggesting that this effect is due to osteoblast inhibiting factors that are secreted by myeloma cells [21]. However, the responsible factors remained unclear.

A recent study suggested that Dickkopf (DKK)-1 could be one of these factors [22]. DKK-1 is an inhibitor of the Wnt signaling pathway, which represents a major signaling pathway in osteoblasts. Wnt glycoproteins bind to the Wnt receptor and its coreceptors LRP5/LRP6 and inhibit the degradation of β-catenin, thus leading to its cytoplasmatic accumulation, translocation into the nucleus and stimulation of expression of osteoblastic target genes. In the absence of a Wnt signal, cytoplasmatic β-catenin is phosphorylated and degraded by the proteasome. Extracellular Wnt antagonists prevent ligand–receptor interactions and can be divided into two functional classes. Members of the secreted frizzled-related protein (sFRP) class, for example sFRP3 (synonym FrzB), bind to Wnt proteins, whereas members of the DKK family bind to the LRP5/LRP6 component of the Wnt receptor complex. Using gene-expression profiles of myeloma patients, Tian et al. found an overexpression of the DKK-1 gene in multiple myeloma patients with focal bone lesions.

In addition to this mechanism, malignant plasma cells are able to induce osteoblast apoptosis. Silvestris et al. found a significantly increased expression of Fas ligand (Fas-L) and tumor-necrosis-factor-related apoptosis inducing ligand (TRAIL) in myeloma cells and an overexpression of Fas and death receptor (DR) 4/5 by osteoblastic lineage cells obtained from patients with extensive osteolytic lesions [23]. Further research on the interaction between myeloma cells and SDF-1α and MIP-1α.
osteoblasts is needed in order to understand the mechanism of osteoblast inhibition and identify possible therapeutic targets in the treatment of myeloma bone disease.

Evaluation of bone disease and biochemical markers

Evaluation of myeloma bone disease requires conventional radiography including X-ray scans of the skull, cervical, thoracic and lumbar spine, ribs, proximal humeri and femora. Another sensitive technique is magnetic resonance imaging (MRI) and can show pathologic results even in patients with normal X-ray scans. However, histomorphometric studies have demonstrated that increased bone resorption can be present even in the absence of radiographic abnormalities. New laboratory parameters that reflect bone metabolism may help to overcome the diagnostic problems in estimating the activity of bone disease.

Amino-terminal collagen type-I telopeptide (NTx) in urine and carboxy-terminal telopeptide of type-I collagen (ICTP) in serum reflect the osteoclastic activity and bone destruction. In myeloma, ICTP correlates with bone resorption and has a prognostic value. As shown by Jakob et al. serum ICTP levels differ significantly between MGUS and myeloma and increase parallel to the myeloma stage according to Durie and Salmon [24]. Another study on myeloma patients demonstrated that serum levels of ICTP were elevated in patients with abnormal bone MRI but lacking lytic bone lesions in conventional radiography [25]. Tartrate-resistant acid phosphatase isoform-5b (TRACP-5b) was described as another novel parameter reflecting osteoclast activity. Terpos et al. found increased TRACP-5b serum levels in myeloma patients, and the levels were associated with the radiographically assessed severity of bone disease [26].

Treatment approaches

Several in vitro and in vivo experiments as well as clinical observations have shown the importance of the positive feedback between myeloma progression and bone resorption for sustaining the disease process [27,28]. The close association between bone resorptive activity and myeloma progression has been highlighted in an experiment using C57BL/KaLwRij mice. In these animals, ovariectomy induced an accelerated bone remodelling, resulting in reduced trabecular bone volume and increased osteoclast number. Non-ovariectomized mice were used as control. Injection of 5T2MM myeloma cells lead to bone disease in both groups with an increased tumor growth and earlier development of osteolytic lesions in ovariectomized mice, thereby confirming the interdependence of osteoclasts and myeloma cells. Therefore, targeting the osteoclast induced bone disease might interrupt the feedback loop and have an additional antmyeloma effect.

Bisphosphonates

Bisphosphonates are currently the most widely used class of antiresorptive drugs in the treatment of myeloma bone disease. They selectively concentrate at the boundary between osteoclast and bone resorption surface. Their main effect is the suppression of osteoclast activity and function. Bisphosphonates are based on a P–C–P structure similar to endogenous pyrophosphate. Two side chains are attached to the carbon, influencing the antiresorptive potency. Nitrogen containing aminobisphosphonates (e.g. pamidronate, ibandronate, alendronate and zoledronic acid) are more potent than the first generation bisphosphonates (e.g. clodronate and etidronate). Several large, randomized, placebo-controlled clinical trials have proved the efficacy of bisphosphonates, i.e. clodronate p.o., pamidronate i.v. and zoledronic acid, in the therapy of myeloma bone disease [29]. Currently, the most potent bisphosphonate is zoledronic acid. Preclinical studies as well as randomized clinical trials (RCTs) showed that zoledronic acid had a superior effect over pamidronate in the treatment of hypercalcemia of malignancy [30]. Long-term follow-up data confirm that zoledronic acid was more effective than pamidronate in reducing the risk of skeletal complications in patients with bone metastases from breast carcinoma and was of similar efficacy in patients with multiple myeloma [31]. Although the main effect of bisphosphonates is the suppression of osteoclast activity, other mechanisms may add to the effects of aminobisphosphonates in multiple myeloma. Phase III studies evaluating the use of bisphosphonates in smoldering myeloma (primary end point: prolongation of time to progression) are ongoing [32].

RANKL antagonists

After the identification of the RANK/ RANKL/ OPG system as the final effector system of osteoclast activation, systemic RANKL blockade has been evaluated in animal models and first clinical trials, using OPG, OPG-Fc fusion protein or RANK-Fc. Treatment of myelomatous SCID-hu hosts with RANK-Fc not only reduced osteoclast formation and myeloma-induced bone resorption, but also resulted in a sustained suppression of paraprotein levels by more than 80%, reflecting a reduced myeloma cell burden. Moreover, studies in the 5T2MM model showed that RANKL blockade by OPG-Fc caused not only inhibition of development of osteolytic bone lesions, but as well a decreased tumor burden and a significant increase in time to morbidity.
The treatment was associated with a decreased number of osteoclasts but had no effect on apoptosis and proliferation of 5T33MM cells in vitro, indicating that the antitymoma effect of RANKL inhibitors is related to inhibition of osteoclast activity.

In humans, trials with an OPG-Fc fusion protein, with a recombinant osteoprotegerin construct and with a human monoclonal antibody to RANKL were performed. These preliminary observations indicate that targeting the RANK/RANKL/OPG system may inhibit the development of myeloma bone disease and also decreases myeloma growth and may therefore offer a new treatment strategy in multiple myeloma.

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References


MULTIPLE MYELOMA

Novel treatments of multiple myeloma

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Over the last decade significant advances in the treatment of multiple myeloma (MM) have been achieved. Most prospective randomized trials indicate that high dose therapy with autologous stem cell transplantation is associated with improved event-free and overall survival. Thalidomide, an oral immunomodulatory and anti-angiogenetic agent is associated with objective responses in 30% of patients with advanced and refractory MM. The addition of dexamethasone to thalidomide is associated with a 50% response rate in refractory patients and with a 70% response rate in previously untreated patients. This oral combination is particularly useful in newly diagnosed patients with features of advanced disease who are candidates for stem cell collection. Bortezomib is a potent and selective inhibitor of the 26S proteasome. Bortezomib has shown activity in 30% of patients with refractory/relapsed MM including patients who have failed thalidomide. The combination of bortezomib with thalidomide and dexamethasone in previously untreated patients has been associated with a response rate exceeding 80%. More recently the Imid lenalidomide combined with dexamethasone has been associated with significantly higher response rates and longer event-free survival than dexamethasone alone. Lenalidomide and dexamethasone is also very active in previously untreated patients. The development of genomics and proteomics provide the basis for a novel molecular classification of MM, can identify unique targets for combination therapy in individual patients and provide the framework for clinical protocols to enhance cytotoxicity and to avoid the emergence of drug resistance.
MULTIPLE MYELOMA

Stem cell transplantation in multiple myeloma

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Single autologous transplantation

High-dose treatment (HDT) with autologous stem cell transplantation (ASCT) has been shown to increase survival and time to progression in multiple myeloma (IMM) compared with conventional chemotherapy. In the French IFM90-study [1], 200 patients were randomised to receive prolonged standard treatment with i.v. combination chemotherapy, or a short phase by induction treatment with the same regimen, followed by high-dose melphalan + TBI and autologous bone marrow transplantation (ABMT). Median survival was significantly better in the ABMT-arm, +60 months, vs. 37 months in the chemotherapy arm, and also event-free survival was superior, 27 months vs. 18 months. These differences are sustained in a long term follow-up, where actuarial survival at 7 years was 43% and 25%, and EFS was 16% and 8% in the ABMT- and chemotherapy arms, respectively. However, these latter differences were only observed in patients under the age of 60 at the time of inclusion in the study, while there was no benefit in older patients [1a]. Similar results have been demonstrated in a more recently published study from the British MRC [2]: 401 patients were randomized to receive either standard chemotherapy (cVAMP) or cVAMP followed by high-dose melphalan and autologous SCT. Median overall survival (OS) was significantly superior in the ASCT-arm — 51 months — compared with 42 months in the cVAMP-group. The difference was most pronounced in patients with a high beta-2-microglobulin (ß-2-m) value at diagnosis, i.e. with a poorer prognosis.

Tandem autologous transplantation

During the last 10 years, there have been indications from non randomized trials and treatment programs that repeated HDT with ASCT, i.e. tandem or double transplantation, can further improve these results [3–5]. These data have been confirmed in recent randomized trials. In the French IFM94-study [6], 399 previously untreated patients were randomized at diagnosis to receive either single or double ASCT following induction treatment. Conditioning in the single transplant arm and for the second transplant in the double transplant arm was melphalan 140 mg/sqm plus TBI 8 Gy total dose in 4 fractions. For the first transplant in the double transplant group, melphalan 140 mg/sqm was used. The rate of CR or VGPR was 49% and 63% in the single and double transplant groups, respectively ($P=0.10$). Median OS was prolonged with 10 months in the double transplant group (58 months, vs. 48 months for the single transplant group, $P=0.01$). The probability of survival at 7 years was 21% vs. 42% and of EFS 10% vs. 20% for the single and double transplant groups, respectively. In an Italian randomized study of 386 patients conditioning for the first transplantation was melphalan 200 mg/sqm and for the second melphalan 120 mg/sqm plus busulfan 12 mg/kg. Median OS was 62 vs. +74 months and median EFS 21 vs. 31 months for the single and double transplant groups, respectively [7]. In contrast to these results, another French randomized study in 230 patients has failed to demonstrate a significant benefit of the double transplant procedure [8]. Concerning the optimal timing of the two transplants, an EBMT registry study has recently demonstrated that the second transplant should preferably be performed before relapse and within 6 to 12 months of the first transplantation [9].

It can be concluded that tandem autologous transplantation seems to improve survival with about one year compared with single autografting, and that it is beneficial to perform the second transplant within a short interval after the first, before disease progression.
Allogeneic stem cell transplantation with full dose conditioning

Allogeneic stem cell transplantation (SCT) with full-dose conditioning has been used in MM for almost 20 years. The treatment results have been hampered by a very high transplant-related mortality (TRM) rate of 25–50%, and furthermore, the curative potential can be questioned since late relapses after several years do occur [10,11], although molecular remissions can be achieved in a way not seen after autologous transplantation [12]. In 1996, the EBMT Myeloma Registry performed a retrospective analysis comparing 189 allogeneic transplant patients with 189 case-matched autologous transplant controls [10]. The results were consistent with previous surveys, with a significantly better survival for the autotransplant group (median survival after transplantation 34 months, vs. 18 months in the allogeneic group). Relapse rate was significantly higher in the autotransplant group (70%) vs. 50% for the allogeneic transplant patients. This difference did not however compensate for the large difference in TRM—41% in the allogeneic vs. 13% in the autologous transplant group, respectively—which was the reason for the poorer survival of the allogeneic transplant patients. In spite of extensive analyses of different prognostic subgroups, no category of patients could be identified where allogeneic transplantation would be more favorable than autologous, although no survival difference was seen in female patients.

A more recent EBMT study compared 225 allogeneic bone marrow transplants performed during 1994–98 with 339 patients transplanted during 1983–93 [11] This analysis demonstrated a decrease in TRM, with a reduction of total TRM from 50 to 30%, and early mortality from 30 to 20%. This resulted in an improvement in outcome, with an actuarial survival of about 50% at 4 years after transplantation, compared to about 30% in the previously transplanted group of patients. Still, although improved, TRM is unacceptably high.

Based on these and other studies with similar results, allogeneic SCT with full dose conditioning cannot be recommended, not even for younger patients, until the lethal complications can be handled more effectively.

Allogeneic stem cell transplantation with reduced intensity conditioning

Based on the high toxicity of allogeneic SCT with full dose conditioning, it is not surprising that MM is one obvious target disease for reduced intensity-conditioning (RIC) or non-myeloablative allogeneic (NMA) transplantation, also called ‘mini-transplantation’ where the rationale is to utilize the antitumor effect of the graft-versus-myeloma reaction, while reducing the treatment-related complications of high-dose conditioning [13,14]. In a retrospective survey of 256 patients reported to the EBMT myeloma registry, TRM was about 24%, but only 13% in low-risk patients, i.e. mainly patients transplanted upfront as a part of first-line treatment [15]. One treatment rationale frequently used is to induce maximal cytoreduction by induction treatment, HDT (mainly high-dose melphalan) and ASCT, followed by consolidation with with RIC allogeneic transplantation and if necessary, also infusion of alloreactive donor T-lymphocytes (DLI). This strategy has been used in a number of phase II-studies, of totally 124 patients [16–19]. The conditioning regimens have been variable and of low or intermediate cytotoxic intensity, and have included fludarabine, low dose TBI, melphalan, cyclophosphamide and ATG. Transplant-related mortality has varied between 4–13% in previously untreated or less treated patients, and about 25% in relapsed and/or heavily pretreated patients. OS and EFS at 2–3 years was 50–100% and 30–60%, respectively in less-treated or relapsed but chemosensitive patients. The incidence of acute and chronic GVHD varied between 10–60% and 40–65%, respectively. As for controlled prospective trials, preliminary data from one study have been presented [20]. Two hundred and eighty-four previously untreated patients have been included, all receiving VAD induction treatment and high-dose melphalan (200 mg/sqm) with ASCT. For inclusion, the patients needed to have poor prognosis criteria with respect to abnormal karyotype and high β-2-m. Sixty-four patients with an HLA-identical sibling donor then received an RIC allogeneic transplantation, while 220 patients with no available donor were randomized to no further treatment or to receive a second autograft after high-dose melphalan 220 mg/sqm. The allogeneic transplant conditioning regimen consisted of fludarabine, low dose busulfan and ATG. Median follow-up time from diagnosis is 23 months (9–44). At 4 years, the probability of survival—on an intention-to-treat basis—was 40% in the autologous vs. 30% in the allogeneic transplant arms (P=0.36), and EFS 20% vs. 15% (P=0.70). When comparing the patients who actually received their planned transplants — 46 in the allogeneic- and 166 in the autologous transplant group, the corresponding results for survival was 50% for the autologous and 35% for the allogeneic transplant patients (P=0.09), i.e. a trend for better outcome after autologous SCT. EFS was essentially identical in both groups – about 18% at 4 years. Transplant-related mortality at 3 months after allogeneic transplantation in the 406 patients who completed all treatment was 6%.

In a retrospective registry study of the EBMT [21], 321 RIC allografts were compared with 196 allotransplants with standard conditioning (SC). The transplants were performed during the same period.
of time, 1998–2002. No case matching was done, and the groups were not completely comparable with respect to prognostic factors. Median survival was 23 and 36 months ($P = N.S$) and PFS was 11 and 17 months ($P = N.S$) for the RIC and SC groups, respectively. Non-relapse mortality at 2 years was significantly higher for the SC group – 37% vs. 23% for the RIC patients. The cumulative relapse incidence at 3 years was higher in the RIC group, 54%, compared with 26% in the group that received SC.

Allogeneic SCT with reduced intensity conditioning is feasible, and TRM is low and acceptable in contrast to SC. However, relapse rate seems to be higher, and long-term outcome does not seem to be improved. High-dose treatment and ASCT followed RIC allogeneic transplantation is feasible, but preliminary results from controlled prospective trials still do not demonstrate a benefit in survival or freedom of progression compared with single or tandem autologous SCT, at least not in high-risk patients. Data from other similar studies, like the EBMT study with a similar design as the French trial but also including patients without high-risk criteria, will be available in the near future.

References

BLEEDING DISORDERS

Diagnostic approaches to bleeding disorders

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Introduction and general approach

The evaluation of a patient with a possible bleeding disorder can be one of the most challenging referrals in hematology practice. In the absence of much evidence-based outcomes data, the practitioner’s experimental judgment will be called upon to formulate a working diagnosis and management plan in many instances. A logical and semi-standardized approach should include the application of a three-part screening process, each of which addresses a specific question, as follows:

1. A comprehensive bleeding history should be obtained with the specific question; ‘what is the pre-test probability that this patient has a bleeding disorder?’ One possible outcome is that no further laboratory evaluation is deemed to be necessary, for example in a patient who complains of minimal muco-cutaneous bleeding symptoms, and who has had many hemostatic challenges without excess bleeding.

2. Global screening tests of hemostasis should then be applied with the specific question; ‘what is the likelihood that there is a hemostatic abnormality to explain (and are the screening tests compatible with) the patient’s history?’ Again, depending on the perceived pre-test probability and the result of these screening test(s), no further evaluation may be judged to be necessary.

3. Specific screening tests of hemostasis may then be applied in order to address the question; ‘what specific hemostatic disorder does this patient suffer from?’

Unfortunately, the available global and specific laboratory assays suffer from many limitations, including sensitivity, specificity, reproducibility, and inconsistency in interpretation between laboratories. Given all these limitations, it is not uncommon to encounter patients with a history that is strongly suggestive of a bleeding disorder yet no diagnosis is established after an exhaustive laboratory evaluation. Because it is generally not feasible to refer every such patient to a research laboratory for advanced investigation, empiric management plans may need to be instituted, using some combination of available hemostatic therapies.

The bleeding history: The best screening test for a bleeding disorder?

The bleeding history should include the age of onset of symptomatic bleeding, the pattern of bleeding both with respect to anatomical distribution (muco-cutaneous vs. deep tissue) and time of onset after hemostatic challenges, and whether hemorrhage has been spontaneous and/or provoked after minor and major hemostatic challenges. A detailed family pedigree, and inquiry about systemic disorders and medication exposure are also important historical aspects. The details of excessive site-specific bleeding should be carefully documented. For example, historical features of menorrhagia that are predictive of an underlying bleeding disorder are an onset at menarche, concomitant iron deficiency anemia, hourly or greater pad/tampon changes, and the passage of clots. Until recently, there have been few published studies on the positive predictive value of site-specific or global bleeding symptoms. Indeed, relatively sparse attention has been paid to the development and validation of user-friendly bleeding scales that are also highly predictive of an underlying disorder.
Possible exceptions are the menstrual blood loss (MBL) scale developed by Kadir et al., although the definition of ‘abnormal’ (>80 mL of blood loss/period) is measured by the alkaline hematin method which is not readily performed in a clinical practice setting. A pictorial chart illustrating blood loss on sanitary napkins with an associated scoring system is more practical, and has been reasonably well validated. A pictorial blood assessment score >100 is frequently used as cut-off to define excessive menorrhagia.

In the ISTH criteria for the diagnosis of von Willebrand disease, a positive muco-cutaneous bleeding history is defined by ≥2 symptoms in the absence of a blood transfusion history, or one symptom requiring blood transfusion, or one symptom recurring on ≥3 distinct occasions. The ongoing European Union collaborative study on type 1 von Willebrand disease (MCMDM-1VWD) has recently developed and validated a questionnaire for muco-cutaneous bleeding that ascribes a severity score based on the clinical impact/outcome of site-specific bleeding; because it is being correlated with laboratory parameters and vWF genotype, it may become the standard assessment tool in the future.

What are the best laboratory global screening tests for a bleeding disorder?

Global screening tests of hemostasis should evaluate for both ‘primary’ and ‘secondary’ hemostatic abnormalities. The Prothrombin and Activated Partial Thromboplastin Times (PT & APTT) are reasonably sensitive and specific assays for factor deficiency states. Furthermore, these tests are reproducible, inexpensive, and easily performed. A platelet count and review of platelet morphology by simple light microscopy (which may pick up abnormalities such as Gray Platelet and Wiskott-Aldrich Syndromes) is mandatory.

More uncertainty revolves around the best laboratory screening strategy for vWD or platelet function abnormalities in patients presenting with a history of abnormal muco-cutaneous bleeding. Formerly, the Bleeding Time (BT) was most commonly used for this purpose, but it is invasive, relatively insensitive, and poorly reproducible. Many laboratories have now either abandoned the BT completely or added the Platelet Function Analyzer (PFA-100™) as a global screen for platelet disorders. The PFA-100 is sensitive to abnormalities that affect high shear-dependent platelet adhesion and aggregation; its utility is significantly better than the BT in the detection of vWD and severe platelet function defects (e.g., Glanzmann’s Thrombasthenia, Bernard-Soulier Syndrome), but like the BT, it suffers from a lack of sensitivity in the detection of mild platelet function disorders, such as storage pool defects and platelet secretion defects. This is an unfortunate weakness as these disorders are relatively prevalent.

In practice, many hematologists elect to screen for vWD in patients with a compatible muco-cutaneous and/or post-surgical bleeding history, and reserve referral for platelet aggregation studies (to evaluate for intrinsic platelet disorders) for those subjects in whom vWD has been ruled out. The PFA-100 pattern of abnormality may be particularly useful in this regard; when both the collagen/epinephrine and collagen/ADP closure times are abnormally prolonged, vWD is more likely. Conversely, when just the collagen/epinephrine closure time is prolonged – and salicylate/NSAID ingestion has been ruled out – platelet aggregometry is more likely to reveal an abnormality of platelet function. A normal PFA-100 study has a reasonably high negative predictive value for vWD, but probably not for intrinsic platelet function defects. Therefore, platelet aggregometry should be considered in any patient with a suggestive history but normal screening studies.

Testing for von Willebrand Disease (vWD)

Testing for vWD using a combination of the factor VIII activity assay (FVIII:C), vW antigen (vW:Ag) and ristocetin cofactor (vW:RCo) activities is indicated in most patients presenting with muco-cutaneous bleeding manifestations. Sub-type classification by analysis of vW multimers can be reserved for patients with suggestive abnormalities on the above screen. Once again, the historical details will provide some measure of pre-test probability of vWD. For example, it has been estimated that 5–20% of women with menorrhagia have underlying vWD. Conversely, 60–95% of women with vWD suffer from menorrhagia. It should be borne in mind that the diagnosis of vWD may be masked by the fact that assays for the vW factor complex may be erroneously high during periods of inflammation and ‘stress’ (e.g., in young children subjected to phlebotomy and in many hospitalized patients) and perhaps in women on estrogen therapy. In women of menstruating age, most studies agree that testing should be performed on days 1–4 of the menstrual cycle, when vW complex proteins may be at their nadir.

Type 1, characterized by a partial quantitative deficiency of vWF, is the most common variant, accounting for 70–80% of all vWD. It has pointed out that the application of commonly used diagnostic criteria for type 1 vWD may include many false positives, since bleeding and low vWF levels often associate by chance. Notwithstanding this proviso, type 1 vWD is defined by a quantitative deficiency of vWF – that is, vW:Ag and vW:RCo levels >2 S.D. below the (ABO blood type population-adjusted) mean, with a normal vW multimer pattern – in a patient with bleeding symptoms and a positive family
history. Any diagnostic algorithm is further complicated by the fact that the disorder, although classically thought of as autosomal dominant, is variably penetrant in many families. Among the causes of acquired vWD, hypothyroidism is probably the most prevalent, and a routine screen of TSH levels as part of a bleeding work-up is appropriate. A FVIII/vW:Ag ratio ≥1.50 (compared to ≤1.10 in healthy subjects) is helpful in the diagnosis of type 1 vWD, although vWF:RCo levels are the single measure of vWF-related activities that best correlate with bleeding severity. Recent data from the ongoing MCMDM 1-VWD study suggest that ≈70% of subjects diagnosed with type 1 vWD have a detectable candidate mutation in the vW gene, the majority of which are missense mutations.

Platelet aggregation tests

Although still considered the ‘gold standard’ for the diagnosis of intrinsic platelet function defects, platelet aggregation studies, which were first introduced in the 1960s, suffer from poor accuracy and reproducibility. Aggregometry is usually performed using ADP, epinephrine, arachidonic acid, collagen, and ristocetin agonists. Patients undergoing platelet function tests (PFTs) should be instructed to refrain from ingestion of all potentially platelet inhibitory drugs for at least 10 days prior to testing. Dietary factors may also affect aggregation responses. Recent studies have begun to re-address some of the standardization challenges of these assays, including pre-analytical variables. In particular, it has been suggested that the addition of platelet-poor plasma (PPP) to platelet-rich plasma (PRP) to adjust the cell count may inhibit aggregation responses, and it may be preferable to simply use unadjusted PRP; however, this is not yet considered to be standard practice. The variability in clinical laboratory practice in the execution of platelet aggregometry (concentration of agonists, use of reference ranges, mode of reporting, etc) is highlighted by a recent survey that included 47 US coagulation laboratories. Some of the variability is explained by a lack of standardized protocols. However, even when provided with a standardized methodology, wide variability still exists among platelet function studies performed at different sites. It is hoped that the pre-analytical and assay variables in platelet aggregometry can be better understood and standardized in the future as a result of recent re-evaluation.

Standard platelet aggregometry is not sensitive to secretion or storage defects, and indeed in classic delta storage pool deficiency (δ-SPD), it is well recognized that platelet aggregation may be normal. Therefore it is recommended that lumi-aggregometry (which evaluates for simultaneous ATP release in response to agonists such as thrombin and ADP) should be performed when possible. ATP release by lumi-aggregometry is considered to be a reasonable screen for δ-SPD.

‘Third Line’ laboratory investigation

Depending on the suspected defect at this stage, specialized laboratories may be capable of performing additional sophisticated studies. These studies should be considered as confirmatory rather than screening. Examples include platelet electron microscopy to examine for the absence of dense granules in suspected δ-SPD. Routine application of this technique has suggested that ‘forme fruste’ variants of this disorder, in which there is a partial deficiency of dense granules, may be relatively prevalent in women with bleeding disorders. Other studies that may be of value in certain scenarios include platelet flow cytometry (e.g., in suspected Glanzmann’s or Bernard-Soulier syndromes), and specific studies of platelet release (e.g., serotonin release), receptor deficiency, or signal transduction.

Tailoring the work-up for bleeding disorders:

As a general rule, the more severe the bleeding disorder, the earlier in life the presentation. However, recent studies have begun to evaluate the clinical and laboratory features of mild bleeding disorders in children. These studies, like those in adults, conclude that platelet function defects (abnormal aggregation and/or ATP release) may account for a relatively large proportion of bleeding diatheses in children. In addition, it is clear that the cause of muco-cutaneous bleeding remains enigmatic in many children referred for evaluation of seemingly abnormal muco-cutaneous bleeding (14). Racial differences may also be an important consideration in the work-up of bleeding disorders. For example, it has been demonstrated that among African-American women presenting with menorrhagia, von Willebrand’s disease is less common, whereas intrinsic platelet function defects are more common.

The approach outlined by this review is recommended primarily for outpatients referred for evaluation. Even more complex is the evaluation of hospitalized in-patients, who are generally referred because of post-surgical bleeding. As already mentioned, it may be difficult or even impossible to establish a diagnosis of vWD in a stressed individual in the post-operative period. Once again, a careful history will provide information on whether the patient has experienced spontaneous or excessive post-surgery/trauma bleeding in the past, or has a positive family history of such. Understanding whether bleeding was uni-focal or multi-focal may suggest a surgical or coagulopathic cause, respectively. Other common acquired causes of bleeding in hospitalized patients, such as vitamin K deficiency, dilu-
tional coagulopathy, or DIC should be considered. However, ultimately, a laboratory evaluation may be indicated; in order to simplify test interpretation, it is recommended that this be postponed until the patient has been discharged if at all possible.

Management of the patient with a positive bleeding history and an unremarkable laboratory evaluation

Even after an extensive tiered laboratory investigation of a subject strongly suspected of having a bleeding disorder, no specific abnormality may be discovered. This outcome may be the case in 40% or more of patients believed to have a bleeding disorder on the basis of the history. This dilemma can present a therapeutic challenge, since many of these individuals are evaluated prior to elective surgery. The literature contains very little information on the management or outcomes in these subjects. Thus, a careful assessment of the risk/benefit and cost/benefit ratios of empirical use of anti-fibrinolytic agents, DDAVP, and/or platelet transfusions, or even recombinant FVIIa, must be implemented. Reminders to the patient to avoid all platelet inhibitory agents prior to the procedure, and to the surgeon to take extra care in securing intra-operative surgical hemostasis are worthwhile.

The future

Studies that aim to better standardize the clinical history and existing laboratory studies in the diagnosis of bleeding disorders will be of particular value. It is clear however that assays of platelet function with greater sensitivity and specificity are required. To this end, proteomics approaches that evaluate the range of proteins expressed by normal and abnormal platelets are a realistic expectation in the next decade. Similarly, it is reasonable to expect that the pharmaceutical armamentarium of hemostatic agents, which is currently quite limited, will be expanded to cope with the shifting paradigm demanding better diagnosis of the causes of bleeding, and avoidance of a simplistic transfusion approach when bleeding does occur.
Antiphospholipid syndrome (APS) is defined as recurrent arterial and/or venous thrombosis and obstetric complications in the presence of antiphospholipid antibodies (aPLA) [1]. APS may affect any system and organ in the body including heart, brain, kidney, skin, lung, and placenta (Table I). This syndrome is predominant in females (female to male ratio is 5 to 1), especially during the childbearing years [2]. It is the most common acquired thrombo philic disorder in the general population, affects both arterial and venous vessels in any diameter [2–7].

**Classification criteria of APS**

Preliminary classification criteria for the classification of ‘definite’ APS were described at an international meeting at Sapporo, Japan [1]. Clinical criteria include vascular thrombosis (one or more episode of arterial, venous, or small vessel thrombosis which should be documented by radiologically or histologically) and pregnancy morbidity (a). three or more unexplained abortions before 10th week of gestation, (b). one or more unexplained fetal death beyond the 10th week of gestation, (c). one or more premature births because of pre-eclampsia, eclampsia, or placental insufficiency. Laboratory criteria are the detection of lupus anticoagulants, and presence of anticardiolipin IgG and IgM antibodies in medium or high titers. Laboratory criteria should be met at two or more occasions at least 6 weeks apart. According to these criteria, ‘definite’ APS is considered if a patient had at least one clinical and one laboratory criteria [1]. Validation studies showed that Sapporo criteria have 71% sensitivity and 98% specificity to diagnose ‘definite’ APS patients [8].

As in the other autoimmune disorders, aPLA and APS may accompany the other autoimmune diseases (most frequently SLE) and certain situations. APS is referred as ‘primary’ when it occurs alone, or 'secondary' when it is associated with other autoimmune disorders especially with systemic lupus erythematosus [9]. Besides these autoimmune conditions, aPLA may be present in healthy individuals, in patients with hematologic and solid malignancies, in patients with certain infections (syphilis, leprosy, HIV, CMV, EBV, etc), and in patients being treated with some drugs (phenothiazines, procainamide, phenytoine, etc). Those antibodies are defined as ‘alloimmune aPLA’, they are generally transient and not associated with the clinical findings of APS [10].

**Anti-phospholipid antibodies (aPLA)**

aPLA are heterogenous antibodies directed against phospholipid–protein complexes. Although several aPLA are defined, in two of those (anti-cardiolipin antibodies (ACLA) and lupus anticoagulant (LA)) clinical studies confirmed an association with the clinical complications of APS. Anticardiolipin antibodies (aCLA) are measured by ELISA, and reported by GPL ad MPL units for IgG and IgM anti-cardiolipin antibodies, respectively [11]. In patients with APS, aCLA are not simply directed to cardiolipin but it recognizes B2-GPI–cardiolipin complexes in the ELISA microplates. Since low titer aCLA may be present in normal population, moderate (20–80 GPL, 20–50 MPL) to high positive (more than 80 GPL or 50 MPL) results are needed for the diagnosis of APS [12]. Lupus anticoagulants are screened by phospholipid-dependent coagulation tests (e.g. activated partial thromboplastin time, kaolin clotting time, Russell’s viper venom time, dilute prothrombin time, or textarin time). A prolonged screening test should not be corrected by mixing of normal plasma (mixing studies), but should be corrected by addition of phospholipid (phospholipid neutralization procedure). For the diagnosis of LA, other coagulopathies including specific factor inhibitors should be excluded [13].
B2-GPI is a plasma protein with high affinity for negatively charged phospholipids. It has been shown that most of the aCLA antibodies are B2 GPI-dependent [14,15]. Although the precise function is unknown, B2-GPI might neutralize negatively charged phospholipids and inhibits coagulation cascade and the presence of anti-B2 GPI antibodies may predispose to thrombosis. Anti-prothrombin antibodies may present in 50–90% of APS patients [4–6]. Anti-prothrombin antibodies may cause hypoprothrombinemia and may be associated with bleeding symptoms in patients with APS. Although it has been shown that both anti-B2GPI and anti-prothrombin antibodies might be associated with clinical features of APS, most of these studies are retrospective, and these antibodies are not included in the Sapporo criteria [16].

Although other aPLA (anti-phosphatidylserine, anti-phosphatidylinositol, anti-phosphatidylylglycerol, antibodies directed to zwitterionic phospholipids, etc) may be detected in patients with APS, the clinical importance of these aPLA are unclear in the absence of a positive LA and/or aCLA test [1].

Autoimmune mechanisms play a role in the generation of aPLA, but the exact pathophysiologic mechanisms causing thrombotic complications of APS have not been clarified. Endothelial cell activation, inhibition of protein C and protein S activity, activation of platelets, increased tissue factor expression, and impairment of fibrinolytic activity have all been proposed as possible mechanisms by which the aPLA may predispose to thrombosis in patients with APS [2,3,6].

In the last decade, more studies have been focused on interaction between aPLA and endothelial cells (EC). It has been shown that binding of aPLA activates EC, which results in the expression of P-selectin, intercellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1, and induces thrombosis in animal models [17–20]. Blank et al. found that synthetic peptides that neutralize aPLA function inhibit the increase of these adhesion molecules on the surface of EC, inhibit monocyte adhesion, and prevent the development of experimental APS in mice [17]. Pierangeli et al. showed that aPLA-induced leukocyte adhesion was completely abrogated in ICAM-1 and P-selectin-deficient (ICAM-1−/−/P-selectin−/−) mice [19]. Combes et al. showed that in vitro generation of endothelial microparticles was increased in patients with lupus anticoagulants [21]. Besides these in vitro studies, we have recently showed that endothelial functions determined by brachial artery ultrasound were impaired in patients with primary APS [22].

On the other hand, some researchers have focused on the interaction between aPLA and monocytes. They have suggested that aPLA increase procoagulant activity of normal donor monocytes, and the mono-

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**Table I. Clinical manifestations of the APS**

<table>
<thead>
<tr>
<th>System</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>Acute coronary syndromes, angina pectoris, cardiac valve involvement, non-bacterial thrombotic endocarditis,</td>
</tr>
<tr>
<td>Arterial system</td>
<td>Atherosclerosis, peripheral arterial disease, myocarditis, claudicatio intermittens</td>
</tr>
<tr>
<td>Venous system</td>
<td>Thrombosis in the large, medium or small arteries</td>
</tr>
<tr>
<td>Central Nervous System</td>
<td>Transient ischemic attack, cerebral arterial occlusion, chorea, convulsions, dementia, transverse myelitis</td>
</tr>
<tr>
<td>Hematological</td>
<td>Thrombocytopenia, autoimmune hemolytic anemia, thrombotic thrombocytopenic purpura, hemolytic uremic syndrome</td>
</tr>
<tr>
<td>Dermatological</td>
<td>Livedo reticularis, Raynaud's phenomenon, leg ulcers, purpura, acrocyanosis, cutaneous infarcts, digital gangrene, Degos's disease, anetoderma</td>
</tr>
<tr>
<td>Ophthalmological</td>
<td>Retinal arterial and venous thrombosis, amaurosis fugax, transient or persistent visual loss, photophobia</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Budd-Chiari's syndrome, mesentery embolism, nodular regenerative hyperplasia of the liver, intestinal vaso-occlusive disease, ischemic colitis, pancreatitis, hepatic or splenic infarct</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>Pulmonary embolism, pulmonary hypertension, alveolar hemorrhage, fibrosing alveolitis, respiratory distress syndrome</td>
</tr>
<tr>
<td>Renal</td>
<td>Renal vein thrombosis, renal arterial thrombosis, acute or chronic renal failure, hypertension, hematuria, nephrotic syndrome</td>
</tr>
<tr>
<td>Psychiatric</td>
<td>Psychosis, cognitive dysfunction</td>
</tr>
</tbody>
</table>

**Thrombosis in the APS**

Thrombotic complications are the major causes of morbidity and mortality in patients with APS. Although the thrombosis may occur in any site of the vascular tree; about 2/3 of the thrombotic events are venous, mainly deep and superficial veins in the lower extremity; remaining 1/3 is seen in the arterial system. In patients with APS, the risk of the thrombotic recurrence is high compared to patients without APS especially after the discontinuation of oral anticoagulant therapy [2–6].
cytes that circulate in patients with APS are in a ‘primed’ state to express tissue factor [23]. Since APS is a clinically heterogeneous disorder with a broad spectrum of presentations, identification of the patients who have high risk of thrombosis is crucial. Although many epidemiological studies showed an increased risk of thrombosis in patients positive for aPLA, some patients with high titers of aPLA did not develop thrombosis, even in long-term follow-up. These facts supported the hypothesis that there are additional inherited or acquired thrombogenic factors, which influence the development of thrombosis in those patients (‘double or multiple hit’ hypothesis) [2].

In the last decades, several genetic factors have been identified in patients with thrombosis, especially with venous thromboembolism [24]. Natural anticoagulant (protein C, protein S, antithrombin) deficiencies, factor V Leiden A506G mutation and prothrombin A20210G mutation were the most common causes of hereditary thrombophilia. It has been shown that natural anticoagulant deficiencies are quite rare in patients with APS. Several studies investigating the role of factor V Leiden A506G mutation and prothrombin A20210G mutation in patients with APS, gave conflicting results [25–33]. It is unclear whether the presence of these mutations increased the thrombosis risk of APS patients.

Factor XIII Val34Leu polymorphism is a newly described polymorphism which is located in the three amino acids away from the thrombin activation site of the factor XIII-A subunit. It has been reported that the presence of Leu allele may decrease both arterial and venous thrombosis risk, and increase bleeding tendency. In a cohort of 60 APS patients with thrombosis, 22 aPLA-positive patients without thrombosis and 126 healthy controls, we could not find any effect of factor XIII Val34Leu polymorphism on the thrombosis in patients with APS [34].

Catastrophic APS (CAPS)
A minority of APS patients may acutely present with multiple simultaneous vascular occlusions affecting small vessels predominantly, and is termed as ‘catastrophic APS (CAPS)’. Precipitating factors have been identified in the majority of the CAPS patients, such as infections, surgery, pregnancy, SLE flares, withdrawal of anticoagulation therapy, and trauma. CAPS definition requires thrombotic involvement of at least three different organ systems over a period of days or weeks. Clinical manifestations of CAPS are related to the extent of organ involvement and cytokine release of affected tissues. Renal dysfunction (70%), pulmonary involvement such as ARDS and pulmonary embolism (66%), skin involvement such as skin necrosis and livedo reticularis (66%), central nervous system manifestations such as cerebral arteriolar and venous thrombosis, convulsions, and encephalopathy (60%), cardiac manifestations such as valve involvements and myocardial infarctions (53%) are the major clinical findings. Usually severe thrombocytopenia is present (60%). Hemolysis and disseminated intravascular coagulation may occur. Recommendations for the treatment of CAPS are intravenous heparin, plasmapheresis, and steroids. Treatment of CAPS should include the precipitating conditions such as the treatment of infections with appropriate antibiotics, and the treatment of SLE flares. The mortality rate is quite high (more than 50%) even in presumably properly treated patients [35,36].

Obstetric complications of APS
The association between the aPLA and fetal loss has been recognized for a long time. Besides the recurrent fetal losses, it is now clear that aPLA may cause pre-eclampsia, eclampsia, fetal growth retardation, uteroplacental insufficiency, preterm birth, and reproductive failure [1,2,8].

Thrombocytopenia in APS
Thrombocytopenia is reported in about 20–40% of patients with APS, is usually mild (70,000–120,000/mm³), and does not require any clinical intervention. Severe thrombocytopenia (lower than 50,000/mm³) is seen only in 5–10% of the patients [37–39]. Although thrombocytopenia has been defined as a clinical criteria in the first classification of APS [40], it was not included in the preliminary classification of definite APS recently proposed in Sapporo [1]. The patients who had APLA and thrombocytopenia as the only clinical manifestation in the absence of other APS findings were defined as ‘probable’ or ‘possible’ APS. In a recent prospective study, however, it has been shown that the ITP patients who presented with thrombocytopenia and had positive tests for APLA had increased risk of thrombosis. It has been proposed that measurement of APLA, especially LA in patients with initial diagnosis of ITP may identify a subgroup of patients with higher risk of developing APS [41].

The pathogenesis of thrombocytopenia in the APS is not clear. Although there is direct evidence that aPLA may bind platelet membranes and cause platelet destruction, the relation between aPLA positivity and thrombocytopenia is still unclear. Some investigators suggest that specific anti-platelet glycoprotein (GP)-antibodies rather than aPLA cause thrombocytopenia in patients with APS, and anti-GP antibodies are rare in patients with APS with normal platelet counts [42,43]. In one study, antibodies directed against the GPIIb-IIIa or GPIb-V-IX complexes were found in about 40% of the patients with APS who had thrombocytopenia [44]. Anti-platelet GP
antibodies in thrombocytopenic patients with APS do not cross-react with antibodies against phospholipids or beta2 GP-I [45]. Immunosuppressive treatment of thrombocytopenia in those patients increases the platelet count and reduces the titers of anti-GP antibodies, but not the titers of aPLA [46]. These data may suggest that thrombocytopenia is a secondary immune phenomenon that may develop at the same time with APS. On the other hand, Fabris et al. showed that platelet antigens in thrombocytopenic patients with APS were different from those in ITP, and surface glycoproteins were not involved. He also found that a 50–70 kDa internal platelet protein had been specifically found in patients with APS and thrombocytopenia but not in patients with ITP [47].

Another issue is the clinical importance of thrombocytopenia in the APS. When the APS patients were divided into three groups according to platelet counts as non-thrombocytopenic, moderately (50,000–100,000/mm³), and severe thrombocytopenic (below 50,000/mm³), the rate of the development of thrombocytopenia was found as 40%, 32%, and 9%, respectively [38]. This data shows that moderate thrombocytopenia does not prevent the development of thrombosis in patients with APS, and anti-thrombotic prophylaxis should be considered in those patients [38,39,41].

Although thrombocytopenia is a common finding in patients with APS, bleeding complications are very rare, even in severe thrombocytopenia. The presence of bleeding symptoms in an APS patient with moderate thrombocytopenia nececcitates a search for the presence of anti-prothrombin antibodies [48], and other diseases which may affect hemostasis such as DIC, uremia etc. Severe thrombocytopenia may require therapy. Treatment strategies in those patients are similar in those with ITP. Glucocorticoids are effective in only 15% of the patients [37]. IVlg and immunosuppressive drugs such as cyclophosphamide may be used in patients who have severe bleeding symptoms and have ‘catastrophic’ APS. Splenectomy produces sustained remission in approximately two-thirds of the patients [49–51]. Preoperative vaccination procedure is the same as ITP. Antithrombotic prophylaxis should be planned to prevent postoperative thrombosis in those patients.

Interestingly there are a few case reports describing the correction of thrombocytopenia with aspirin [52,53], warfarin [54,55], and anti-malarial drugs [56]. It has been suggested that inhibition of platelet activation, aggregation, and platelet consumption may help to increase platelet count in those patients.

**Management of thrombotic complications in APS**

Management of patients with aPLA mainly depends on the presence of clinical symptoms and findings:

a. **aPLA-positive individuals with no APS symptoms or findings**: It is known that aPLA may be present in 1–5% of healthy individuals [57]. If aPLA-positive individuals have no history or findings of APS, treatment should not be considered [3,4,58,59]. Although some investigators recommend prophylactic therapy for aPLA-positive individuals if they face to acquired thrombotic risk (puerperal period, surgery, immobilisation etc.) [7,58], there are no prospective or controlled studies investigating the effectiveness of anti-thrombotic prophylaxis in those individuals.

b. **Primary prophylaxis of thrombosis in aPLA-positive patients**: Although many experts recommend to use anti-thrombotic prophylaxis in aPLA-positive patients who fulfill Sapporo APS criteria and have no history of thrombosis, there are only few studies addressing this issue. The difficult point is to define thrombotic risk in aPLA-positive patients who had no thrombosis. Erkan et al. showed that APS patients with fetal losses were also at high risk of thrombosis [60], and they recommended prophylactic aspirin therapy, 325 mg/day. Petri et al. reported that hydroxychloroquine might have a protective effect against thrombosis in secondary APS (SLE-APS) patients [61].

c. **Treatment of venous thrombosis in APS**: Treatment of the first venous thrombotic attack is similar to those with idiopathic venous thrombosis. Heparin and oral anticoagulation therapy are routinely used for this purpose. Recurrency of thrombosis is higher in patients with APS compared to patients with no aPLA and venous thrombosis [62], especially in the first 6 months of thrombosis and after the cessation of the therapy [62–64]. However, the duration and the intensity of the treatment are not clear. In the first studies, it has been suggested that high intensity (INR > 3) oral anticoagulant therapy should be used in APS [62,64]. Recent studies however, showed that high intensity oral anticoagulant therapy is not superior to conventional dose oral anticoagulant therapy (INR 2–3) for the prevention of thrombosis in those patients [2,3,7,63–66,68]. Ames et al. also showed that high intensity oral anticoagulant therapy might increase bleeding complications in patients with APS [68]. Coexistence of thrombocytopenia is the major cause for bleeding. The duration of oral anticoagulant therapy is an another debated issue. Although life-long therapy was recommended in the first studies; recent prospective studies yield no firm conclusions.

d. **Treatment of arterial thrombosis in APS**: Acute coronary syndromes, transient ischemic attacks, and cerebral arterial thrombosis are the most
common causes of arterial involvement in patients with APS. Morbidity and mortality are high in arterial involvement. In APS patients with acute coronary syndromes, high intensity oral anticoagulant therapy is recommended. The effectiveness of additional aspirin therapy is debated [7]. In APASS (The Antiphospholipid Antibody in Stroke Study Group) study [68], APS patients with stroke were randomized to either aspirin (325 mg daily) or warfarin (targeted INR 2.2), and were compared for the risk of recurrent stroke. APASS study found no significant difference between two arms, although the study population was older than the APS patients in most of the cohorts. It is important to determine other disorders to decide for the treatment modalities in APS patients with stroke. In stroke patients who have atrial fibrillation and cardiac valve disorders are recommended to use warfarin therapy with moderate to high intensity.

Conclusion

APS is an acquired autoimmune disorder with thrombotic tendency, obstetric complications and multi-systemic symptoms. The identification of the APS is extremely important since thrombotic complications may cause severe morbidity and mortality. Although many studies showed the association between aPLA and clinical findings of APS; exact pathophysiological mechanisms are still unclear. The elucidation of pathogenetic mechanism of APS may help to identify patients who are at high risk for thrombosis, and may improve management of the patients.

References

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Hypercoagulable states

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Abstract
The incidence of venous thromboembolism (VTE) in the USA is approximately 1 per 1000 with over 200,000 new cases per year. Despite improved methods for antithrombotic prophylaxis, the incidence of VTE has remained constant for the last 20 years and about 30% of patients developing VTE die within 30 days. Risk factors for VTE include advancing age, obesity, recent surgery or trauma, hospital or nursing home confinement, malignancy, and stroke; among women, pregnancy, oral contraceptives, and hormone replacement therapy play a significant role. Prior to 1993, a cause of thrombophilia was detectable in a relatively small percentage of patients presenting with VTE. Hereditary abnormalities were found in only 5 to 15% of patients and were confined to deficiencies of antithrombin, protein C, and protein S. Discovery of the factor V Leiden and prothrombin G20210A mutations has greatly increased the percentage of Caucasian patients in whom venous thrombosis can, in part, be attributed to hereditary thrombophilia. It has also been demonstrated that elevated plasma homocysteine levels constitute a risk factor for venous as well as arterial thrombosis. These abnormalities, along with the presence of markers of the antiphospholipid antibody syndrome can be identified in a substantial percentage of patients presenting with a first episode of idiopathic venous thromboembolism (i.e. in the absence of a triggering risk factor such as surgery, immobolization, or active malignancy). Testing for the hereditary thrombophilias is warranted in selected subsets of patients for testing, the choice of tests to perform, and the timing of the testing are important issues to consider. Routine testing of unselected patients with venous thromboembolism would be warranted if the identification of abnormalities led to an alteration in the type or duration of initial anticoagulant therapy. However with the exception of the antiphospholipid antibody syndrome, the available data do not indicate that the anticoagulation management of the majority of patients with hereditary thrombophilia should be any different from those without identifiable abnormalities. The risk of recurrent venous thromboembolism in patients with an initial episode of venous thromboembolism will be discussed along with the impact of a thrombophilic defect and the likely benefit of prolonged anticoagulation versus the associated bleeding risk associated with prolonged anticoagulation.

Keywords: Hypercoagulability, Thrombophilia, Venous Thromboembolism
Inflammation and coagulation

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Inflammation is associated with thrombotic predisposition in patients. The mechanisms by which inflammation contributes to the thrombosis involve both humoral and cellular changes. Inflammation impacts all phases of blood coagulation: initiation, propagation and the inhibition. Inflammatory mediators like endotoxin and tumor necrosis factor alpha induce expression of tissue factor on blood cells, particularly monocytes. Tissue factor then triggers the initiation of coagulation. Normally, negatively charged membrane surfaces are limiting so that even if some activated coagulation factors are generated, they fail to propagate coagulation. C reactive protein, an acute phase reactant, levels increase in response to inflammation. The C reactive protein can then induce tissue factor formation. Complement membrane attack complex provides a potent stimulus for cells to express negatively charged phospholipid on their outer membrane leaflet. Alternatively, exposure to collagen in combination with thrombin provides a potent stimulus to platelets eliciting the formation of microparticles and the subsequent exposure of negatively charged phospholipid membrane surfaces that can propagate coagulation. Even when these two events both occur to augment coagulation, potent natural anticoagulant mechanisms limit the thrombotic response. Inflammatory mediators, however, can depress these potent natural anticoagulant mechanisms. Of the natural anticoagulants, the protein C pathway is one of the systems down regulated by inflammation. Thrombomodulin and the endothelial cell protein C receptor are both required for optimal protein C activation in response to thrombin generation. In severe inflammatory situations, both proteins are down regulated resulting in a decreased anticoagulant response. Furthermore, free protein S levels often decrease, further impairing the pathway. In immune mediated inflammatory disease, the pathway may also be down regulated. Some anti-phospholipid antibodies severely impair the protein C pathway. Clinically relevant thrombosis is minimized naturally either by inhibiting the blood clotting or by rapid lysis of the blood clot or both. In addition to shifting the hemostatic balance in favor of clot formation, inflammation elevates plasminogen activator inhibitor levels resulting in decreased fibrinolytic activity. Procoagulant impacts of inflammation are also expressed at the cellular level. Interleukin 6 can increase platelet numbers and their responsiveness to agonists like thrombin. All of these events tend to shift the hemostatic balance in favor of clot formation.
PLATELETS

Giant platelet syndrome

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Introduction

Platelets are small, disk-shaped, anuclear cells with a mean diameter of 2 to 3 μm. They are derived from cytoplasmic fragmentation of megakaryocytes, released into the circulation and survived for 7–10 days. Megakaryocyte development and platelet formation are regulated by thrombopoietin and other cytokines. It is now generally assumed that platelets are released by extension and fragmentation of cytoplasmic processes (proplatelet) of megakaryocytes through sinus endothelial cells in the bone marrow. It is not known, however, how the number and the size of the platelet are controlled at this step [1].

Giant platelet syndrome is a group of unique disorders characterized by the presence of abnormally large platelets, and is usually accompanied by thrombocytopenia. Thus, it is also called macrothrombocytopenia. Giant platelets are occasionally observed as an incidental finding in routine blood smear examinations. Most of them are due to acquired disorders such as idiopathic thrombocytopenic purpura (ITP) and myelodysplastic syndrome (MDS) (Table I). In contrast, inherited giant platelet disorders are rare. The mechanisms of giant platelet formation and thrombocytopenia are not fully understood in both inherited and acquired disorders. It is important from a clinical standpoint that congenital disorders are distinguished from acquired disorders, especially ITP, to avoid unnecessary treatments. We will discuss the molecular basis, diagnosis, and management of some major inherited giant platelet syndromes.

Inherited giant platelet syndrome

In recent years we have seen remarkable progress in the molecular understanding of some giant platelet syndromes. The Table I lists some major inherited giant platelet syndromes according to the possible underlying cause: abnormalities in the platelet cytoskeleton, GPIb/IX/V, and transcription factors. Clinical and laboratory features as well as responsible genes and chromosomal localizations are also shown. There are still many inherited disorders in which the underlying genetic abnormality has not yet been elucidated.

Autosomal dominant macrothrombocytopenias with leukocyte inclusions (MYH9 disorders)

May-Hegglin anomaly (MHA), first described a century ago, is the prototype of these disorders (Figure 1) [2]. Sebastian (SBS), Fechtner (FTNS), and Epstein (EPS) syndromes belong here. All four disorders have macrothrombocytopenia however, each of these disorders is distinguished from others by the presence or absence of granulocyte inclusion bodies and the presence or absence of a variable combination of Alport manifestations, including nephritis, deafness and cataracts (Table II). Although these four disorders were previously considered to be separate clinical entities, a recent positional cloning approach disclosed that these disorders are caused by mutations in the same gene, MYH9, which encodes the nonmuscle myosin heavy chain-A (NMMHCA) [3–5]. Thus, they appear to represent the same entity with different genetic penetrance and variable phenotypic expression. The bleeding tendency is usually mild.

The diagnosis of macrothrombocytopenia with leukocyte inclusions has been conventionally made on the basis of hematomorphological examinations. It is, however, not always easy to detect granulocyte inclusions on Wright stained smear. Immunofluorescence analysis of neutrophil NMMHCA localization on Wright stained smear. Immunofluorescence analysis of neutrophil NMMHCA localization has revolutionized the diagnosis of MYH9 disorders [6]. Abnormal NMMHCA aggregates and accumulates in the neutrophil cytoplasm, and this abnormal...
subcellular localization of NMMHCA is present in every neutrophil from individuals with MYH9 mutation. The localization pattern of neutrophil NMMHCA in MYH9 disorders can be classified into three groups according to the number, size, and shape of the fluorescence-labeled NMMHCA granules: type I, II and III (Figure 2). In type I, NMMHCA forms one or two large and intensely stained cytoplasmic foci. Type II neutrophils consist of several cytoplasmic spots with circular to oval shape. Type III or speckled staining is detected in patients with EPS and isolated macrothrombocytopenia, in which Wright or May-Grünwald-Giemsa (MGG)-stained inclusion bodies have never been identified [6].

An MYH9 mutation is strictly associated with the hematological abnormalities. Although the molecular mechanism of the production of giant platelets has not been elucidated, it is suggested that abnormal NMMHCA, by interfering with the formation of myosin thick-filament, affects proper proplatelet formation in megakaryocytes. Genetic analysis of many kindreds with MYH9 disorders revealed that there is no clear relationship between clinical phenotypes and the sites of the MYH9 mutations. It is likely that the MYH9 mutation alone does not cause associated Alport manifestations and that unknown genetic and/or epigenetic factors might influence the phenotypic consequences of MYH9 mutations [7,8].

We have recently generated and analyzed MYH9 knock-out mice [9]. No homozygous mice were born, suggesting that MYH9 expression is required for

<table>
<thead>
<tr>
<th>Disease</th>
<th>Inheritance</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Clinical and laboratory features</th>
</tr>
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<tbody>
<tr>
<td>Acquired</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ITP</td>
<td></td>
<td></td>
<td></td>
<td>almost normal RBC and WBC</td>
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<tr>
<td>MDS</td>
<td></td>
<td></td>
<td></td>
<td>anemia, abnormal WBC</td>
</tr>
<tr>
<td>Inherited</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormalities in platelet cytoskeleton</td>
<td>AD</td>
<td>MYH9</td>
<td>22q12-13</td>
<td>Macrothrombocytopenia, granulocyte inclusions with/without Alport manifestations (Table II)</td>
</tr>
<tr>
<td>Abnormalities in GPIb/IX/V</td>
<td>AR</td>
<td>GP1BA</td>
<td>17pter-p12</td>
<td>No ristocetin-induced platelet agglutination</td>
</tr>
<tr>
<td>Bernard-Soulier syndrome</td>
<td></td>
<td>GP1BB</td>
<td>22q11</td>
<td></td>
</tr>
<tr>
<td>Mediterranean macrothrombocytopenia</td>
<td>AR</td>
<td>GP9</td>
<td>3q21</td>
<td>No bleeding tendency</td>
</tr>
<tr>
<td>Bernard-Soulier syndrome carrier</td>
<td>AD</td>
<td>GP1BA</td>
<td>17pter-p12</td>
<td></td>
</tr>
<tr>
<td>DiGeorge/Velocardiofacial syndrome</td>
<td>AD</td>
<td>GP1BB</td>
<td>22q11</td>
<td>Mild thrombocytopenia with normal ristocetin-induced platelet agglutination</td>
</tr>
<tr>
<td>Abnormalities in transcription factors</td>
<td>XL</td>
<td>GATA1</td>
<td>Xp11</td>
<td>Dyserthropyoeis with/without β-thalassemia trait</td>
</tr>
<tr>
<td>X-linked macrothrombocytopenia with dyserthropyoeis</td>
<td>AD</td>
<td>FLI1</td>
<td>11q23</td>
<td>Contiguous gene syndrome due to chromosome 11q23 microdeletion</td>
</tr>
<tr>
<td>Paris-Trousseau thrombocytopenia/ Jacobsen syndrome</td>
<td></td>
<td></td>
<td></td>
<td>Growth and psychomotor retardation</td>
</tr>
<tr>
<td>Mediterranean macrothrombocytopenia/ Bernard-Soulier syndrome</td>
<td></td>
<td></td>
<td></td>
<td>Dysmegakaryopoiesis associated with giant α-granules</td>
</tr>
<tr>
<td>Unknown cause</td>
<td>AR, AD</td>
<td>unknown</td>
<td></td>
<td>Gray or colorless platelets due to absent α-granules</td>
</tr>
</tbody>
</table>

*See Table II for details.
AD: autosomal dominant, AR: autosomal recessive, XL: X-linked.
embryonic development. By an antisense oligonucleotide-directed suppression of transcription, NMMHCA is involved in a rearrangement of the actin cytoskeleton and loss of cell adhesion [10]. \( MYH9 \) \(-/-\) embryos, therefore, appeared to fail to develop a normal patterned embryo. Such fetal lethality may partly explain the absence of naturally occurring homozygous mutations in human subjects. In contrast, heterozygous mice (\( MYH9 \) \(+/-\)) were viable and fertile without gross anatomical, hematological, and nephrological abnormalities. Immunofluorescence analysis showed the normal cytoplasmic distribution of NMMHCA. Interestingly, we found that some but not all mice have hearing loss. The distribution of \( MYH9 \) expression in the inner ear has been studied in the developing fetal, neonate, adult mice [11], suggesting that \( MYH9 \) may have important roles in the development and maintenance of auditory function. On the other hand, the unconventional myosins, Myosin VIIA is localized in the stereocilia and cell body of hair cells and is critical in differentiation, formation, and/or maintenance of sensory hair cell structure [12–14]. Myosin VIIA have been linked to nonsyndromic and syndromic hearing loss. Therefore, the requirement of NMMHCA in the mammalian auditory system might be limited and could be compensated by other conventional myosins. Thus heterozygous loss of NMMHCA might result in the phenotype that does not appear uniformly, or the severity of phenotype might be unrecognizable.

Bernard-Soulier syndrome

Bernard-Soulier syndrome (BSS) is an autosomal recessive bleeding disorder characterized by giant platelets, thrombocytopenia and prolonged bleeding time (Figure 3), originally described by Bernard and Soulier in 1948 [15]. BSS is caused by quantitative or qualitative abnormalities in the glycoprotein (GP) Ib/IX/V complex, the platelet receptor for von Willebrand factor [16,17]. As a result of the absence of GPIb/IX/V complexes on the platelet membrane, platelets cannot stick to the damaged blood vessel walls, and consequently patients bleed. In the central cytoplasmic domain, GPIb associates with the actin cross-linking protein, filamin A. Thus, the defective linkage between GPIb/IX/V and cytoskeleton is the proposed molecular cause of the giant platelets.

The classical diagnostic features are a prolonged bleeding time, moderate to severe thrombocytopenia, and giant platelets. Especially, giant platelets and the absence of ristocetin-induced platelet agglutination are the laboratory hallmarks of BSS (Table I). Flow cytometric determination of platelet GPIb/IX expression is a convenient method for diagnosis of BSS in a clinical laboratory.

Thus far, 39 mutations in the genes for GPIb\(\alpha\), GPIb\(\beta\), and GPIX have been described. Approximately half of the mutations were found in the GPIb\(\alpha\) gene (17 mutations), and the remaining were found in the GPIb\(\beta\) (12 mutations) and GPIX genes (10 mutations). A majority of the mutations correspond to single-base substitutions or small base deletion and insertion mutations. Recent studies have shown that the phenotypes caused by mutations in the subunits of GPIb/IX span a wide spectrum, from

Table II. Macrotrombocytopenia with leukocyte inclusions/MYH9 disorders

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Macrothrombocytopenia</th>
<th>Leukocyte inclusions</th>
<th>Alport syndrome*</th>
</tr>
</thead>
<tbody>
<tr>
<td>May-Hegglin anomaly (MHA)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sebastian syndrome (SBS)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Fechtner syndrome (FTNS)</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Epstein syndrome (EPS)</td>
<td>+</td>
<td>–</td>
<td>–</td>
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</tbody>
</table>

*Nephritis, deafness and cataracts.
the normal phenotype to isolated macrothrombocytopenia with normal platelet function, to full-blown BSS and platelet-type von Willebrand disease. It is important to note that although heterozygous BSS carriers are generally asymptomatic with subnormal platelet number and function, they have giant platelets. Several individuals with heterozygous GPIb/IX mutation have been initially identified as having undifferentiated thrombocytopenia or refractory ITP. In Italy, a mutation in GPIbα (Ala156Val) was found to be a founder mutation responsible for the autosomal dominant macrothrombocytopenia previously known as a Mediterranean macrothrombocytopenia [18]. In addition, patients with DiGeorge/velo-cardio-facial syndrome due to a heterozygous chromosome 22q11.2 microdeletion, which includes the GPIbβ gene, have macrothrombocytopenia [19] (Table I).

X-linked macrothrombocytopenia with dyserythropoiesis

Recently, several unrelated families with X-linked macrothrombocytopenia with mild to moderate dyserythropoiesis have been found to have mutations in the GATA-1 gene. GATA-1 is a megakaryocyte- and erythroid-specific transcription factor required for normal growth and differentiation of both lineages. Defective GATA-1 function due to missense mutations causes reduced transcription and thus protein expression of its target genes, including GPIbα, GPIbβ, GPIX, and GPV. The concomitant decrease in the expression of GPIb/IX/V and other platelet-specific gene products is related to the macrothrombocytopenia and bleeding tendency in this disorder [20].

Paris-Trousseau syndrome/Jacobsen syndrome

Paris-Trousseau syndrome/Jacobsen syndrome is a contiguous gene syndrome characterized by mental retardation, and facial and cardiac abnormalities due to a heterozygous 11q23 deletion. The platelets contain giant α granules on peripheral blood smears, and in the bone marrow megakaryocytes are increased with many micro megakaryocytes. Hemizygous deletion of the transcription factor Fli1 contributes to the hematopoietic defects in this disorder [21].

Inherited macrothrombocytopenias of unknown cause

Gray platelet syndrome

Gray platelet syndrome (GPS) is characterized by thrombocytopenia and abnormal giant platelets with absent platelet α-granules. Patients with GPS have a bleeding tendency of variable severity. The gene(s) responsible for the disease are currently not known. The most characteristic feature and thus the laboratory hallmark of the syndrome is agranular platelets. On Wright- or MGG-stained peripheral blood smears, platelets appear gray or colorless due to the absence of platelet α-granules and their constituents. Because platelet α-granule proteins such as platelet-derived growth factor are synthesized but not properly stored in the granules and released from megakaryocytes into the bone marrow, myelofibrosis is present in most cases [22].

Type 2B von Willebrand disease

Patients have a prolonged bleeding time, decidedly low vWF activity measured as ristocetin cofactor activity, a mild deficiency of vWF antigen level, and enhanced ristocetin-induced platelet aggregation at low concentrations of ristocetin. Although the molecular mechanisms remain to be elucidated, some patients with type 2B vWD have been reported to have giant platelets [23].

Approach to patients with macrothrombocytopenia [24]

First of all, acquired causes of macrothrombocytopenia, including ITP and myelodysplastic syndromes, should be ruled out. Complete history and physical examination should be carefully performed. In syndromic forms, patients show complications of physical abnormalities such as facial, cardiac, skeletal anomalies and/or mental retardation. If the patient previously had normal platelet counts, acquired rather than congenital conditions are more likely to be the underlying cause. In inherited macrothrombocytopenias, platelet counts are constantly decreased, ranging from as low as 10 × 10^9/l to near normal 150 × 10^9/l. On a peripheral blood smear, the majority of platelets are large, being similar to or larger than red blood cells or small lymphocytes. In contrast, in patients with the much more common ITP, large platelets are present but the majority are of normal size. Because routine automated blood cell counting systems differentiate blood cells by their size and do not recognize giant platelets as platelets, these instruments underestimate platelet counts in patients with macrothrombocytopenia. The mean platelet volume, usually calculated as a parameter of the complete blood count, also does not reflect actual platelet size in the case of giant platelets. Platelet count should therefore be determined manually in a calculating chamber or on peripheral blood smears. Careful examination of a smear also allows morphological assessment of leukocytes and erythrocytes. If granulocyte inclusion bodies are obscure or absent, immunofluorescence analysis for neutrophil NMMHCA localization is helpful to make a clear distinction. Flow cytometric analysis of
platelet GPIb/IX expression can differentiate BSS heterozygotes from patients with “true” isolated macrothrombocytopenia. Patients with congenital macrothrombocytopenia generally do not respond to standard ITP treatments, including corticosteroids, intravenous immunoglobulin, and splenectomy. If treatment for bleeding is clinically indicated, the administration of antifibrinolytic agents such as α-aminocaproic acid or tranexamic acid and recombinant activated factor VII may transiently improve the episodes [25]. Transfusion of platelets is effective for serious bleeding and as prophylaxis prior to major surgery, but may be complicated by the development of alloantibodies. In certain instances, hematopoietic stem cell transplantation (SCT) may be a curative therapeutic option [26]. It is important to make a proper diagnosis to avoid unnecessary treatment. Affected families should be educated about their diagnosis to avoid unnecessary medications and potentially dangerous treatments for presumed ITP. When evaluating patients with refractory ITP or undifferentiated thrombocytopenia, congenital macrothrombocytopenias should be included in the differential diagnosis.

Conclusions

Inherited giant platelet syndromes are rare conditions, yet the study of them has been instrumental in elucidating the structure and function of normal platelets as well as the mechanisms of thrombopoiesis. Further research is needed to understand the pathogenesis of many congenital disorders with unknown causes.

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References


PLATELETS

The role of ADAMTS13 in the new pathogenesis of TTP

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Thrombotic Thrombocytopenic Purpura (TTP) is a severe microvascular occlusive microangiopathy characterized by thrombocytopenia, Coombs-negative hemolytic anemia and ischemic symptoms localized mainly but not exclusively in central nervous system and resulting from diffuse platelet thrombi in microcirculation [1]. To understand the broad clinical spectrum of TTP it is important to read the original case report prior to effective treatment, when the full natural history of this fatal illness was appreciated. In 1924 Dr. Moschowitz [2] described the first case in a previously healthy 16-year-old girl who died with multiple organ failure and post-mortem examination showed widespread thrombi in the terminal circulation of several organs, composed mainly by platelets. These thrombi remain the hallmark of pathologic diagnosis.

The current era in our knowledge began with the observation by Moake and his colleagues in 1982 [3] of unusually large multimers of von Willebrand factor (ULVWF) in the plasma of patients with chronic relapsing TTP. They are similar to those normally contained in vascular endothelial cells and platelets but absent in plasma. These ULVWF multimers promote platelet-dependent microvascular thrombosis, suggesting that patients lacked a VWF protease able to reduce the size of VWF multimers by cleavage. The next key step was the discovery made 14 years later, when Furlan, Tsai et al. [4,5] isolated a VWF-cleaving protease that had properties consistent with the activity that had been postulated to be absent in patients with TTP. These two groups subsequently published evidence that the consistently very low or undetectable plasma levels of this protease were indicative of TTP [6,7]. The VWF-cleaving protease is now known as ADAMTS13, a member of the ADAMTS metalloprotease family (A Disintegrin And Metalloprotease with Thrombospondin-1 repeats), thanks to the results obtained by Fujikawa [8], Gerritsen [9] and Zheng [10] in 2001. In the same year Levy identified the ADAMTS13 gene and described in seven unrelated families 12 distinct mutations, underlying the molecular mechanism responsible for congenital TTP [11]. At this point everything seemed to become clear: an enzyme deficiency allows ULVWF multimers to circulate; the unusually large multimers cause disseminated platelet clumping in the microcirculation; the deficient protease can be replaced by plasma infusion, which is sufficient in patients with congenital deficiency; the inhibitor of ADAMTS13 activity in patients with acquired disease can be removed by plasmapheresis, making plasma exchange a more effective treatment than plasma infusion. However, as clinical studies have extended beyond the initial translational research to community studies [12], the inevitable increase in complexity of these syndromes has been appreciated. The main finding of these subsequent studies is that only approximately 50% of patients with overt clinical disease have low plasma levels of the protease. Simultaneously an enormous number of in vitro studies have been initiated in order to comprehend the pathophysiology of TTP. Between 2002 and 2004 it has been demonstrated that ULVWF multimers are cleaved by ADAMTS13 between Tyr1605-Met1606 residues located within the A2 domain as they are secreted in long “strings” from stimulated endothelial cells [13,14]. The ULVWF strings are anchored to the endothelial cell membrane via P-selectin molecules that are secreted concurrently with the ULVWF multimers [15]. Specifically, ADAMTS13 enzyme binds under flow conditions to accessible A3 domains in the monomeric subunit of ULVWF multimers and then cleaves Tyr 1605-Met 1606 peptide bonds in adjacent A2 domain. Majerus et al. [16] have shown that the spacer and TSP1-1 like domains are required for ADAMTS13 binding to VWF, that may be modu-
lated by CUB and TSP1-2/8 like domains. As a consequence of ADAMTS13 deficiency, ULVWF multimers are not cleaved after their secretion from endothelial cells, but remain anchored to the cells. Passing platelets adhere via their GpIb and GpIIb/IIIa to the A1 and the A3 domains of the monomeric subunits of ULVWF strings anchored to P-selectin to form large, potentially occlusive, platelet thrombi.

Moreover, considering that severely deficient ADAMTS13 activity may not always be sufficient to cause TTP, in the last two years some researchers have started to investigated other proteins that might be involved in the mechanisms underlying TTP. Pimanda and their colleagues have been demonstrated that the multimeric size of VWF can be controlled by disulfide bonds-reduction by thrombospondin-1 (TSP-1) [17]. VWF reductase activity was centered around Cys974 in the C-terminal sequence of TSP-1 but the role of TSP-1 in the aetiology of TTP is under investigation. Many other aspects of ULVWF processing are now rapidly coming into focus, such as the involvement in ULVWF secretion of such cytokines as IL-8, TNF-α and IL-6 in complex with its receptor. This might explain the role of inflammation as triggering agent in TTP [18]. Furthermore different authors are studying the regulation of ADAMTS13 activity by different factors as thrombin and plasmin, that cleave ADAMTS13 at specific sites, resulting in the loss of ADAMTS13 activity [19]; or chloride ions which, binding to VWF and causing its conformational change, have specific inhibitory effect on ADAMTS13 activity [20]. Finally it has been demonstrated that the VWF A1 domain inhibits the cleavage of A2 domain by ADAMTS13 and this inhibition can be relieved by interaction of A1 domain with platelet GpIIb or certain glycosaminoglycans (heparin) [21].

Until now it has been paved the way to understand the exact role of ADAMTS13 and the other modulators in developing TTP but many unresolved issues still remain.

References
Genetic and molecular techniques have provided the means to gain increasing insights into the biology of acute myeloid leukemia (AML). These investigations demonstrated that AML is not a homogeneous disease but a heterogeneous group of biologically different subentities. These subentities are currently primarily defined by cytogenetic and molecular analyses. Based on cytogenetics three main subgroups of AML can be discriminated: AML with balanced translocations, AML with unbalanced aberrations and AML without cytogenetically detectable aberrations. Within the latter group molecular alterations are identified in more than half of all cases such as NPM-mutations, FLT3- mutations, MLL-duplications and mutations of CEBP-α.

These different subtypes reveal a different prognosis. Based on these insides recent treatment strategies try to adapt the knowledge about the biology to develop biology adapted treatment strategies. This process is still under development and is determined by the availability of new agents and their evaluation in phase II-studies but also on the more conservative approach to direct currently established protocols to biologic risk groups. The AMLCG has thoroughly investigated the outcome of biologically different subgroups on the basis of a prospective randomized trial asking three main questions: The comparison of double induction therapy with either HAM–HAM versus TAD–HAM, priming with G-CSF and the impact of autologous stem cell transplantation versus long term maintenance as treatment in remission.

The new strategy of the AMLCG is directed to a more biology oriented treatment. Hence, besides AML with the t(15;17) which is been treated in a different protocol for a long time already also other subtypes will receive risk adapted regimens. Hence, in patients with complex karyotype early transplantation with those reduced conditioning will be applied. In patients with standard risk AML intensification in way of the S-HAM regimen is currently explored. In addition a prospective randomized comparison of conventional versus dose-reduced conditioning has been initiated. In patients with core binding factor leukemia transplantation will not be performed but diseases will be monitored on a molecular level. Transplantation is only initiated as soon as a molecular signal is redetected or reveals rising transcript levels.

The step towards the biology adapted treatment of AML is long and requires the combined efforts of researchers, clinicians and the pharmaceutical industry. The first steps towards this goal, however, have been taken.
ACUTE MYELOID LEUKEMIA

The treatment of AML: Current status and novel approaches

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The epidemiology of AML suggests that in Western countries the median age of patients is around 65 years. Convention in recent years has often resulted in collaborative groups designing trials for patients under or over 60 years. In the former group treatment options included consolidation with stem cell transplantation of matched sibling allograft or autologous cells collected earlier in remission, which was not considered feasible in older patients.

Treatment in younger patients

It is now expected that complete remission (CR) can be obtained in 75–90% of patients under 60 years, usually with one course of treatment. Treatment mortality should be <10% with optimal supportive care. About 50% of patients who achieve complete remission will relapse, mostly over the next two years, so the overall 5 year survival at five years is around 50%. This represents an improvement of 1−1.5% per annum over the last 20 years. No single treatment modality accounts for this although intensification of treatment made possible by better supportive care seems the most plausible explanation [1,2].

Stem cell transplantation (both autograft and matched sibling allograft) has been carefully assessed in a number of major collaborative group trials [3–7]. This approach undoubtedly reduces the risk of relapse even in patients who have been treated intensively; however the impact on survival is less clear. The risk of relapse is dominated by a number of validated prognostic factors such as cytogenetics, FLT3 mutation status, the extent of blast clearance from the bone marrow after treatment course 1, presenting WBC and patient age [8,9]. Favourable risk cytogenetics includes (inv16); t(8;21) and t(15;17) and has an overall cure rate of 70−75%. Poor risk cytogenetics (abnormalities of Chs 5,7 3q-complex, t(9;22)) have a survival of 15%. All other lesions and normal have a survival of 50%. The presence of a FLT3 internal tandem repeat (ITD) mutation which itself significantly increases the relapse risk [10], adds refinement to the cytogenetic risk group – particularly the intermediate risk group (Relapse risk ITD+ve 70%; ITD-ve 45%). Mutations of RAS are present in 12% of patients, but do not appear to be prognostically important. As a general rule these prognostic factors are independent of treatment, including transplantation. Our recent analysis has been unable to show that there is benefit from transplant in FLT3 positive cases. Other mutations – although relatively rare, are being identified which may have prognostic value.

Numerous trials have been conducted over the years with the aim of determining which drug and dose combination is the most effective. Comparisons of anthracyclines, doses of Ara-C, the presence or not of a 3rd drug, have all been assessed without any convincing evidence of overall superiority for one schedule. Given the intensity of induction and consolidation, maintenance treatment is not indicated in younger patients. The precise total number of treatment courses required is not fully established but MRC studies suggest that the maximum should be 4 [11].

Once a patient relapses, his/her outcome is again dictated by prognostic factors more than treatment. These are age, length of first remission and cytogenetic risk group [12]. Patients who fail a transplant do less well, but this may in part be due to a reluctance to treat such patients intensively. Old age, short first remissions and adverse cytogenetics are virtually unsalvageable. Overall the outcome from relapse is about 20% without transplant, 35% with an autograft and 45−50% with an allograft (matched sibling or volunteer donor) [13].
Potential for improvement in younger patients

With a remission rate as high as it currently is, it will be difficult to show a benefit on remission rate of a new treatment with adequate statistical power. However it is well established but induction treatment – while not necessarily impacting on remission rate – can improve disease free survival by improving the “quality” of remission.

One of the interesting approaches currently in trial is adding in the immunoconjugate Gemtuzumab Ozogamicin (Mylotarg™). Initial studies at full dose (9 mgs/m²) suggested that unexpected liver toxicity would be problematic [14]. However a large study currently being conducted by the MRC confirms that adding Mylotarg in a reduced dose (3 mgs/m²) is feasible [15], however any benefit will not be known for 2–3 years.

Poor risk cases are particularly refractory, and although there is not unreal consistency in the evidence base such patients are usually offered allogeneic SCT (including MUD transplants) as soon as the risk status is known.

With the recognition of different molecular abnormalities, the expectation is that an era of molecularly target therapy – most likely in combination with existing treatments is imminent.

Treatment of older patients

The problem of treatment of older patients with AML has a number of facets [16,17]. First there is a substantial population of older patients who are not considered fit for the usual combination chemotherapy approach on offer due to age, frailty or comorbidity. Second, the success of effective treatment in younger patients is less successful in substantial part due to aggregation of adverse prognostic factors in older patients e.g., cytogenetics, prior MDS, and molecularly defined chemo-resistance. Chemoresistance is defined by a number of proteins several of which may be coexpressed in the same patient. The most common and predictive is over expression of Pglycoprotein. Attempts to modulate the efflux action of the protein using the cyclosporine analogue PSC-833 have been unsuccessful [18,19]. Third, the survival has not – unlike in younger patients – improved much in the last two decades. Overall the current expectation of CR in an older patient is 15% at 5 years. The relapse rate is high so overall the survival is 10–15% at 5 years.

Many randomized trials with large numbers have tried to improve outcome, with little clear success. This raises a further strategic issue in older patients, which is the question as to whether we should continue to focus clinical research and large numbers of elderly patients in large phase 3 trials as opposed to searching for novel against with a larger number of randomized phase 2 studies.

Transplantation in the older patient

Until recently it was accepted that standard allogeneic or autologous SCT in older patients carried a prohibitive mortality. However interest has been rekindled by the development of reduced intensity transplantation. This is clearly feasible with stable establishment of a full chimeric state. There does not yet seem to be a best choice preparative protocol. The slightly less reduced intensity will be strong enough to achieve full chimerism, but will often be accompanied by acute or chronic GVHD. More reduction in preparation intensity will reduce GVHD but may be associated with rejection.

Initially it was not thought that this transplant approach would be suitable for AML since several weeks are required to establish full chimerism. However a number of phase 2 experiences now suggest that durable survivals are seen in AML. However there is nothing to suggest that this approach has a survival advantage and it is important that this approach develops within the context of a prospective clinical trial. The improvements in tissue typing brought about by molecular matching of unrelated donors, has brought unrelated transplants to be a very practical option. Limited data suggests that this is also the case in older patients. These developments mean that allogeneic transplant is a viable option for older patients and is ready to be evaluated in clinical trials.

Novel agents

Given the lack of progress in older patients, and the probability that the molecular understanding of AML will bring new agents to the clinic, it is appropriate to direct new strategies and agents to the older patient.

Gemtuzumab ozogamicin [Mylotarg]

This immunoconjugate links a humanized anti-CD33 monoclonal antibody to the powerful intercalator calicheamicin. The key features are that the complex of CD33 antigen and antibody is rapidly internalized making it a potentially specific targeting vehicle for a chemotherapeutic agent. Since 90% of AML cases express CD33 and that expression is primarily on haemopoietic tissue this target is appropriate. A unique feature is that the clinical link joining the antibody to calicheamicin is only lysed intracellularly. Free calicheamicin is too toxic to give as a free agent so this is an important property.

Although only licences in the US and Japan for relapsed disease in the elderly. There is much interest in either combining it with chemotherapy – either simultaneously or sequentially – in or as maintenance
treatment in older patients. Pilot studies have confirmed the feasibility of this approach [15] but randomized studies will not be available for 3–5 years.

### Targeting FLT-3 mutations

About 30–35% of younger patients and a lower proportion of older patients will have a FLT-3 mutation. As well as providing valuable prognostic information, FLT-3 mutation could provide a target for therapeutic targeting. At least 5 agents with in vitro activity against cells carrying a FLT-3 mutation have had some clinical evaluation (PKC412; CEP701; MLN518, SU5416) [20–24]. PCK 412 and CEP701 have been assessed in unrandomized phase 2 trials in patients with mutations. Although a degree of disease control was seen, no CR’s were obtained with it seems likely that in future these agents will be combined with chemotherapy. Second generation agents may be more effective but no clinical data is yet available.

### Farnesyl migration

A number of cytoplasmic proteins, including RAS, which are involved in signal transduction, proliferation, angiogenesis and apoptotic pathways require to be prenylated. This process is mediated by the enzyme Farnesyl Transferase (FTase). FTase adds a 15-mer fatty acid chain to the C-terminal residue of substrate proteins which bear a – CAAX motif (farnesylation). This allows attachment to the inner surface of the membrane so facilitating binding of guanine nucleotide. While RAS may be an obvious target in AML, but disruption of other known or unknown proteins, may also be relevant. Tipifarnib (Zarnestra) is the agent which is most developed clinically in AML. Phase 1 in relapsed or refractory disease trials established a tolerable daily dose of 600 mg which can be given orally for 21 days a month. An unrandomized phase 2, involving trial patients, confirmed activity with a remission rate of around 25% in older patients not considered fit for intensive treatment. Activity was not restricted to patients with RAS mutations [25–27].

In spite of the convenience of an oral agent with activity as monotherapy, future interest will be in combination therapy. Clofarabine (2-chloro-2'-fluoro-deoxy-β-D-arabinofuranosyl adenine is a rationally designed purine analogue. The design is intended to harness the potentially favourable profile of Fludarabine while avoiding the neurotoxicity associated with the dose of Fludarabine that is effective in AML, and resisting deamination. With the potential to become orally available Clofarabine, activity in advanced paediatric ALL lead to approval in the USA. Activity in AML has been established in a phase 2 trial in relapsed disease [28]. An unrandomized phase 2 study using a reduced dose as first line treatment patients who were not considered fit for intensive therapy [29]. A remission rate of 56% was observed with an acceptable toxicity profile although the degree of myelosuppression suggests that even a reduced dose was in fact intensive treatment. Nevertheless Clofarabine seems worthy of development either as a component of intensive treatment or at reduced dose for older patients.

### References

The treatment of AML: Current status and novel approaches


ACUTE MYELOID LEUKEMIA

Management of APL

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Acute promyelocytic leukemia (APL) has become a curable disease by all-trans retinoic acid (ATRA)-based induction therapy followed by two or three courses of consolidation chemotherapy. Currently more than 90% of newly diagnosed patients with APL achieve complete remission (CR) and over 70% of patients are curable. To further increase the CR and cure rates, detection and diagnosis of this disease at its early stage is very important, hopefully before the appearance of APL-associated coagulopathy.

In induction to ATRA, concomitant chemotherapy is indispensable, except for patients with low initial leukocyte counts. Prophylactic use of intrathecal methotrexate and cytarabine should be done, particularly for patients with hyperleukocytosis. If patients relapse hematologically or even molecularly, arsenic trioxide (ATO) will be the treatment of choice under careful electrocardiogram monitoring. Am80, liposomal ATRA or gemtuzumab ozogamicin may be used at this stage or later. Whether ATO may be used for newly diagnosed APL should wait for the results of on-going randomized trials, and probably for the long-range outcome including possible secondary malignancy due to ATO. Allogeneic stem cell transplantation will be the treatment of choice after patients of age <50 years have relapsed, provided that they have HLA-identical family donors or DNA-identical unrelated donors.
ACUTE LYMPHOCYTIC LEUKEMIA

A broad and integrated diagnostic work-up for a modern management of Acute Lymphoblastic Leukemia (ALL)

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Acute lymphoblastic leukaemia (ALL) represents a biologically and clinically heterogeneous group of diseases characterized by the abnormal proliferation and accumulation of immature lymphoid cells within the bone marrow and lymphoid tissues. Malignant transformation is a consequence of somatic mutations in a single lymphoid progenitor cell and this mutation might occur at different stages of B- or T-cell development. The diagnosis and classification of ALL is currently a multistep procedure based on morphology, cytochemistry, immunophenotype, cytogenetics, molecular genetics, immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements, multidrug resistance (MDR), genomic profiling and relies on the simultaneous application of multiple techniques [1–4]. Increasing evidence suggests that chromosomal defects and molecular abnormalities are consistently present in patients with ALL, and progress in our understanding of the biologic and genetic characteristics of ALL has not only improved our knowledge of leukemogenesis, but has also allowed the identification of prognostic groups with specific cellular and molecular features [5,6]. Despite the effort to develop risk classification systems that are both reproducible and comparable, a need to refine the ability to distinguish between higher and lower risk patients still remains. In addition to their biologic relevance and their potential prognostic impact, cytogenetic and molecular abnormalities, as well as the immunophenotypic combinations, may offer additional tools for the detection of minimal residual disease (MRD) during the clinical course of the disease. Evidence suggests that a broad and integrated characterization of adult ALL in the context of multicenter protocols is essential for an optimal clinical management [7]. Detailed studies of individual patients need to be conducted at specialized centers, where preservation of viable cells, DNA, RNA, protein lysates, etc. is possible. An integrated biologic approach aimed at identifying prognostic factors implies a coordinated effort through central handling of all patients’ samples so that all the necessary investigations can be consistently performed in each individual case and the patients can be enrolled in the same therapeutic protocols. Besides, an integrated approach, using cytogenetic and molecular analysis and leukemia-associated immunophenotypes, can allow to identify suitable markers for monitoring MDR in virtually all childhood and adult ALL cases [8,9]. Recent advances in genome technologies have opened the way for the analysis of gene expression profiles in human leukemias that may lead to innovative genomic-based classifications of hematologic malignancies, as well as to the design of innovative therapeutic strategies.

Morphology and cytochemistry

ALL has been defined by the presence of more than 30% lymphoblasts in the bone marrow (BM) or peripheral blood (PB) according to the French-American-British (FAB) Co-operative group classification system [10]. In the recently proposed World Health Organization (WHO) classification scheme, [11] a blast count above 20% is sufficient for a diagnosis of acute leukemia. The morphologic/cytochemical examination recognizes three morphologic types: L1, L2 and L3 (Table 1). The prognostic significance between the L1 and L2 morphologic subtypes of ALL has never been fully proven; simi-
larly, immunophenotypic profiles that may be of prognostic significance do not correlate with the L1 or L2 morphology. Only the L3 type of ALL still holds as a distinct entity characterized by its morphology and also by its unique immunophenotypic and genotypic features. Unlike acute myeloid leukemia (AML), no single cytochemical test is specific for ALL; by definition, however, ALL is negative for myeloperoxidase (MPO) in cytochemistry studies and lacks staining with the anti-MPO monoclonal antibody (MoAb). According to the FAB criteria, cases of acute leukemia with more than 3% MPO positive blasts should be classified as AML. More than 95% of cases of L1 and L2 ALL are positive for terminal deoxynucleotidyl transferase (TdT) expression and its detection is useful in distinguishing reactive lymphocytosis from ALL; nevertheless, TdT expression can be found in some cases of AML. Most cases of ALL have a characteristic localized periodic acid-Schiff (PAS) staining pattern, but this finding is not specific for ALL and 'block' reactivity can be seen in some AML. Reactivity for nonspecific esterase can be detected in a subset of ALL and is usually weaker than that seen in acute monocytic leukemia. Acid phosphatase and alpha-naphthyl-acetate-esterase (ANAE) reactions give a strong positivity localized to the Golgi region in more than 80% of the blast cells in cases of T-lineage ALL; nowadays, however, they are no longer routinely utilized. Even though no single cytochemical reaction is specific, cytochemistry still represents a relevant component in the integrated diagnostic work-up of acute leukemias that helps to differentiate between ALL and AML.

**Immunophenotype**

The immunophenotypic characterization of blast cells has several objectives: (a) lineage assignment, (b) evaluation of cell maturation, and (c) assessment of phenotypic aberrations [12–14]. Lineage assignment of blast cells by immunophenotype may still represent a major challenge in some acute leukemias; this is mainly due to the cross-lineage antigen expression and it emphasizes the need to use combinations of several lineage-associated markers to establish the lineage affiliation of the blast cells. Flow cytometry is a powerful technique for the characterization of normal and neoplastic hematopoietic cells, the use of highly specific MoAb that recognize distinct epitopes of surface and intracellular antigens has improved the definition of the origin and level of differentiation of acute leukemias. There is no consensus on the best way to report the analyzed data; it is customary to report percentage of blasts expressing each antigen tested and to consider any marker present on more than 20% of blasts as positive: the cut-off level of 20% is arbitrary, however. Another point to consider in leukemia immunophenotyping is the intensity of antigen expression; since differences in fluorescent intensity may be important in distinguishing leukemic cells from normal cells and in discriminating among subtypes of leukemia, quantitative flow cytometry (QFCM) may now be used to measure antigen-binding sites on cells more objectively and this approach may be useful both at diagnosis and during the monitoring of MRD [15]. As shown in Table 2, B-lineage ALL (70–80% of cases) can be classified into four groups according to the expression of B-cell differentiation antigens and cytoplasmic and surface immunoglobulins (Ig); also T-ALL (15–25% of cases) can be classified into four groups based on the level of thymocyte maturation and antigen expression [16]. T-ALL can be further classified according to the subtypes of T-cell receptor (TCR) molecules. Although the affiliation of ALL cases to the B- or T-cell lineage is relatively easy, about 5% of cases remain difficult to classify as ALL or AML; these cases coexpress several lymphoid and myeloid antigens, either on the same cells (biphenotypic leukemia) or on two different populations (hybrid leukemia). There is no consensus regarding diagnostic criteria for such cases. The European Group for the Immunological Characterization of Leukemia (EGIL) [16] has suggested the use of a scoring system based on different combinations of B, T and myeloid antigen expression. According to a strict scoring system, four groups can be identified; the most common group is that in which the blasts coexpress myeloid and B-lymphoid antigens, and less commonly myeloid and T-lymphoid antigens. Cases coexpressing T- and B-lymphoid markers and those with trilineage differentiation are rare. The clinical significance of biphenotypic acute

<table>
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<tr>
<th>FREQUENCY</th>
<th>Pro-B</th>
<th>Common</th>
<th>Pre-B</th>
<th>B</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td>20–25%</td>
<td>50%</td>
<td>2–5%</td>
<td>20–25%</td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>50–70%</td>
<td>90%</td>
<td>25%</td>
<td>2–5%</td>
<td>15%</td>
</tr>
</tbody>
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**Table 1. Blast cell characteristics of ALL subtypes**
leukemia has not been determined and there has been a lack of uniformity in treatment; for example, there is no agreement as to whether induction therapy should use anti-lymphoid and/or anti-myeloid drugs [17]. To allow reproducible conclusions to be drawn about the optimal treatment of biphenotypic leukemias, increased numbers of patients are required for an objective analysis; due to the rarity of the disease, this will only be possible through multicenter studies.

Other markers are used to identify the maturation level of the blast cells and eventually establish atypical or aberrant phenotypes indicative of specific underlying genetic lesions. A variable proportion of ALL express apparently nonlineage associated markers, e.g., myeloid antigens and CD34. Expression of myeloid associated markers in ALL is well known, but its clinical significance is controversial. The expression of myeloid-associated antigens is a component of the initial diagnostic evaluation of acute leukemias and is also a valuable tool for monitoring disease after therapy and for the detection of MRD. The quantification of the level of expression of given antigens on the leukemic population may have therapeutic implications; MoAb have, in fact, reached clinical utilization in different lymphoproliferative disorders. This applies, in particular, to antibodies directed against CD20, CD22 and CD52. All three antigens on the leukemic population may have therapeutic implications; MoAb have, in fact, reached clinical utilization in different lymphoproliferative disorders. This applies, in particular, to antibodies directed against CD20, CD22 and CD52. All three antigens may be expressed by ALL cells. Thus, the percent of positivity and the degree of expression by the leukemic population at diagnosis and at relapse is important when considering the potential clinical utilization of such antibodies for the management of ALL patients.

**Table 2. Immunologic classification of ALL**

<table>
<thead>
<tr>
<th>B-lineage ALL:</th>
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<tbody>
<tr>
<td>CD19 + and/or CD79a + and/or cyCD22 +  pro-B ALL (B-I)</td>
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<tr>
<td>CD10 + cyIg-</td>
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<tr>
<td>cyIg + slg-</td>
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<tr>
<td>slg +</td>
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<tr>
<td>common ALL (B-II)</td>
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<tr>
<td>pre-B ALL (B-III)</td>
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<tr>
<td>mature-B ALL (B-IV)</td>
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<table>
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<tr>
<th>T-lineage ALL:</th>
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<tbody>
<tr>
<td>cyCD3+ + CD7+</td>
</tr>
<tr>
<td>CD2 + and/or CD5 + and/or CD8 +</td>
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<tr>
<td>CD1a +</td>
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<tr>
<td>CD1a- mCD3 +</td>
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<td>Anti-TCR γ/δ +</td>
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<td>cortical T ALL (T-III)</td>
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<tr>
<td>mature T ALL (T-IV)</td>
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<tr>
<td>γ/δ + T ALL (group a)</td>
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<td>γ/δ + T ALL (group b)</td>
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All with expression of one or two myeloid markers (My+ ALL)

Cytogenetic and molecular analyses

Cytogenetic and molecular analyses are important in identifying prognostic markers in ALL [5,6,27,28]. The study of cytogenetic abnormalities is the basis for unraveling molecular events that may be involved in the disease, such as the role of fusion transcripts that derive from translocations, tumor suppressor genes from deletions, or the control of cell cycle regulatory genes. There are some limitations associated with cytogenetic studies in ALL: the leukemic cells do not always produce good metaphases and important abnormalities can be missed. Reverse-transcriptase polymerase chain reaction (RT-PCR), DNA flow cytometry, fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH), among other techniques, have made it possible to detect more precisely the molecular and chromosomal defects in common subtypes of ALL. However, detection of chromosomal abnormalities by classic
karyotypic analysis or by molecular techniques has its advantages and inconveniences. Only through a karyotypic analysis can an overall evaluation of the whole genome be carried out and the results obtained can direct further investigations; on the contrary, molecular techniques allow detection of specific abnormalities in situations where karyotyping is difficult (e.g., insufficient metaphases or detection of submicroscopic abnormalities). The chromosomal abnormalities in ALL can be categorized as numerical in nature or structural, with or without numerical abnormalities. Hyperdiploidy is the gain of additional chromosomes so that the total number of chromosomes in a single cell exceeds 46; in ALL, this process seems to be nonrandom and two forms are usually distinguished: ALL with 47–51 chromosomes and ALL with 52 or more chromosomes. Hyperdiploidy is seen in 5–15% of cases of adult ALL and the association with a favorable outcome is less obvious than in childhood ALL. Hypodiploidy (chromosomes <46) is found in 2–8% of cases of ALL and is associated with a poorer outcome. The majority of chromosomal abnormalities found in ALL are structural, usually translocations. More than 30 different nonrandom translocations have been identified in ALL. Since only a relatively limited number of patients have so far been studied and many of these translocations are uncommon, the prognostic implications for most of them have still to be conclusively defined. Most of the more common karyotypic structural rearrangements have been studied at the molecular level. In molecular terms, chromosomal abnormalities or their submicroscopic equivalents are of two general types: those in which the breakpoint occurs within the involved genes, leading to the production of a fusion RNA transcript and a chimeric protein (qualitative change), and those which represent Ig/TCR rearrangement errors (quantitative change). Qualitative abnormalities are found to produce functional fusion genes; one of the most common is the t(9;22)(q34;q11) which forms the BCR-ABL fusion gene; another is t(1;19)(q23;p13), where the E2A gene fuses with PBX1. The rearrangement involving the MLL gene on chromosome 11 in the q23 region results in a fusion gene with AF4 on chromosome 4, band q21. Quantitative abnormalities result from Ig/TCR rearrangement errors which juxtapose the proto-oncogene to regulatory Ig/TCR sequences, leading to deregulated protein expression, for example the SIL-TAL1/tal deletions on chromosome 1p32 in T-ALL [29]. A list of the main molecular genetic abnormalities identified in ALL and currently used for molecular diagnosis is reported in Table 3, even if this list is not exhaustive and represents a compromise between the current most appropriate molecular method to detect or exclude an abnormality and the most widely used technique. Qualitative fusion transcripts predominate in B-lineage ALL and recombinant errors are rare; in contrast, they are much more frequent in T-ALL, where they represent the majority of molecular abnormalities. Identification of recurring cytogenetic abnormalities and molecular alterations in ALL has had a major impact on risk assessment and a number of structural and chromosomal changes have been incorporated into existing classification systems [5,6]. Within our multicenter GIMEMA ALL 0496 protocol, a central handling of biologic material at presentation is required for all registered cases. This has proven feasible and adequate metaphases could be obtained in over

<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene Involved</th>
<th>Abnormality</th>
<th>Incidence</th>
<th>Molecular detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-ALL</td>
<td>BCR ABL</td>
<td>t(9;22)(q34;q11)</td>
<td>Adults: 30%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Childern: 3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c-MYC IgH</td>
<td>t(8;14)(q24;q32)</td>
<td>1%</td>
<td>FISH</td>
</tr>
<tr>
<td></td>
<td>E2A PBX1</td>
<td>t(1;19)(q23;p13)</td>
<td>5%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>IL3 IgH</td>
<td>t(5;14)(q31;q32)</td>
<td>&lt;1%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>MLL AF1P</td>
<td>t(1;11)(p32;q23)</td>
<td>&lt;1%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>MLL AF4</td>
<td>t(4;11)(q21;q23)</td>
<td>&lt;1%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adolescents: 5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLL AF9</td>
<td>t(9;11)(q21;q23)</td>
<td>&lt;1%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>MLL ENL</td>
<td>t(11;19)(q23;p13)</td>
<td>&lt;1%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>TEL AML1</td>
<td>t(12;21)(p13;q22)</td>
<td>&lt;1%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>T-ALL</td>
<td>c-MYC TCRαδ</td>
<td>t(8;14)(q24;q11)</td>
<td>2%</td>
<td>FISH</td>
</tr>
<tr>
<td></td>
<td>HOX11 TCRαδ</td>
<td>t(10;14)(q24;q11)</td>
<td>5–10%</td>
<td>Southern</td>
</tr>
<tr>
<td></td>
<td>LMO1 TCRαδ</td>
<td>t(11;14)(p15;q11)</td>
<td>1%</td>
<td>Southern</td>
</tr>
<tr>
<td></td>
<td>LMO2 TCRαδ</td>
<td>t(11;14)(p13;q11)</td>
<td>5–10%</td>
<td>Southern</td>
</tr>
<tr>
<td></td>
<td>SIL TAL1</td>
<td>Normal 1p32</td>
<td>Adults: 10%</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>TAL1 TCRαδ</td>
<td>t(1;14)(p32;q11)</td>
<td>1–3%</td>
<td>Southern</td>
</tr>
<tr>
<td></td>
<td>TCL1 TCRαδ</td>
<td>inv(14)(q11;q32)</td>
<td>&lt;1%</td>
<td>FISH</td>
</tr>
</tbody>
</table>

Table 3. Main chromosomal abnormalities characterized at the molecular level in ALL.
70% of enrolled cases. The opportunity offered by this protocol to combine molecular and cytogenetic data in the framework of a therapeutic trial, has allowed an integrated molecular-cytogenetic classification to be proposed [30] which categorizes adult ALL cases into subgroups which are as homogeneous as possible based on defined genetic alterations and has also enabled a group of patients to be identified without known cytogenetic or molecular changes. The results obtained by this integrated classification have enhanced the importance of a broad genetic characterization of patients with ALL and offer a further biologic basis for stratified treatment approaches.

**Multidrug resistance**

Some investigators have studied MDR-1 gene expression in leukemic cells from ALL patients in an attempt to demonstrate a correlation with treatment response and/or patient follow-up. All studies reached the conclusion that the overexpression of the gene is probably partially implicated in the chemoresistance phenomenon; this resistance can be expressed at diagnosis and can also be acquired after treatment. Expression of MDR-1 at diagnosis has no effect on the probability of entering CR in pediatric ALL patients, but the CR rate in adult ALL appears significantly lower in MDR positive cases compared with MDR negative cases [31]. The MDR-1 gene encodes for a membrane P-glycoprotein p170 (P-gp) that acts as an adenosine triphosphate (ATP) dependent efflux pump. The expression of this gene confers resistance to some chemotherapeutic agents such as vinca alkaloids, anthracyclines, etc. However, the exact prognostic significance of this resistance mechanism is still unclear [32]. Several studies have found a correlation between high P-gp expression levels and/or P-gp function, and poor response to chemotherapy in AML [33] and, to a lesser extent, in ALL [31,34]. One of the main reasons for these contradictory results may be methodologic problems in P-gp detection. The methods currently used are: (a) measurement of P-gp function by efflux studies, (b) P-gp expression levels by MoAb, and (c) MDR-1 gene expression encoding for (P-gp) by RT-PCR. However, each of these methods has disadvantages. MDR-1 detection could thus represent a valuable biologic parameter in the diagnostic screening of ALL patients. The inclusion of this parameter may result in the design of biologically based risk adapted therapeutic strategies for the management of adult ALL. The adoption of protocols based on drugs that are not P-gp substrates may offer therapeutic advantages for CR achievement for ALL patients expressing the MDR-1 protein.

**Minimal residual disease**

One of the most important challenges in leukemia treatment is to accurately distinguish patients who require more intensive (and potentially more toxic) therapy from those in whom high cure rates can be achieved with less intensive therapy. MRD studies can provide a direct measurement of leukemic cell responses to chemotherapy. This information can be used to improve strategies of risk assessment and treatment selection in the management of ALL patients. Nevertheless, before using MRD data to guide therapy, further analysis is required to conclusively establish the predictive value of MRD findings. Leukemia cells can be potentially distinguished from normal hematopoietic progenitors on the basis of morphologic and cytochemical properties, immunophenotype, karyotypic or genetic abnormalities, and Ig/TCR gene rearrangements. These different characteristics have been exploited in an attempt to detect small numbers of blasts among normal cells and a variety of techniques have been developed for the detection of residual disease. The conventional criteria for remission in patients with acute leukemia are based on the morphologic examination of BM samples and patients are considered to be in CR when BM aspirates contain less than 5% blasts. At the time of morphologic CR, however, the extent of MRD varies considerably. The methods for MDR analysis include cytogenetics, FISH, Southern blotting, immunophenotype and PCR techniques. The applicability of these techniques for MRD detection depends on three parameters: (a) specificity to discriminate between malignant and normal cells without false positive results, (b) sensitivity limit of at least $10^{-3}$, and (c) reproducibility and applicability (easy standardization and rapid collection of results for clinic application) [8,9,35,36]. Only a proportion of leukemias have specific markers such as chromosomal translocations, e.g., t(9;22), t(4;11) or t(1;19), and conventional karyotypic analysis may be used to monitor MRD if an abnormal karyotype is present at diagnosis; however, its low specificity and the risk of analyzing metaphases from normal cells represent major obstacles in its routine use. The main advantage of FISH is that it provides interpretable information based on interphase cells with a low proliferative rate. Nonetheless, the sensitivity of FISH analysis for MRD monitoring is limited. Immunophenotyping techniques using multicolor-gated flow cytometry are based on the aberrant expression of antigens by the leukemic cell population and on the identification of markers that may be found on malignant cells in combinations that are normally not observed in normal BM and PB cells. For a productive detection of MRD in ALL, it is necessary to distinguish leukemic lymphoblasts from their normal counterparts and the intensity of expression may also help in distinguishing leukemic cells
from normal progenitors [15,37]. Some immunophenotypic combinations present on leukemic cells are confined to certain normal tissues and are not found in normal BM or PB cells. Overall, flow cytometry can be utilized to monitor MRD in about 85-90% of cases.

The detection of leukemia-associated clonal genetic changes at the karyotypic and genetic levels has been extensively tested by molecular biology techniques, based on PCR analysis. Two types of PCR targets can be used to detect MRD in ALL patients: leukemia-specific breakpoint fusion regions of chromosome rearrangements (translocations, deletions or inversions) or junctional regions of leukemia clone-specific rearranged Ig/TCR genes. The presence, at diagnosis, of one of these transcripts allows the monitoring of rearranged Ig/TCR genes. The presence, at diagnosis, of TCR gene rearrangements occur in 95% cases.

BCR-ABL MRD during the clinical follow-up (e.g., detection of one of these transcripts allows the monitoring of specific breakpoint fusion regions of chromosome 22. Correlative studies have demonstrated that distinct gene expression profiles could be found in each of the prognostically important leukemia subtypes, based on immunophenotypic and cytogenetic/molecular features [41]. In a study dedicated specifically to childhood T-ALL, microchip analysis could identify previously unrecognized molecular subtypes of T-ALL and associate the activation of particular oncogenes to defined stages of normal thymocyte development [42]. Hierarchical clustering of all adult ALL samples based on gene expression profile identified two well-defined groups which correlated precisely with the T- or B-cell immunophenotype of the leukemic cells. Further analysis identified gene expression profiles associated with the presence of either ALL1-AF4, BCR-ABL or E2A-PBX1 gene rearrangements. Furthermore, an integrated analysis of childhood and adult ALL highlights a strong similarity between cases which harbor specific rearrangement regardless of patient’s age [44].

With the use of these technologies, it has been shown that genetically defined subgroups express different sets of genes. In individual cases, the genetic lesion could be classified by microarray analysis, while being negative by RT-PCR [41]. Evidence has also been provided that the lineage affiliation of rare cases with unique phenotypic features may be clarified on the basis of the genomic profile [45]. Times are mature to verify whether these innovative technologies will change our approach to the characterization of leukemias. Should this be the case, it is foreseeable that in the near future all new cases will undergo a rapid gene chip analysis that may possibly substitute many of the analyses routinely carried out nowadays through the efforts of numerous laboratories. As our knowledge increases, it is also likely that ad hoc chips will be designed that contain a number of genes sufficient for an adequate classification of given diseases, rather than using the broad chips presently available. It appears realist to hypothesize that we are

Gene expression profiling

Genomic profiling is becoming a reality that may profoundly modify the management of ALL patients. Several studies have identified unique gene expression signatures characteristic of various hematologic and non-hematologic cancers [39,40]. The potential exploitation of microchip analysis is manifold: it can define the genetic signature of given neoplastic populations, it can help define the lineage affiliation of the tumor, it can identify sets of genes that characterize subsets of patients with distinct responses to treatment and, ultimately, have a prognostic impact, it may identify new targets for future therapies based on the under- or overexpression of given genes, it may allow definition of drug susceptibility or resistance, etc. These innovative technologies have been recently utilized in both childhood and adult ALL [41–44]. In pediatric ALL, it has been shown that distinct gene expression profiles could be found in each of the prognostically important leukemia subtypes, based on immunophenotypic and cytogenetic/molecular features [41]. In a study dedicated specifically to childhood T-ALL, microchip analysis could identify previously unrecognized molecular subtypes of T-ALL and associate the activation of particular oncogenes to defined stages of normal thymocyte development [42]. Hierarchical clustering of all adult ALL samples based on gene expression profile identified two well-defined groups which correlated precisely with the T- or B-cell immunophenotype of the leukemic cells. Further analysis identified gene expression profiles associated with the presence of either ALL1-AF4, BCR-ABL or E2A-PBX1 gene rearrangements. Furthermore, an integrated analysis of childhood and adult ALL highlights a strong similarity between cases which harbor specific rearrangement regardless of patient’s age [44].

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in the verge of a time when subgroups of patients will be classified on the basis of the genomic profile and that the latter will direct the therapeutic strategy in terms of both drug decisions and treatment aggressiveness. Finally, in the very near future, great attention will be paid to the identification of new therapies aimed at targeting the specific regulatory pathways operational in the different leukemia subtypes.

Conclusions

ALL are classified on the basis of the presumed cell of origin and do not represent a single disease but rather a heterogeneous collection of diseases with different genetic profiles and differences in clinical progression, treatment and outcome. Following a diagnostic work-up, prognostic data are routinely achieved through physical examination, serum biochemical profiles, peripheral blood count and bone marrow morphology. Over the years, information obtained through karyotype, molecular genetics, extensive immunophenotype, multidrug resistance and, more recently, genomic profiling is progressively contributing to a better understanding of the biology of this complex disease, to the identification of subgroups of patients with a different clinical outcome, to the more precise monitoring of MRD, to the use of different therapeutic protocols based on prognostic indicators and, recently, also to the design of innovative and specific treatment strategies.

References

STEM CELL TRANSPLANTATION

Current issues in allogeneic stem cell transplantation

GÉRARD SOCIÉ

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Abstract

Hematopoietic stem cell transplantation (SCT) has evolved as a central treatment modality in the management of different hematological malignancies. However, 2 major issues still impact overall mortality and morbidity post transplant.

1. Despite adequate post-transplantation immunosuppressive therapy, acute graft-versus-host disease (GvHD) remains a major cause of morbidity and mortality in the hematopoietic stem cell transplantation setting, even in patients who receive human HLA identical sibling grafts. Up to 30% of the recipients of stem cells or bone marrow transplantation from HLA-identical related donors and most patients who receive cells from other sources (matched-unrelated, non-HLA-identical siblings, cord blood) will develop Grade II or more acute GvHD despite immunosuppressive prophylaxis. Thus, GVHD continues to be a major limitation to successful hematopoietic stem cell transplantation.

2. With improvements in outcome, an increasing number of patients survive free of the disease for which they were treated. Today, about 60% of patients will survive 5 years after diagnosis. Therefore, immediate survival is no longer the sole concern. The aim of the allogeneic SCT now is to cure a patient’s underlying disease and, at the same time, to minimize the incidence of post-treatment complications and ensure the best possible long term quality of life. The long time span between initial therapy and late effects, the multiple factors influencing cancer related health risk and the unknown effect of treatment on aging are common characteristics of late effects. While the treatment strategy of a cancer patient depends widely on the type and extension of the disease, considerations for a long term survivor depend much more on the type of treatment applied, age of the patient, its general health status, as well as his familial and social integration.

I will discuss, based on the most recent knowledge, advances and current issues in GvHD and some typical examples of late effects in survivors, and the practical recommendations that could assist practitioner and patient decision about appropriate healthcare for specific clinical circumstances.
Besides chemotherapy, immunotherapy represents an approach based on the more or less specific recognition of tumoral cells. Allogeneic stem cell transplantation (ASCT) has been used now for more than 3 decades in the treatment of malignant hemopathies. Several lines of evidence could show that donor-derived immuno-competent effectors can exert a potent long lasting graft-versus-tumor activity. Today, this approach represents the most efficient and the most widely form of immunotherapy used worldwide.

However allogeneic transplantation suffers from major weakness: Despite a common HLA identity between matched donor and recipient, the allogeneic recognition of some normal tissues, expressing epithelial antigens, is associated with a high rate of morbidity and mortality. Usually, the indications of allogeneic transplantation are still limited to the youngest patients (age under 45 or 50). Because of its high toxicity, ASCT has also been limited for years to diseases for which a potent antitumoral effect had been documented in the early years. Interestingly, the world experience of this approach in malignant diseases relies mainly on patients with leukemia (chronic or acute) and younger than 50 years which represent only a part of the wide spectrum of cancer population.

Given the strength and curative potential of allogeneic transplantation, new strategies aiming to widen the use of this approach to other cancers, were urgently needed. On these bases, we and others started by the end of the 90s to develop new approaches that would allow overcoming the above cited limitations: The first step has been the switch from bone marrow to peripheral blood stem cells (PBSC) as source of graft [1]. We established that PBSC transplantation is associated with a quicker hematological recovery, lower transplant-related cost and identical incidence acute GVHD in comparison to bone marrow, despite a higher number of immuno-competent cells infused [1]. However chronic GVHD appears to be more frequent and severe [1,2]. The second step considered the preparative or so-called conditioning regimen. While it has been postulated for many years that transplant conditioning has to be myeloablative to allow long-term engraftment of allogeneic cells, different investigators succeeded to perform allogeneic transplantation after a non-myeloablative (NMA) or reduced intensity regimen (RIC) [Storb, 1997 #545] [3,4]. Early after the first reports, we started to develop our own strategy using a combination of fludarabine, low dose busulfan and anti-thymocyte globulin (ATG), as initially described by Slavin et al. Our initial work was focused on the optimization aspects [5,6]. We have now in hands a well defined platform allowing rapid engraftment, lower supportive care [7], low transplant related-infections [8] and mortality while preserving a high anti-tumor effect [5]. Simultaneously, we investigated the applicability of this strategy to new populations of cancer patients. We could confirm the feasibility in older populations essentially between 50 and 55 years [5], and participated to the demonstration of its activity in non-leukemic malignancies such as multiple myeloma (MM) [9], ovarian carcinoma [10] and different other metastatic solid tumors [11]. These clinical achievements leaded us to some important biological observations related to post-graft anti-infectious activity [8], some aspects of the GVHD reaction [12] and to study post graft immune reconstitution [13–15].

Presently, with the dramatic reduction of transplant-related mortality, ASCT has probably entered the era of global immunotherapy applicable to the largest number of patients. Despite these tremendous...
advances achieved in less than 5 years, further investigations are urgently needed in 2 major directions:

- Anti-tumor effect exists but tumoral escape is still the major cause of failure of the procedure in patients with advanced diseases: further work and novel complementary approaches are necessary to further enhance the allogeneic anti-tumor effect.
- Procedure-related mortality has decreased notably but still exists: optimization of the procedure is still to be pursued. This is notably the case in the oldest patients.

References

ADVANCES IN MOLECULAR HEMATOLOGY

Split-signal FISH for detection of chromosome aberrations

J. J. M. VAN DONGEN, M. VAN DER BURG, & A. W. LANGERAK

Department of Immunology, Erasmus MC, Rotterdam, The Netherlands

Abstract
Chromosome aberrations are frequently observed in hematopoietic malignancies. These aberrations can deregulate expression of an oncogene, resulting in aberrant expression or overexpression, or they can form leukemia-specific chimeric fusion proteins. Detection of chromosome aberrations is an important tool for classification of the malignancy and for the definition of risk groups, which need different treatment protocols. We developed rapid and sensitive split-signal fluorescent in situ hybridization (FISH) assays for frequently occurring chromosome aberrations. The split-signal FISH approach uses two differentially labeled probes, located in one gene at opposite sites of the breakpoint region. In normal karyotypes, two co-localized green/red signals are visible, but a translocation results in a split of one of the co-localized signals. Split-signal FISH has three main advantages over the classical fusion-signal FISH approach, which uses of two labeled probes located in two genes. First, the detection of a chromosome aberration is independent of the involved partner gene. Second, split-signal FISH allows the identification of the partner gene or chromosome region if metaphase spreads are present, and finally it reduces false-positivity.

Keywords: Fluorescence in situ hybridization (FISH), split-signal FISH, chromosome aberrations, fusion gene, peptide nucleic acid, leukemia, lymphoma

Chromosome aberrations in hematopoietic malignancies
Chromosome aberrations play an important role in hematological malignancies [1]. Most of these aberrations concern balanced translocations involving genes that play key roles in the development and function of hematopoietic cells, such as transcription factors, cell cycle regulators, and signal transduction molecules. Chromosome translocations can result in deregulated expression of (onco)genes as a direct consequence of a translocation to a regulatory element, e.g., an immunoglobulin (Ig) or T-cell receptor (TCR) enhancer [2,3]. Ig and TCR-gene related chromosome aberrations are particularly found in mature B-cell malignancies, such as various types of B-cell non-Hodgkin lymphomas (B-NHL), and in immature T-cell malignancies, mainly T-cell acute lymphoblastic leukemias (T-ALL). Alternatively, translocations can result in fusion of two genes that encode leukemia-specific chimeric (fusion) proteins. Fusion genes with expression of aberrant fusion proteins are particularly found in precursor-B-ALL, acute myeloid leukemias (AML), and chronic myeloid leukemia (CML). The fusion proteins have functional features that differ from the corresponding wild type proteins and mostly play a role in oncogenesis. In addition to the new features of the fusion protein, loss of wild type activity due to the translocation (in some translocations enhanced by deletion of the second allele) might contribute to oncogenesis.

Several clinical studies have demonstrated that chromosomal translocations are useful markers contributing to classification of the malignancies and to the definition of risk groups, that need different treatment protocols. In precursor-B-ALL MLL gene translocations and t(9;22) with the BCR-ABL fusion gene are associated with a poor prognosis, while t(12;21) with the TEL-AML1 fusion gene is associated with a good outcome. Analogously, in AML inv(16) with CBFB-MYH11, t(8;21) with AML1-ETO, and t(15;17) with PML-RARA are associated...
with good prognosis, while 11q32 (MLL gene) aberrations show poor outcome in AML as well [1].

### Detection of chromosome aberrations

Several techniques can be used for the detection of chromosome aberrations, each having its inherent advantages and disadvantages (Table I). An advantage of conventional karyotyping is that it is highly informative as virtually all abnormalities can be detected. This includes not only structural abnormalities, but also numerical abnormalities such as hypo- or hyperploidy. However, the interpretation may be difficult if the karyotype is complex. Another disadvantage is that for some samples no reliable results can be obtained because of a low mitotic index or poor chromosome morphology. In addition, some chromosome abnormalities are cryptic, i.e. they cannot be identified via conventional karyotyping, because changes in chromosome banding patterns are too marginal to be detected, such as t(12;21), t(5;14) and SIL-TAL1 fusions [4].

Chromosome aberrations can also be identified via Southern blot or PCR analysis on genomic DNA. Southern blotting is considered to be technically demanding and laborious and the applicability to the detection of chromosome translocations is limited, because the breakpoints in many translocations are scattered over large regions (>25kb). Nevertheless, Southern blot analysis has proven to be useful for detection of MLL translocations. As the MLL gene can have many translocation partner genes and the breakpoint region is relatively small (6.5 kb), Southern blot analysis is suitable for the detection of MLL rearrangements, independent of the partner gene [5,6]. Southern blotting has also been used for detection of E2A (ETV6) gene rearrangements, since the majority of E2A (ETV6) breakpoints are located in a breakpoint region of 15 kb [7].

**PCR analysis on the DNA level** is relatively easy for detection of SIL-TAL1 fusion genes [8,9]. but much more complex for other translocations, mainly because PCR analysis needs multiple primers, if genomic breakpoint regions are larger than 2 to 4 kb [10–12].

An alternative approach, which is suitable for detection of chromosome translocations resulting in formation of fusion genes, is detection of fusion genes or fusion gene transcripts via (nested) RT-PCR analysis [13]. The advantage of this approach is that it reaches sensitivities of 1 cell in $10^4$ to 1 cell in $10^6$ cells, enabling detection of minimal residual disease.

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**Table I. Techniques for detection of chromosome aberrations**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyotyping</td>
<td>Detection of virtually all abnormalities (structural and numerical abnormalities)</td>
<td>Interpretation may be difficult if the karyotype is complex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sometimes low mitotic index or poor chromosome morphology</td>
</tr>
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<td></td>
<td></td>
<td>Some chromosome abnormalities are cryptic</td>
</tr>
<tr>
<td>Southern blotting</td>
<td>Detection of chromosome aberration independent of partner gene/chromosome region</td>
<td>Technically demanding and laborious</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Detection of chromosome aberrations is limited, if breakpoints are scattered over large regions (&gt;25 kb)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Focused on a specific type of aberration, determined by the probe</td>
</tr>
<tr>
<td>PCR</td>
<td>Can be easy for some chromosome aberrations e.g. SIL-TAL1 gene fusion</td>
<td>Detection of chromosome translocations is limited, if breakpoints are scattered over large regions (2 to 4 kb)</td>
</tr>
<tr>
<td></td>
<td>Sensitive, allowing detection of minimal residual disease</td>
<td>Variant translocations can easily be missed, if these variants are not covered by the used primers</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Suitable for detection of chromosome aberrations with formation of fusion genes</td>
<td>Sensitive, allowing detection of for minimal residual disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Variant translocations can easily be missed, if these variants are not covered by the used primers</td>
</tr>
<tr>
<td>FISH</td>
<td>Dividing cells (metaphase nuclei) and non-dividing cells (interphase nuclei) can be analyzed</td>
<td>Focused on a specific type of aberration, determined by the applied probe set</td>
</tr>
<tr>
<td>general</td>
<td>Detection of cryptic aberrations</td>
<td>Translocations with other partner genes are missed</td>
</tr>
<tr>
<td>fusion-signal</td>
<td>Detection of the fusion of two partner genes involved in the translocation</td>
<td>5–10% false-positivity</td>
</tr>
<tr>
<td>split-signal</td>
<td>Detection of chromosome aberration independent of partner gene/chromosome region</td>
<td>Partner gene/chromosome region can not be identified if metaphase spreads are absent</td>
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</tbody>
</table>
<p>Abbreviations: MCL, mantle cell lymphoma; FCL, follicular lymphoma; DLBL, diffuse large B-cell lymphoma; ALCL, anaplastic large cell lymphoma; B-NHL, B-cell non-Hodgkin lymphoma; MALT, mucosa-associated lymphoid tissue; LPL, lymphoplasmacytic lymphoma (immunocytoma).

Fusion-signal FISH versus split-signal FISH

There are two main approaches of FISH probe design for use on (interphase) nuclei, i.e. fusion-signal FISH and split-signal FISH [18]. The classical fusion-signal FISH approach uses two differentially labeled probes, red and green, which flank the breakpoint regions of the two genes, which are involved in the translocation (Figure 1A). In normal karyotypes, i.e. without chromosome aberration, two red signals and two green signals are detectable. In case of a translocation, a red and a green signal will be juxtaposed giving rise to a co-localized green/red signal, which will generally appear as a yellow signal. In addition, separate green and red signals of the unaffected chromosomes will be visible.

The split-signal FISH approach also uses two differentially labeled probes, but these probes are located in only one of the two involved genes, hereafter called the target gene, and are positioned at opposite sides of the breakpoint region of the target gene (Figure 1B) [19]. In normal karyotypes, i.e. without chromosome aberration, two red signals and two green signals of the unaffected chromosomes will be visible. A translocation will result in a split of one of the co-localized signals, resulting in a separate green and red signal together with a fused signal of the unaffected chromosome [19,20].

The split-signal FISH approach has several advantages over the more traditional fusion-signal FISH. First, the detection of a translocation is independent of the involved partner gene. This is particularly of great interest for target genes with multiple partner genes such as MLL and E2A (ETV6) (Figure 2) [19]. Although the detection is independent of the involved partner gene or partner chromosome, split-signal FISH in principle allows the identification of the partner chromosome, if metaphase spreads are present on the slide. As a result of the translocation, one</p>
of the probes moves to the partner chromosome, i.e. der(partner), while the other probe remains on the der(target) chromosome. The split-signal approach therefore also allows the detection of new partner chromosomes or chromosome regions. Further molecular analysis can then be performed to identify the new partner gene, such as panhandle PCR or long distance inverse PCR [21,22].

Another advantage of split-signal FISH is absence of the traditionally high levels of false-positivity as observed via the fusion-signal FISH approach, which range between 5 and 10%. False-positivity occurs as a result of coincidental co-localization of two signals, which actually represent two separate signals in a three-dimensional nucleus, but due to the two-dimensional analysis of the nucleus, are visible as a single co-localized signal. On the other hand, one could argue that split-signal FISH can give rise to low frequencies of false-negativity due to the same type of coincidental co-localization of two separate signals making these cells indistinguishable from normal nuclei. However, 5–10% false-negativity (percentage deduced from fusion-signal FISH) within the leukemic cell population will not alter the result in diagnostic material where the percentage of malignant cells is virtually always over 25%. Consequently, 10% reduction from 25% to 22.5% has no diagnostic meaning [18].

Reduction of background staining using PNA-based blocking

The successful use of large genomic probes for FISH is dependent on blocking of the undesired background staining derived from repetitive sequences present throughout the human genome. The finishing of the human genome project has shown that a large proportion of the human genome is comprised of tandem repeated sequences (i.e. arranged in blocks) and interspersed tandem repeated sequences (distributed all around the genome).
Previously, heat denaturation and reannealing studies on DNA of higher organisms have distinguished three populations of genomic DNA: a slowly reannealing component (45% of the total DNA) containing unique sequences of protein-encoding genes, and intermediate and quickly reannealing components (30% and 25% of the total DNA, respectively) representing repetitive sequences [23]. The fast component contains small (a few nucleotides long), highly repetitive DNA sequences, while the intermediate component contains the interspersed repetitive DNA that can be classified as either SINEs (Short Interspersed Nuclear Elements), LINEs (Long Interspersed Nuclear Elements) or LTRs (Long Terminal Repeats) [24–27]. The repetitive units of the intermediate reannealing component the major reason that large genomic nucleic acid probes are not well suited for hybridization analysis without blocking the repetitive elements to prevent undesired staining.

Blocking of repetitive sequences can be achieved using a component of the total DNA, Cot-1 DNA, enriched with repetitive sequences [28]. Recently, a novel method has been developed based on selection of specific Peptide Nucleic Acid (PNA) oligos, directed against the Alu sequences, which is the most frequent repetitive element within and around genes. PNA is a DNA analogue in which the deoxyribose phosphodiester backbone is replaced by a pseudo-peptide backbone of N-(2-aminoethyl)-glycine units to which the nucleobases are attached through a methylene carbonyl linker (Figure 3) [29,30]. The charge of the pseudo-peptide backbone of PNA is neutral, whereas the charge of the deoxyribose phosphodiester backbone of DNA is negative. Because of lower electrostatic repulsion, a PNA-DNA interaction occurs faster and is stronger than a DNA-DNA interaction [31]. Different PNA oligos were selected in such a way that they cover both the upper and lower strand of the repetitive sequences and could therefore be used as a blocking reagent [32].

This novel PNA-based method for suppression of background staining is now included in our FISH procedure (DakoCytomation, Glostrup, DK, EU). A paraformaldehyde pre-treatment is used to improve the brightness of the fluorescence signals. The pre-mixed ALL probe sets contain PNA oligos and the fluorescently labeled DNA probes, and are denaturated together with the target DNA before hybridization in a humified environment overnight. Excess of
probe and PNA oligos is removed by washing under stringent condition, before embedding and examination of the hybridization area (Figure 4).

Concluding remarks

Split-signal FISH probe sets each consists of two differentially labeled probes (generally composed of several BAC/PAC clones), which are located in the target gene at opposite sides of the breakpoint region [17–20,33]. Directly labeled FISH probes work smoothly in combination with the newly developed PNA-blocking system, which allows combined blocking and hybridization in a single step. This single-step hybridization procedure makes split-signal FISH an easy, rapid, and sensitive tool for molecular cytogenetics (Figure 4).

The split-signal FISH approach has three major advantage over fusion-signal FISH. First, translocations involving the target gene can be detected independent of the involved partner gene. Second, split-signal FISH allows identification of the partner gene or partner chromosome region, if metaphases are present. The third advantage is the absence of high levels of false-positivity due to coincidental co-localization, as observed in the traditional fusion-signal FISH approach. One could argue that split-signal FISH can give rise to similar frequencies of false-negativity due to the same type of coincidental co-localization, but 5–10% false-negativity as deduced from fusion-signal FISH within the leukemic cell population will not alter the result in diagnostic material where the percentage of malignant cells is virtually always >10%.

![Figure 4. Protocol for FISH with PNA-based suppression of background staining. Slides with tissue or cytology preparation are pre-treated to increase the access of target DNA for the labeled probes. The probe mixture containing PNA oligos and fluorescent labelled DNA probes is applied to the target DNA and co-denaturated, before hybridization. Unspecifically bound probe is removed by washing before the slide is scored with a fluorescent microscope. Normal cells present on the slides serve as control cells.](image-url)
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ADVANCES IN MOLECULAR HEMATOLOGY

Regulation and dysregulation of hematopoiesis by a cytokine-induced antiapoptotic molecule anamorsin

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Many growth factors and cytokines have a crucial role in hematopoiesis, and the cytokine-receptor system is known to involve in normal and abnormal hematopoiesis. Upon binding to receptors, cytokines initially induce activation of cell surface tyrosine kinases that transmit mitogenic and anti-apoptotic signals through simultaneous activation of downstream signaling molecules including Ras/MAPK, PI3-K/Akt, and STATs. However, it is not fully understood yet how cytokines mediate anti-apoptotic effects. To define molecule(s) that mediates anti-apoptotic effect of cytokine and confer resistance to apoptosis induced by cytokine deprivation, we have established a subline named Ba/F3-Ad that can grow and survive under IL-3-deprived conditions from a murine IL-3-dependent cell line, Ba/F3. In order to identify the molecule(s) that conferred the resistance to factor-deprived apoptosis on Ba/F3, we performed the expression cloning by constructing a retroviral cDNA library from IL-3-starved Ba/F3-Ad. In short, we infected the retrovirus library into parental Ba/F3 (5×10^5 clones, the average size of the inserts, 1.3 kb; the infection efficacy was estimated at about 35%), and screened the clones that can survive under IL-3-deprived condition. After isolation of several clones that can survive under IL-3-deprived condition, we isolated the integrated cDNA from genomic DNA extracted from one clone by the PCR method.

By sequencing the integrated cDNA, we found that the coding region of murine AM cDNA consists of 930 bp. Comparison with the DNA data base search revealed that the sequence of AM does not exhibit homology with any known anti-apoptotic molecules, including Bcl-2 family proteins, caspase inhibitors, or signal transduction molecules. Also, we found a human homologue of AM in EMBL/GenBank data libraries, which revealed 82.6% similarity to murine AM at a DNA level and 81.9% similarity at an amino acid level. Human homologue of AM was originally found by Lotfus et al. as a molecule with unknown function on chromosome 16. AM encodes an about 37-kDa protein and the protein sequence data base indicates that AM had generic methyltransferase motif around amino acids 60–99.

At first, we examined whether AM was indeed involved in the resistance to factor-deprived apoptosis of Ba/F3-Ad. For this purpose, we stably expressed murine AM cDNA in parental Ba/F3 cells, and investigated their sensitivities to IL-3-deprived apoptosis. In control Ba/F3 cells, the subdiploid fraction formed from apoptotic cells emerged as early as 24 h after IL-3 depletion, which was effectively reduced in AM-transfected Ba/F3 cells (% of apoptotic fraction: control Ba/F3, 25% at 24 h, 57% at 36 h, 87% at 48 h; AM-transfected Ba/F3, 3% at 24 h, 4% at 36 h, 12% at 48 h). In agreement with this finding, the activation of caspase-3, which is detected as a conversion of left-sided peak to right-sided peak in fluorescent intensity, was significantly suppressed in AM-transfected Ba/F3 cells as compared to that in control Ba/F3 cells. However, it should be noted that AM alone could not support the growth of Ba/F3 cells, since proliferating cells drastically decreased in AM-transfected Ba/F3 cells under IL-3-deprived condition (% of the cells in S-G2/M phase before and after IL-3 depletion: 68% at 0 h vs. 10% at 48 h). We also confirmed that AM can confer resistance to apoptosis caused by IL-3 depletion in another murine IL-3-dependent cell line, 32D.
Next, we examined the expression profile of human AM homologue in various organs using MTA panels. AM was expressed ubiquitously in various tissues, especially with high expression levels in heart, liver and pancreas. As for hematopoietic tissues, it was abundantly expressed in fetal liver and spleen. Also, we found that the expression of AM was detectable from the early stages (7 day) of embryogenesis by the PCR method.

Since AM was supposed to exert anti-apoptotic effects in Ba/F3-Ad under IL-3-deprived condition, we examined its expression levels in parental Ba/F3 and Ba/F3-Ad after IL-3 depletion. As expected, AM was still expressed in Ba/F3-Ad after 18-h IL-3 deprivation, while its expression was hardly detectable in parental Ba/F3 cells. However, the addition of IL-3 recovered it expression in parental Ba/F3 cells from after 3 h. We next investigated the expression of AM was also regulated by other cytokines in Ba/F3 cells. After IL-3 depletion, clones of Ba/F3 each expressing the receptors for EPO, SCF (c-Kit), and TPO (c-Mpl) were cultured with the corresponding cytokines for the time indicated. EPO, SCF, and TPO individually induced the expression of AM as efficiently as IL-3, suggesting that the expression of AM would be regulated by cytokines(s) shared by various cytokines. Based on the fact that Ras is activated by various cytokines and was constitutively activated in Ba/F3-Ad, we speculated that Ras might control the expression of AM in Ba/F3 cells. To examine this possibility, we stably expressed oncoprotein H-ras (H-rasG12V) in Ba/F3 cells. Also, we prepared the clone from Ba/F3, in which dominant negative H-ras (H-rasS17N) was inducibly expressed by the IPTG treatment. In Ba/F3 cells transfected with H-rasG12V, the expression of AM was maintained after IL-3 depletion for up to 36 h, while its expression declined to an undetectable level in the mock clone transfected with an empty vector. Furthermore, the induced expression of H-rasS17N led to the reduction of AM expression even in the presence of IL-3. Collectively, these results suggest that the expression of AM is completely dependent on cytokine stimulation, and, at least partially, regulated by Ras signaling in Ba/F3 cells. Next, we investigated the intracellular localization of AM by immunofluorescence staining with an anti-AM monoclonal antibody (mAb). AM was exclusively localized to the cytoplasm in Ba/F3 cells irrespective of the stimulation with IL-3.

To assess in vivo roles of AM, we tried to generate AM null (AM−/−) mice. We constructed a targeting vector, in which the first exon was replaced by the neomycin-resistant cassette (a positive selection marker). The vector also included the diphtheria toxin as a negative selection marker. The targeting vector was introduced into an ES cell line R1 by electroporation, and the transfected cells were cultured with 150 µg/ml of G418. We confirmed the homologous recombination in the selected ES cell lines with Southern blot and PCR analyses. The wild type allele was detected as a 5-kb SacI fragment in Southern blot analysis with a 5’ flanking probe, while the targeted allele was detected as a 13-kb SacI fragment. Also, in PCR analyses, the 310-bp fragment was amplified from the wild type allelic genomic DNA with a primer pair 5’/3’ or the 730-bp fragment was amplified from the targeted allelic genomic DNA with a primer pair 5’/3’. To generate chimeras, we injected three ES cell lines, in which the homologous recombination was confirmed, into blastocysts of C57BL/6J mice. Then, male mice with a high degree of chimerism were crossed with C57BL/6J females to generate AM+/− mice. Genotyping was performed by Southern blot and PCR analyses using tail- and embryo-derived DNA. Finally, we confirmed that the expression of AM protein in the limb was partially reduced in AM+/− embryos and completely lost in AM−/− embryos by Western blot analysis using an anti-AM mAb.

Genotypic analysis of embryos from AM+/− mice intercrosses revealed that AM−/− embryos started to die between E12.5 and E14.5; the rate of dead AM−/− embryos increased after E14.5 (0% until E12.5, 18.8% at E14.5, 36.4% at E16.5, and 44.4% at E18.5); and all AM−/− mice died at birth, suggesting that AM−/− mice expire in late gestation. AM−/− embryos were apparently smaller in body size than AM+/+ embryos, while AM+/− embryos displayed phenotypes similar to AM+/+ embryos. Despite no significant difference in the formation of blood islands in yolk sac, the fetal liver (FL) and spleen of AM−/− embryos were remarkably smaller than those of AM+/+ embryos; the size of FL of AM−/− embryos was almost one third of that of AM+/+ embryos, and the spleen of AM−/− embryos appeared scar. Furthermore, AM−/− embryos later than E14.5 showed anemic; AM−/− embryos at E18.5 showed half values of red blood cells (RBC), hemoglobin (Hb) and hematocrit (Ht) in the peripheral blood (RBC: 365±50.8, 335.5±50.6 and 210±67.0×10⁴/mm³; Hb: 11.7±1.5, 10.6±1.3 and 6.7±2.3 g/dl; Ht: 39.7±5.8, 37.8±5.3 and 26.3±7.6% in AM+/+ (n=10), AM+/− (n=22) and AM−/− (n=8) embryos, respectively). Moreover, RBC of AM−/− embryos were macrocytic (mean corpuscular volume in AM+/+, AM+/− and AM−/− embryos were 108.8±4.3, 113.1±9.5 and 128.2±16.6, respectively). In addition to the defect in hematopoietic organs, heart walls of AM−/− embryos were thinner than those in AM+/+ or AM+/− embryos, whereas AM−/− embryos displayed no apparent macroscopic or histological abnormalities in other organs.

In order to clarify the mechanism of anemia seen in AM−/− embryos, we analyzed the FL, the main
hematopoietic organ at late embryonic days. The number of morphologically identifiable small-sized erythroid cells (erythroblasts) with condensed chromatin nucleus was apparently reduced in FL of AM−/− embryos compared to that of controls. Furthermore, erythroblasts were larger in AM−/− embryos than in AM+/+ embryos, and more mature erythroblasts (i.e., polychromatic and orthochromatic erythroblasts) markedly decreased in AM−/− embryos. In addition, TUNEL assays showed that a substantial fraction of FL cells led to apoptosis at E14.5 in AM−/− embryos, while it was hardly detected in AM+/+ embryos. To determine which type of cells undergo apoptosis, we performed flow cytometric analysis using AM−/− and AM−/− FL cells at E14.5. Although Annexin V-positive apoptotic cells were scarcely (only 0.5%) detected in AM+/+ FL cells, 60.2% of the isolated cells were positive for Annexin V in AM−/− FL. Furthermore, most importantly, almost all of these apoptotic cells were Ter-119-positive erythroid cells but not Ter-119-negative cells mainly composed from hepatocytes. Since neither the absolute number of hematopoietic stem/progenitor cells (CD34+ c-kit+ or CD34 low CD44 high) nor that of very immature proerythroblasts (Ter-119+c-kit+) did not decrease in AM−/− FL, it was speculated that immature hematopoietic cells of AM−/− mice may succumb to apoptosis, and fail to attain terminal differentiation.

We next examined whether hematopoietic stem/progenitor cells of AM−/− FL cells could survive, proliferate and differentiate in response to cytokines in vitro. When E14.5 AM+/+, AM+/− or AM−/− FL cells were cultured in methylcellulose with the combination of cytokines SCF, IL-3, IL-6 and EPO, the number of myeloid (granulocyte/macrophage and granulocyte) colonies formed from AM−/− FL cells was one third to one fourth of those by AM+/+ FL cells. Notably, AM−/− FL cells gave rise to little or no mix (myeloid/erythroid) and erythroid colonies, while these colonies did develop from AM+/− and AM+/+ FL cells. Furthermore, BFU-E colony did not develop when E14.5 AM−/− FL cells were cultured with SCF and EPO (Fig. 5E). These results suggest that AM is indispensable for cytokine-dependent survival and growth of hematopoietic stem/progenitor cells in vitro, especially with erythroid lineage.

To characterize the anti-apoptotic function of AM, we compared the gene expression profiles between AM−/− and AM+/+ FL cells at E14.5 using a cDNA microarray. Among 4289 genes, including Bcl-2 family, caspases, cytokines and signal transduction molecules, 184 genes were significantly down-regulated and 40 were up-regulated in AM−/− FL. Concerning apoptosis related genes, Bcl-xL and Jak2 were down-regulated most significantly. We also confirmed that their expression was decreased in AM−/− FL cells compared with AM+/+ FL cells by semiquantative RT-PCR assays. Furthermore, AM was found to express in a part of leukemia and malignant lymphoma cells at a significantly high level.

These results suggest that AM may play a crucial role in hematopoiesis through mediating anti-apoptotic effects of various cytokines, and also that AM may contribute to abnormal growth of leukemia/lymphoma cells.
WT1 overexpression: A clinically useful marker in acute and chronic myeloid leukemias

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Monitoring of acute leukemia patients during and after treatment for the presence of remaining leukemic cells minimal residual disease (MRD) have been shown to give major insight into the effectiveness of treatment. However, so far applicability of this strategy has been limited to those leukaemia subsets characterized by genetic markers amenable to sensitive detection by PCR. Although PCR for immunoglobulin and T-cell receptor gene rearrangement represents the gold standard for MRD detection in most cases of acute lymphoblastic leukemias (ALL) lacking the availability of fusion gene transcripts as molecular markers, the situation in AML is more complicated because, at present, more than 50% of them lack any sort of clonality markers suitable for MRD monitoring. Thus, a number of studies have been performed in the attempt to identify cytogenetic and molecular abnormalities associated with leukemic transformation.

The Wilms Tumor Gene (WT1) represents a molecular marker for the detection of the leukemic clone useful for monitoring the presence of leukemic cells in all the patients affected by acute and chronic leukemias as well as myelodysplastic syndromes. The WT1 gene, cloned in 1990 by Call et al. [1] encodes for a protein with the characteristics of a zinc finger transcription factor. WT1 expression is restricted to a small number of tissues [2] including testis, ovaries, myometrium, stromal cells of the uterus, heart, lung, intestine, liver and in the supportive stroma and splenic capsule of the spleen [2]. In contrast, several other tissues and cell lines were negative for WT1 expression. Although the role of the WT1 gene in the development of malignancies in the kidney appears quite well defined, currently its potential function in human hematopoiesis still needs to be clarified. The role of WT1 in the leukemogenesis process appears controversial. The majority of human acute myeloid and lymphoblastic leukemias express high levels of wild type WT1, [3,4] suggesting that this tumor suppressor might have paradoxical oncocentric activity in the hematopoietic cells. Although the potential usefulness of WT1 expression as a panleukemic marker was envisaged by Inoue et al. [5] several years ago, its introduction in the clinical practice was limited principally by the background expression level normally detected in bone marrow (BM) samples from healthy volunteers. More recent data obtained using quantitative RT-PCR methods established that sorted populations of normal progenitors express WT1 at very low levels, sometimes even undetectable by very sensitive methods of nested RT-PCR [6–9].

The introduction of a precise method of quantitative PCR allow to distinguish the levels in normal controls and in leukemic samples, so overcoming the obstacle represented by the low amount of WT1 transcript present in normal hematopoiesis. Using a Real Time quantitative PCR, we were able to demonstrated the presence of high levels of WT1 in the majority of cases of acute myeloid and lymphoblastic leukemias at onset of disease as well as in the different phases of chronic myeloid leukemias (ML) [5]. In addition also the patients affected by myelodysplastic syndromes express increased amount of WT1 transcript with variable level according to the subtypes of MDS [10].

By analyzing a large number of normal BM and peripheral blood (PB) samples we also established that the majority of the PB samples score negative and the median number of WT1 copies detected in the
positive samples is very low. By contrast, all the normal BM samples score positive, but, even if higher than in PB samples, the median number of WT1 copies remains low. To gain a further insight into the theory which attributes the abnormal levels of WT1 expression to the presence of cells with a high degree of immaturity, we tested WT1 expression in regenerating BM samples obtained from AML patients in complete remission during recovery from chemotherapy-induced aplasia. The median value of WT1 was similar to that detected in normal BM. Similar results were obtained by analyzing several samples of enriched CD34-positive cells obtained from normal PB cell donors. These data confirm that the CD34-positive cell compartment is not responsible for the increased WT1 expression detected in leukemias. These results lead to the conclusion that the WT1 overexpression found in leukemic samples is not due to the degree of immaturity of the cell population, but intrinsically related to the presence of leukemic cells.

Conversely, in AML, MDS and CML BM and PB samples, a significant higher level of expression can be detected. Since the RQ-PCR methods clearly distinguish between the level of WT1 transcripts in normal and leukemic cells, WT1 expression can represent a molecular marker extremely useful in the clinical setting of MRD assessment. In particular in cases lacking cytogenetic lesions, it may help to establish the response to therapy and to monitor the behavior of the leukemia clone during follow-up.

To validate the role of WT1 as a marker of MRD, we studied a number of patients bearing a fusion gene transcript suitable for the quantitative assessment of the MRD amount by RQ-PCR and performed a simultaneous analysis of the WT1 amount at sequential time intervals during the follow-up [6]. The WT1 levels were shown to strictly parallel the behavior of the other molecular markers (fusion gene transcripts) used for the MRD monitoring. Furthermore, increased WT1 expression above the range found in normal BM and/or in normal PB samples during follow-up of AML patients was always found to be predictive of an impending hematological relapse even in AML patients lacking additional molecular markers. The increase of the WT1 levels could also precede the occurrence of the overt hematological relapse of some months, although the kinetics of the relapse appears highly variable. On the opposite, normal WT1 values have always been found associated with persisting remissions. Therefore, evaluation of WT1 expression could represent a sort of universal marker that can allow a rather sensitive evaluation of the MRD in all AML patients with a degree of sensitivity that can be estimated to reach in most cases between $10^{-3}$ and $10^{-4}$ [6]. Furthermore, the finding of extremely low and often undetectable WT1 levels in the PB of normal individuals and in leukaemia patients in CCR, suggests that PB could even more sensitive than BM in revealing impending relapses. Although this point still needs to be demonstrated, replacement of BM with PB sampling could greatly improve patients' compliance and considerably simplify molecular monitoring.

The clinical application of WT1 gene as a molecular marker for MRD detection was already suggested by Inoue et al. [11] in 1996 and confirmed later on by different studies. Recently published data obtained in a pediatric group of AML patients in which the presence of MRD was assessed by flow cytometric analysis for the blast cell count. They found a strict correlation between the levels of WT1 transcript and the percentage of blast cells detected by flow cytometric analysis. Their results confirm the finding that clinical remission is constantly associated with low levels of WT1 while increasing values are associated with relapse.

Finally, similarly to what demonstrated for AML, [6,9] WT1 levels seem to represent a good marker for MRD detection also in MDS patients treated with intensive therapies aimed at the disease eradication [10].

Moreover, it has been demonstrated that even in the transplant setting, as already demonstrated for acute leukemia patients treated with intensive chemotherapy, the determination of the WT1 amount can represent a useful marker to monitor the persistence or the reappearance of leukemic cells and that the finding of increasing amounts of WT1 transcript during follow-up is predictive of relapse.

References


The word haemovigilance is derived from the word pharmacovigilance, which covers activities and systems to collect information in medicinal products, especially for adverse drug reactions in humans. Haemovigilance, initially created in France in the beginning of 90s has Greek and Latin roots: “haema”: blood and “vigilans”: paying special attention. Haemovigilance concerns blood components. Pharmacovigilance concerns plasma derivatives such as clotting factor concentrates, immunoglobulins, albumin and other fractionated products. Since 1993, in European legislation plasma derivatives are considered to be pharmaceuticals, and the manufacturers have to comply with the European regulations on pharmacovigilance.

Transfusion reports from US and United Kingdom showed that most of the incidents were caused by clerical errors. Not only identification but also administrative errors also plays an important role. In France, by a law in 1994, the first definition of hemovigilance was introduced. Various number of definitions have been written since then. In the European Blood Directive, it is defined as a group of organized surveillance procedures relating to serious, adverse or unexpected events or reactions in donors or recipients, and the epidemiological follow-up of donors. The definition given by the European Haemovigilance Network (EHN) is the one most widely used. “Haemovigilance is a set of surveillance procedures covering the entire transfusion chain (from the donation of blood and its components to the follow-up of recipients of transfusions), intended to collect and assess information on unexpected or undesirable effects resulting from the therapeutic use of labile blood products, and to prevent the occurrence or recurrence of such incidents” [1].

Haemovigilance and the European Blood Directive

On February 8, 2003 the European Blood Directive 2002/98/EC was published and came into force. In this Directive, Chapter V is dedicated to haemovigilance and there are two operating articles dealing with haemovigilance; “Article 14: Traceability

- Member States shall take all necessary measures in order to ensure that blood and blood components collected, tested, processed, stored, released and/or distributed on their territory can be traced from donor to recipient and vice versa. To this end, Member states shall ensure that blood establishments implement a system for identification of each single blood donation and each single blood unit and components thereof enabling full traceability to the donor as well as to the transfusion and the recipient thereof. The system must unmistakably identify each unique donation and type of blood component. This system shall be established in accordance with requirements referred to in Article 29(a). With regard to blood and blood components imported from third countries, Member states shall ensure that the donor identification system to be implemented by blood establishments permits an equivalent level of traceability.
- Member States shall take all necessary measures in order to ensure that the system used for the labelling of blood and blood components collected, tested, processed, stored, released and/or distributed on their territory complies with the identification system referred to in paragraph 1 and the labelling requirements listed in Annex III.
European haemovigilance network (EHN)

Five countries took the initiative in 1998 to work together in the field of haemovigilance: Belgium, France, Luxembourg, Portugal and The Netherlands and the EHN was born. Later, Austria, Denmark, Finland, Greece, Ireland and the United Kingdom joined to the EHN and Canada, Croatia, Norway and Switzerland were adopted as associate members.

EHN was established to increase blood safety at a European level and has the following objectives [2,3]; (a) favour exchange of valid information between members, (b) increase rapid alert and/or early warning between the members, (c) encourage joint activities between the members, (d) undertake educational activities in relation to haemovigilance, (e) standardization of processes and forms, (f) comparison and analysis of data, generated by national systems, (g) assistance in the implementation of the European Blood Directive, in relation to legal provisions.

This European network focuses on two systems; Rapid Alert/Early Warning system (RAS) and a reporting system for adverse reactions to blood component transfusion. Reporting forms has been developed in order to standardize the information process needed and to take appropriate action to prevent the occurrence or recurrence of adverse events.

All EHN activities can be followed on the EHN Web-site (www.ehn-org.net). Ten Members States of the EU – Belgium, France, Denmark, Greece, Ireland, Portugal, Luxembourg, Finland, Netherlands, and United Kingdom-are 10 full members of EHN. Non-EU Members States as Australia, Canada, Switzerland and Norway are associate member, and Brazil, Spain and Romania have expressed their wish to join as associate members.

Haemovigilance systems around the world

There are significant differences in haemovigilance around the world, in terms of definition, organizational schemes, state of development and implementation [4]. Although mostly developed in EU countries, there are some countries, even basic traceability causes a problem. Most of these systems are on a volunteer basis but there are a few which the report of the reactions are mandatory like the one in France.

Basically the existing systems can be classified according to their legal status (mandatory vs. voluntary), their field of application (all events vs. very serious reactions), their organization (centralized vs. less decentralized), and their financing. These two programmes represent two different models for other countries.

In Germany, the labile blood components are considered as medicinal products and come under the German Drug Law. According to the national legal provisions covering medicines, side effects have to be reported according to the rules of pharmacovigilance. Nevertheless it should be mentioned that the recent German Transfusion Law also established haemovigilance as a separate entity.

Little is known about the haemovigilance systems of the new EU countries by 2004.

French haemovigilance system

In 1993 by law, in France, haemovigilance became a national system of surveillance and alert, from blood collection to the follow-up of the recipients, gathering and analysing all adverse events of blood transfusion in order to prevent their recurrences. In France, unlike the other European countries, the reporting of all adverse reactions is mandatory, regardless of their severity and their transfusion imputability. The law states that “anyone, doctor, chemist, dental surgeon, midwife or nurse, who notices an unexpected or untoward effect due or possibly due to a blood product, must report it at once” [5].

The agencies that take part in haemovigilance system in France: The French Control Authority on Health Products (AFSSaPS), -The French National Blood Service (EFS) and The Institute of Sanitary Surveillance (INVS). The National French Blood Agency was disbanded and the control of blood transfusion activity was transferred to the AFSSaPS, effective for haemovigilance in 1999 [5,6].

The French Haemovigilance Network is built on three levels [1,7]. Local level with 2000 public and private hospital correspondents and one correspon-
dent in each of the 18 regional blood centres helped by a colleague mostly physicians or pharmacists, in each distribution site. Every public hospital must also have an Haemovigilance and Transfusion Safety Committee, with technical, medical and administrative members. Regional level with 25 haemovigilance regional coordinators that are physicians located in each French administrative region, under the authority of the prefect and regional director of social and sanitary affairs (DRASS). They supervise investigations and implementation of corrective actions, when required. At national level AFSSaPS defining haemovigilances main features, leading and coordinating the actions of the different actors, taking the appropriate measures to ensure transfusion safety and passing on to the Ministry of Health all the epidemiological information necessary to take action.

The global traceability of blood products improved from 95% in 2000, to 99% in 2003. On average 7000 adverse reactions are reported each year, from shivers or pruritus to death. Currently, more than 68,000 adverse reactions are in the national database. In 2003, 6933 adverse reactions were reported, among which 2911 (42%) had a strong association with transfusion. There were 1898 acute (65%) and 1013 (35%) delayed adverse reactions. More than 50% of acute reactions were allergic and 22% were FNHTR. Almost all the 1013 delayed adverse reactions (98.5%) were red cell immunization. Seven viral infections (6 HCV and 1 CMV) with strong transfusion imputability was recorded [5].

Turkey
In general, the blood system is decentralized with high degree of more than 300 blood banks, most of them are associated with a hospital. So it is not surprising not to set up a national centralised and efficient surveillance system. Legislations and regulations are not ready yet.

China
The Chinese Government has decided to undertake a major upgrade of the system around blood transfusion. In the coming years there will be important changes towards a comprehensive system that will include haemovigilance.

References
TRANSFUSION POLICIES IN ANEMIA

Blood transfusion and alternatives in elderly, malignancy and chronic disease

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Introduction

Blood transfusion is an essential part of modern medicine and health care, however it should be prescribed only to treat conditions associated with significant morbidity or mortality that cannot be prevented or managed by other means [1].

Both blood transfusion service and clinical users should share efforts to establish and implement policies and strategies aiming at reducing the need for transfusion, minimize unnecessary transfusions and ensure a safe, rational and appropriate use of blood and blood components. Blood should not be transfused unless it has been obtained from appropriately selected donors, has been tested for transfusion transmissible infections and tested for compatibility between donor's red cells and patient's plasma [1].

Anemia of chronic disease is the second prevalent anemia after anemia caused by iron deficiency, occurs in patients with acute or chronic activation [3].

Definition

Anemia is defined as a hemoglobin concentration in blood that is below the expected value, when age, gender, pregnancy and certain environmental factors, such as altitude, are taken into consideration [1].

However anemia in general results as a consequence of one or more of the following generic causes:-

- Increased loss of red blood cells.
- Decreased production of red blood cells.
- Increased destruction of red blood cells.
- Increased demand of red blood cells.
- Increased production of abnormal red blood cells.

When anemia develops it leads to various physiologic changes, the most important of which is the compensatory response which aims at preserving the oxygen supply to the tissues. When anemia is severe enough that the patient is de-compensated, it is very important that red cells transfusion is considered to increase the oxygen carrying capacity needed for the tissues. De-compensation might happen in case of significant cardiovascular disease, fever or infections which increase the demand for oxygen.

General common features associated with anemia related to elderly, malignancy, and chronic disease

The rate at which anemia develops determines the severity of the symptoms and accordingly the consequences. Subsequently anemia can be classified as:-

- Mild
- Moderate
- Severe

Moderate anemia may cause no symptoms; nevertheless it reduces the patient's reserves to adjust to any event such as infection.

Severe anemia is an important factor in reducing the patient's tissue supply to critical levels. In this situation, urgent treatment is required and the need for transfusion should be assessed. Severe anemia is usually a sign of an advanced underlying disease.

The clinical picture of anemia in the above mentioned three conditions are due to either anemia itself or the underlying cause. However chronic anemia in these situations may have some symptoms which will increase greatly if the patient develops sudden hemolysis, bleeding or during other physiological conditions such as pregnancy or labor.
Anemia has been associated with relatively poor prognosis among patients with various conditions, including cancer, chronic kidney disease and congestive heart failure [3].

Symptoms such as fatigue and shortness of breath are subjective, but are still useful in determining the need for red cell transfusion in patients with chronic anemia [4].

Anemia of chronic disease is not itself usually a cause of symptoms. The anemia is mild and well tolerated unless it is superimposed on other threatening conditions. The importance of recognizing anemia of chronic disease is in identifying its underlying cause [5].

Special considerations when transfusing an elderly, malignancy and chronic disease

Blood transfusion in general should be prescribed, administered and monitored very carefully by a competent medical person. This is due to the fact that it carries potential risks and hazards. The patient may have several causes of anemia, such as malnutrition deficiency, infection, malignancy or hemoglobinopathy. There are few considerations which should be considered in the transfusion management of the following three conditions:-

- **Elderly**: the rate of red cell transfusion must be slower than in younger age patients. Elderly patients are subjected to heart failure due to blood volume overload if the rate of transfusion is fast. Diuretics should be considered in the appropriate dose when transfusion is inevitable. Moderate anemia warrants correction in patients older than 65 years old, especially those with additional risk factors [such as coronary artery disease, pulmonary disease, or chronic kidney disease] or combination of these factors [3].

- **Malignancy**: usually patients with malignant disorders who need red cell transfusion are likely to need other blood components as well such as platelet concentrates. Due to the frequency of this therapy, one should consider at the beginning of transfusion protocol several issues as leukodepletion, irradiation and erythropoietic agents.

- **Chronic disease**: same considerations of malignancy are applied in cases of anemia due to chronic disease. In patients with renal failure who are receiving dialysis and in patients with cancer who are undergoing chemotherapy, correction of anemia up to levels of 12 g/dl is associated with an improvement of quality of life [3].

Red cells transfusion

In cases such as elderly, malignancy and chronic diseases, red cell concentrate is the component of choice, as whole blood carries the risk of volume overload. Red cells are prepared by centrifugation and separation from whole blood. It should be stored at 4°C and has to be administered within 60 minutes of issuance from controlled storage temperature. Red cell concentrate should be administered through standard [170 micron] giving set.

One red cell concentrate unit should increase the hemoglobin level in an adult by 1 g/dl.

Red cell concentrate [packed red cells, or plasma reduced red cells] is the simplest red cell component. This component can further be leukodepleted using leukocyte filters, irradiated by gamma irradiation, washed by normal saline to remove the plasma proteins or suspended in additive nutritive solution to extend its shelf life and reduce its hematocrite. It is advisable to leukodeplete all red cell concentrate units used for patients who are anticipated to receive multiple transfusions. This is to avoid all the untoward consequences of leukocytes. Immune-compromised patients such as malignancy under chemotherapy should receive irradiated cellular components to avoid the possibility of developing GVHD.

Compatibility of red blood cells with the intended recipient must be verified by suitable pre-transfusion testing [2]. Cross-matching [compatibility testing] is done between the donor’s red cells and patient’s serum for every unit for transfusion. It is recommended to transfuse fully ABO, Rh and K compatible red cells. Red cell antibody screening should be performed in every transfusion; this is to avoid any hemolytic reaction which in these conditions might lead to very serious consequences.

The medical person who gives the transfusion to a patient is responsible for the control of identity and other safety measures. Verification of identity shall be carried out either by asking the patient to tell his/her name or other identification details on a wrist band which has been attached to the patient according to well-specified rules [2].

Close monitoring of patients during transfusion is essential in order to manage any adverse effects in due time. The dose and frequency of red cell transfusion has to be appropriate to the patient’s age, weight, and clinical condition.

Because of the risk of damage to the blood components, no medical products or infusion solutions may be added to blood units [2].

Alternatives to red cell concentrate transfusion

Transfusion is usually not needed for chronic anemia, and the unnecessary transfusions in these situations
may lead to unavoidable complications. Simple preventive measures and the use of oral iron replacement can greatly reduce the prevalence of iron deficiency anemia and reduce the need for blood transfusion [1].

Erythropoietic agents for patients with anemia of chronic disease are currently approved for use by patients with cancer who are undergoing chemotherapy, patients with chronic kidney disease, and patients with HIV infection who are undergoing myelosuppressive therapy [3].

Transfusion guidelines

The philosophy of the appropriate clinical use of blood [ACUB] is a rational move to be adopted by clinicians. In order to achieve this practice, health care providers and systems should implement effective and sustainable health programs and services. This leads to the control and standardization of the blood transfusion practice.

Establishing national guidelines for blood transfusion protocols is a very important tool for proper management of all conditions that need blood transfusion or alternatives, including anemias. Government support is very useful in this policy.

National guidelines should elaborate issues such as information on different blood components, blood ordering policies, compatibility, handling, monitoring and management of adverse effects to blood. Many countries have considered this and produced their national guidelines. WHO has issued a group of publications regarding the use of blood components which are very reasonable and can be applied in many health systems.

Management

One should always bear in mind that blood transfusion is an adjuvant therapy, and it is always accompanied by other types of treatment measures. These other treatment measures have to be taken into consideration while ordering, administering and monitoring blood transfusion. It is very rational that every patient is managed individually and golden rules to be avoided.

Blood transfusion management in the above mentioned cases depends on various factors such as age, underlying illness, duration & rate at which anemia developed and the degree of compensation. Red cell transfusion is considered only when anemia is severe enough to cause reduced oxygen supply to tissues leading to de-compensated situation. The aim of red cell transfusion in this case is to supply the patient with sufficient hemoglobin to improve the hypoxia and not to increase the lab hemoglobin level.

In order that blood transfusion becomes effective, it is very important to treat the underlying cause of anemia whenever it is possible. The need for transfusion can often be avoided by either preventing or early diagnosis or treatment of anemia and conditions that lead to anemia, or correction of anemia and the replacement of depleted iron stores by prescribing oral iron in the relevant dose and frequency.

Transfusion are particularly helpful in the context of either severe anemia [ < 8.0 g/dl] or life threatening anemia [ < 6.5 g/dl] particularly when the condition is aggravated by complications that involve bleeding [3].

Clinicians who are managing anemia in the above mentioned situations should have good knowledge about red cell components specially indications, risks, storage and benefits. They should as well inform their patients about the potential hazards and benefits of red cell transfusion and alternative if relevant.

Recommendations

- Red cell transfusion should be considered only when anemia has caused inadequate oxygen supply to the needs of the patient. Otherwise patients should be managed early enough to avoid this situation.
- Treatment of the underlying cause of anemia usually prevents further decrease of oxygen carrying capacity, hence making management easier and avoids blood transfusion that carries potential hazards.
- National training programs should be in place which aims at standardizing the practice of blood transfusion among all clinicians.
- Decisions to transfuse blood should always be based on careful assessment of clinical and laboratory indicators that transfusion is necessary to save life or prevent significant morbidity [1].
- Do not transfuse more than necessary, in other words do not restore laboratory hemoglobin level. It should be raised to relieve clinical condition.

Conclusions

Transfusion can be a life saving intervention, however like all other treatments; it may result in acute or delayed complications and carries the risk of transfusion transmissible infections [1].

There are no reliable parameters to guide the need for red cell transfusion. The decision to transfuse red cell is a complex one and depends on factors such as the cause of the anemia, its severity and chronicity, the patient’s ability to compensate for anemia, the likelihood of further blood loss and the need to provide some reserve before the onset of tissue hypoxia [4].
Clinical experience of blood prescribers is crucial to the management of anemia as there is no reliable trigger or golden rule to be followed.

References
TRANSFUSION POLICIES IN ANEMIA

In search of the transfusion threshold

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Introduction

Every medical decision involves weighting the risk versus benefit. The risks related to red blood cell transfusion include the adverse effects of allogeneic blood and the risks of anemia. The potential benefits of blood transfusion are reduction of mortality and morbidity and improvement in functional recovery. In this lecture, I will focus on risks related to anemia and what is known about the benefits of allogeneic transfusion. I will complete the presentation with a description of a new clinical trial called FOCUS.

Risk from anemia

Animal data

The heart is the most vulnerable to the effects of anemia. The heart extracts a high percentage of oxygen. When anemia develops coronary blood flow must increase to maintain oxygen delivery. Blood flow increases because of decrease blood viscosity that results from anemia.

A series of experiments have been performed in canines to evaluate the effect of anemia (Table I). After hemodilution, healthy animals survive hemoglobin concentrations between 3 and 5 gdl\(^{-1}\) [1–3]. However, myocardial ischemia is detectable by electrocardiograph changes at hemoglobin concentrations below 5 gdl\(^{-1}\). At hemoglobin levels around 3 gdl\(^{-1}\), severe physiologic derangement develops. This is manifested by anaerobic metabolism (lactate production) myocardial dysfunction, and death. Some animals survive with hemoglobin levels as low as 1 to 2 gdl\(^{-1}\).

Animals with experimentally induced coronary artery disease poorly tolerate anemia. In the presence of coronary stenosis from 50% to 80%, myocardial ischemia and/or reduced cardiac function develop at hemoglobin levels between 7 to 10 gdl\(^{-1}\). This finding suggests animals with coronary artery disease are less tolerant of anemia than animals with normal hearts.

Human data

Studies in patients who decline blood transfusion for religious reasons provide critical insights into risks associated with anemia. In a small study of 125 patients, [4] mortality rose with lower preoperative hemoglobin levels and greater operative blood loss. The fatality rate was 61.5% in patients with preoperative hemoglobin concentrations below 6 gdl\(^{-1}\), but only 7.1% in patients with preoperative hemoglobin concentrations greater than 10 gdl\(^{-1}\). In this small case series, patients with a hemoglobin level above 8 gdl\(^{-1}\) and operative blood loss below 500 ml, none of the patients died.

The largest study in patients who refused blood transfusion confirmed animal data that patients with cardiovascular disease are more susceptible to anemia than patients without cardiovascular disease. In a cohort study in 1,958 adult surgical patients who underwent a surgical procedure in an operating room, [5] mortality rose as preoperative hemoglobin levels fell. However, patients with cardiovascular disease had a much higher risk of death as the hemoglobin concentration fell below 10 gdl\(^{-1}\) than patients without cardiovascular disease (Figure 1).

A recent study combined data from two cohorts of Jehovah’s Witness patients and examined the morbidity and mortality associated with postoperative hemoglobin concentrations below 8 gdl\(^{-1}\)[6]. Of 2083 consecutive patients, 300 (15%) had postoperative Hgb levels <8 gdl\(^{-1}\). None of the patients with postoperative hemoglobin levels between 7.1–8.0 died although 9.4% had significant morbidity. The 30 day mortality was 34% when the postoperative hemoglobin level fell to 4.1–5.0 gdl\(^{-1}\).
The effect of anemia was assessed in normal volunteers undergoing isovolemic anemia to 5 gdl\(^{-1}\). Transient electrocardiogram changes occurred with hemoglobin levels between 5\(^{-1}\) and 7 gdl\(^{-1}\) in 5.7% of volunteers [7,8]. Subtle changes in cognition were found in young volunteers with hemoglobin levels between 5\(^{-1}\) and 7 gdl\(^{-1}\) [9]. Fatigue developed when the hemoglobin level fell to 7 gdl\(^{-1}\) and increased as the hemoglobin level dropped to 5 gdl\(^{-1}\) [10]. Heart rate is linearly related to hemoglobin concentration [11]. These studies suggest that important clinical effects can be measured in young, normal humans with hemoglobin levels between 5\(^{-1}\) and 7 gdl\(^{-1}\). It is unclear how these results relate to sick patients undergoing surgery but it seems possible such patients would be even less tolerant of anemia.

**Efficacy of transfusion**

**Clinical trial data**

Clinical trials are essential to establish efficacy of any treatment including blood transfusion. Unfortunately, there is limited evidence. Of the 10 randomized clinical trials, all but one was too small to evaluate clinical outcomes [12]. A meta-analysis that combined the results of these trials [12] found the Transfusion Requirements in Critical Care (TRICC) trial contributed 83% of the information for the analysis of mortality in the meta-analysis [13]. The other trials were too small to reliably evaluate the effect of transfusion thresholds on clinical events.

The TRICC trial evaluated two transfusion triggers in 838 volume resuscitated patients admitted to intensive care unit [13,14]. In the restrictive arm of the trial, patients were randomized to a transfusion strategy in which blood was administered when the hemoglobin concentration fell below 7.0 gdl\(^{-1}\) (and maintained between 7.0 to 9.0 gdl\(^{-1}\)). In the liberal transfusion group, patients were transfused to maintain hemoglobin concentration between (10.0 gdl\(^{-1}\) and 12.0 gdl\(^{-1}\)). Surprisingly, the 30-day mortality was slightly lower (not significant) in the restrictive transfusion group than the liberal group (18.7% vs. 23.3%). Overall, these findings were not statistically significant, although in patients less than 55 years of age and with Apache scores less than 20 (less ill patients), mortality was significantly better in patients randomized to the restrictive transfusion group. The results were different in patients with cardiovascular disease. In patients with ischemic heart disease (defined as myocardial infarct, angina, congestive heart failure, and cardiogenic shock), the liberal transfusion group had a small and non-significant improved outcome.

**Observational studies**

The seven large observational studies that evaluated the transfusion thresholds came to different conclusions and must be interpreted very cautiously. Studies in critically ill patients [15,16], patients undergoing coronary artery bypass surgery, [17] and surgical patients undergoing hip fracture repair [18] came to different conclusions. A very large study in acute myocardial infarction suggested [19] mortality was reduced by transfusion in patients with hematocrit levels less than 33%. However, a more recent analysis of clinical trials in acute coronary syndrome found that blood transfusion increased mortality [20]. Three small studies found higher rates of cardiac events in anemic patients. Thus, there is great heterogeneity of the study populations and results. Furthermore, the results of these observational studies should be interpreted very cautiously because statistical approaches to adjusting for differences between patients receiving transfusion versus those not transfused may not be successful. This point is emphasized by the results of recent clinical trials evaluating hormone replacement therapy where the results on cardiovascular risk were opposite of observational studies. Similarly, the

<table>
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<th>Table I. Effects of anemia in animals undergoing hemodilution.</th>
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<td>Group</td>
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<td>Normal</td>
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<td>Death</td>
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<td>Coronary Artery Disease</td>
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In search of the transfusion threshold
TRICC investigators found difference in their clinical trial than their observational study [15].

**FOCUS: A new clinical trial**

Given the limited high quality evidence, we have initiated a new NIH funded trial called FOCUS. The transfusion trigger trial for Functional Outcomes in Cardiovascular Patients Undergoing Surgical Hip Fracture Repair (FOCUS) is a randomized clinical trial designed to test the hypothesis that higher blood transfusion threshold improves functional recovery and reduces morbidity and mortality. Patients who undergo surgery for hip fracture, have a history of cardiovascular disease, and have a postoperative hemoglobin level less than 10 g/dl below 10 g/dl/C28 are eligible. Patients will be randomized to receive enough blood to raise the hemoglobin level above 10 g/dl above 10 g/dl/C28 any time the hemoglobin level is detected to be below 10 g/dl below 10 g/dl/C28 during the hospitalization or to receive transfusion if symptoms of anemia develop. Transfusion is permitted but not required if hemoglobin level is less than 8 g/dl less than 8 g/dl/C28. The primary outcome is ability to walk 10 feet (or across a room) without human assistance at 60 days. The most important secondary outcome is postoperative unstable angina, myocardial infarction or death. Medical records will be reviewed while the patient is in the hospital. Patients will be telephoned at 30 and 60 days after entry into the study to determine functional capacity and vital status. Long term mortality will be determined by searching vital statistic registries in US and Canada. The pilot study for the trial has been published [21].

**Summary**

We have very limited data to guide the transfusion decision. The best evidence suggests that mortality and morbidity rises as the blood count falls but that in patients without cardiovascular disease a 7 g/dl 7 g/dl/C28 level is tolerated in most patients. Below 5–6 g/dl 5–6 g/dl/C28, the mortality and morbidity rises rapidly. In patients with cardiovascular disease, animal and human studies suggest that a higher hemoglobin concentration may be necessary but there is limited evidence from clinical trials to inform this question. We expect that results from the new clinical trial called FOCUS will provide important new information to help guide transfusion decisions.

**References**


Combined use of oral chelators and desferrioxamine in thalassemia

A. PIGA, S. ROGGERO, F. MARLETTO, L. SACCHETTI, & F. LONGO

The standard iron chelation therapy is based on the use of deferoxamine (DFO) [1]. A subcutaneous infusion of 20–50 mg/kg/day over 8–12 hours 6–7 days a week promotes a total iron excretion of 0.15–0.5 mg/kg/day [2]. This may counterbalance the mean iron input from standard transfusional regimens of 0.25–0.5 mg/kg/day [3]. The effectiveness of this treatment is mainly determined by the compliance, that may vary significantly [4]. During these last few years the oral chelator deferiprone (DFP) has been approved in many countries and gained room at least as a second line option for patients where DFO is not tolerated or inadequate [5,6]. The relative lower efficacy is partially counterbalanced by the advantages in compliance due to the administration route. Recent results from independent studies suggest that deferiprone may be more cardio protective than deferoxamine. Patients on long-term treatment with deferiprone have a better myocardial MRI pattern [7], and less chance to develop a new cardiac disease or to worsen an existing one [8].

Rationale

The efficacy and tolerability profiles of DFO and DFP are very different, due mostly to the different physicochemical and pharmacological properties: molecular weight, Fe:chelator molar ratio, Fe affinity, Fe binding stability, and excretion. Some in vitro data suggested the possibility of an additive effect of combining the two chelators. A recent study from Link showed that the combination of DFO and DFP improved significantly the iron depletion rate from rat heart cells [9]. Clinical data are scarce. Grady performed the first exhaustive study on iron balance in thalassemia, assessing in each patient the fecal and urinary iron excretion with each chelator and finally in combination. The preliminary results show that in most patients an additive effect may be reached combining the two chelators. In some subject a synergistic effect has been observed. A “shuttle” hypothesis has been formulated to explain the described synergistic effect: DFP, crossing cell membranes more easily, could bind excess intracellular iron and mobilize it into plasma, where DFO, with its higher affinity, may take it and speed up its excretion.

Clinical studies

Several studies have been published or presented at meetings on long-term use of the two drugs [10–18]. Unfortunately none of these is a randomized controlled trial and most do not satisfy high quality standards, nor the safety has been investigated systematically. Furthermore many different treatment schemes have been used, making difficult to summarize findings. Anyway the results seem to indicate a higher efficacy of combination therapy, in terms of urinary iron excretion, lowering serum ferritin levels, trend to normalize in iron-related MRI abnormalities both in the liver and in the heart, and in systolic function parameters. These observations, as the hypothesis that deferiprone could be faster or more efficient in removing excess iron from the heart have raised a lot of attention from clinicians, but should be verified with formal controlled studies.

Definition

A problem of terminology exists: in papers and presentations sometimes the prescription and dose timing is not fully described and under the umbrella ‘combination’ are associated very different treatments such as the daily taking of both drugs at full doses and simultaneously, or alternating the two drugs in some
way during the week. An approach to a common terminology could be the following:

- **Mono-therapy**: a single chelator is prescribed and taken for more than three months
- **Alternate therapy**: in a single day a single chelator is taken; the two chelators take turn on a weekly, monthly or quarterly basis (e.g., DFP five days a week and DFO two days a week)
- **Combination therapy**: prescription of more than one chelator, to be taken in the same day at least for a significant part of the period
  - **Sequential**: in a single day two chelators are taken in sequence; no substantial overlapping of the two drugs in the plasma (e.g., DFP thrice a day and DFO night time)
  - **Simultaneous or concomitant**: in a single day two chelators are taken at the same time; substantial overlapping of the two drugs in the plasma (e.g., DFO infusion starts at 7 PM and ends at 7 AM; DFP taken at 8PM, 11 PM and 7 AM).

Some other forms of combination exist, as a course of intravenous DFO at each transfusion to reinforce the iron chelation done at home.

**Potential applications**

Combination treatment should be potentially considered every time there is a need to search for an additive or synergistic effect. This include the reversal of severe iron-related complications, the most important of which is heart disease. At a new onset of heart disease it is important to provide a full protection from cardio-toxicity with a continuous treatment, to minimize the presence of non-transferrin bound iron in the plasma. Intensive DFO chelation with continuous intravenous infusion has been demonstrated to be effective. The addition of deferiprone seems to enhance the efficacy and to reduce the duration of intensive treatment.

Also for the prevention of iron-related complications may be an important field of application. The recent development of MRI techniques to quantify the heart iron and the relationship of MRI signal to systolic function give the rationale for a more effective prevention of this complication.

Another indication may be the achievement of safe tissue levels, in any patient where the iron overload is severe or in special conditions, such as the preparation to pregnancy or to a stem cell transplant or to antiviral treatment for hepatitis.

Another potential application regards patients with dose-related side effects to DFO or DFP. Individually tailored combination of the two drugs may minimize side effects, maintaining efficacy.

**Side effects**

This page is still to be written, as the experience with combination treatment is relatively recent. From the published studies it does not come out a trend to increase of the known side effects of both drugs nor the evidence of new ones. A raised prevalence of agranulocytosis has not been confirmed.

**New iron chelators**

The development of new iron chelators may enhance greatly the possibilities of combination treatment. In particular ICL670, a new tridentate oral chelator has good chances to be approved in the near future, due to the positive results of extensive clinical studies [19,20]. Its pharmacokinetics and pharmacodynamics offer interesting perspectives of potential combination with DFO or DFP. Well designed randomized controlled trials may answer important questions, but the sponsorship of combination therapy trials from industry is unlikely.

**Conclusions**

For the clinical practice: there is growing evidence that DFO and DFP combination treatment is more effective than s.c. DFO or DFP alone and may give benefit in certain clinical conditions such as heart disease. Little is known as regards the safety.

For the research: the results of many observations are large randomized controlled trials on combination therapy compared to mono-therapy. Clinically relevant outcomes should be studied, as liver iron concentration on short-term and heart disease and survival on long-term.

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Combined use of oral chelators and desferrioxamine in thalassemia


Sickle cell disease: A multigenic perspective of a single gene disorder

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Half a century has elapsed since the elucidation of the molecular basis of the sickle hemoglobin mutation in the mid 50s. Although significant progress has been made in our understanding of the disease and in the development of new therapies, many questions are still unanswered, and a cure remains elusive. This is particularly evident in the clinical heterogeneity of sickle cell disease (SAD), which ranges from a mild clinical course with survival into the 6th and 7th decades to a very severe presentation with significant organ damage and death at relatively young ages [1].

Most of the advances in unraveling the phenotypic heterogeneity of SCD have been made in the past 30 years, after the dawn of the molecular era. Studies in this period have clearly shown the importance of high hemoglobin F (Hb F) determinants and α-thalassemia as modifiers of disease severity. The impact of these modifiers has been well established and will not be discussed in detail here [1]. We will rather try to summarize recent advances on the effect of non-globin genetic modifiers on the phenotype of SCD. This process has been greatly facilitated by the completion of the Human Genome Project, which has identified a large number of single nucleotide polymorphisms (SNPs) in and around many genes that can potentially impact on different aspects of SCD pathophysiology. The realization that α-thalassemia and variation in Hb F levels alone do not account for the vast clinical diversity of the disease has provided further impetus to such an endeavor.

The well-known consequences of the sickle mutation and its downstream effects are: (1) a chronic hemolytic anemia, (2) episodic vasoocclusion with resultant characteristic painful episodes, and (3) chronic organ damage. The clinical phenotype of SCD is highly variable; complications such as acute chest syndrome, stroke, leg ulcers, and avascular necrosis do not occur in all of the patients suggesting that factors beyond the β6 Glu → Val mutation play a role in the pathogenesis of these syndromes. In the past two decades, contributions by Hebbel et al. and others, has established that SCD is an “inflammatory state” and that the endothelium is activated [2]. In more recent years, evidence has accumulated that decreased nitric oxide (NO) bioavailability, contributed by scavenging of NO by cell free hemoglobin, a product of the hemolytic process, plays a significant role in the vascular pathobiology of SCD [3]. Thus, the emerging view of the pathogenesis of many complications of SCD involves complex interactions between sickle reticulocytes, neutrophils, monocytes, and the endothelium. It therefore follows that many factors that are important in the pathways leading to inflammation, cell adhesion, NO metabolism, vascular reactivity and coagulation will be important in the pathophysiology of the disease and that variations in the expression of a number of molecules in these pathways will contribute to the heterogeneity of SCD.

The approach to the study of this complexity in the post-genome era should involve several different methodologies including: (1) analysis of polymorphisms in “candidate genes” by high throughput methods and the association of these polymorphisms with distinct phenotypic features of the disease, (2) the study of differential gene expression in various tissues using cDNA microarrays, and (3) the application of proteomics. The tissues and cells that are particularly important in this regard include blood cells (neutrophils, monocytes, reticulocytes), bone marrow, endothelial cells, and liver tissues. A candidate gene approach should include a large number of patients with and without a certain complication (e.g., stroke) to determine the role of these polymorphisms as risk factors. Pooling studies to discover the role of genome wide SNPs as risk factors for certain complications of the disease is also emerging as a viable approach. In all of these studies, careful characterization of the phenotype remains of critical importance. A study of
sickle cell patients from different populations is likely to yield important information because of the differences in the genetic backgrounds in these populations and its potential implications on the disease phenotype. The important disease related “phenotypes” should include features such as stroke and CNS disease, frequency of vasoocclusive episodes (VOEs), frequency of the acute chest syndrome, avascular necrosis of the hips and shoulders, gallbladder disease and cholecystectomy, leg ulcers, renal involvement, priapism, and retinopathy. Table I lists these important “sub-phenotypes” of SCD and their frequency in the SCD patient population in the U.S.

For the past several years, our center and many other centers have focused on the study of non-globin genetic modifiers of sickle cell disease. Some of the findings of these early studies will be summarized below.

**Hb F and Hb F response to hydroxyurea**

It is well established that fetal hemoglobin has an ameliorative effect on SCD because the gamma chains of Hb F are excluded from the deoxyhemoglobin S polymer. Because of this potent anti-sickling effect, enhancing Hb F production has been a major therapeutic goal in SCD. In fact, this goal has been achieved to a large extent by the use of the S-phase specific chemotherapeutic agent, hydroxyurea [4].

Several genetic determinants are known to contribute to the heterogeneity of baseline Hb F levels in SCD. Haplotypes of the β6 chromosomes are among the most extensively studied. Senegalese and Asian–Indian haplotypes are associated with higher Hb F levels, and therefore a milder clinical and hematologic phenotype compared to the other African haplotypes (Benin, Bantu, and Cameroon). The common feature of the Senegal and Asian haplotypes is the presence of a C→T polymorphism in the promoter of the Gγ gene (−158 C→T, detectable by the restriction enzyme Xmn I). The search for other cis-acting regulatory elements in the β-globin cluster has failed to yield consistent results. Thus, it has become clear that β6 haplotypes and variations in cis-acting elements associated with different haplotypes are only partially responsible for the variation seen in Hb F levels among SCD patients. This has prompted the search for other trans-acting regulatory elements controlling Hb F levels. One of these putative elements is a quantitative trait locus (QTL) on the X-chromosome (Xp22), which presumably controls the production of F-cells. This F-cell production locus (FCP) and the −158 C→T polymorphism in the Gγ promoter are estimated to account for ~50% of the variation in Hb F in SCD [5]. Other QTLs that influence Hb F levels have been located at 6q22.3−23.2, and 8q [6–8]. In a study of 180 single nucleotide polymorphisms (SNPs) in 38 candidate genes in 280 SCD patients, the strongest association with Hb F levels was found with SNPs in the 6q 22.3–23.2 region. Detailed analyses of this region, identified 12 SNPs in the introns of four genes, associated with a 20–30% variation in Hb F [9]. The genes in question were phosphodiesterase 7 (PDE7), microtubule-associated protein 7 (MAP7), peroxisomal biogenesis factor 7 (PEX7) and mitogen-activated protein kinase 5 (MAPK5). Although the precise mechanisms of this effect have not yet been identified, there is some data to suggest that the product of these genes may be involved in the regulation of γ-globin gene expression in various cellular systems (K562 cells).

In a collaborative study between Boston University and the Medical College of Georgia Sickle Cell Centers, genetic determinants of Hb F response to hydroxyurea was studied in 214 African–American SCD patients. Two hundred twenty six SNPs in 46 candidate genes associated with Hb F regulation and drug metabolism were analyzed. SNPs in two genes, in CYP2C9, a member of the cytochrome P 450 family and in Aquaporin 9 (AQP9), a membrane channel that stimulates urea transport and transports uncharged solutes, were associated with a good response to hydroxyurea [10].

**Genetic polymorphisms as risk factors for stroke**

Stroke occurs in 11% of US sickle cell patients by 20 years of age [11]. Increased TCD (Transcranial Doppler) velocities of blood flow (>200 cm/sec) in major intracranial arteries has been shown to be a predictor of stroke risk in children with SCD. The value of TCD as a surrogate marker of cerebrovascular disease and a predictor of stroke risk has been validated in the randomized STOP trial, and a therapeutic intervention based upon this risk stratification (prophylactic transfusions in children with high TCD velocities) has been proven to reduce the stroke risk [12]. Despite the validation of the utility of TCD as a clinical tool for predicting stroke risk, biologic factors underlying the development of cerebrovascular disease and stroke in the SCD population are poorly understood. The presence of α-thalassemia has been shown to be protective of cerebrovascular disease and
stroke [11]. Several recent studies have sought an association between certain genetic polymorphisms and stroke risk in SCD. Driscoll et al. [13] found a higher rate of stroke in siblings with SCD (P = 0.0012), suggesting the role of genetic factors. A recent study has found an association with HLA genotypes and stroke (HLA DRB1*0301 and HLA DRB1*0302 genotypes were associated with increased stroke risk); of particular interest is the association of certain genotypes with distinct subtypes (large vessel vs. small vessel stroke) in a retrospective study of the CSSCD population [14,15]. This latter study found an association between an Interleukin-4 receptor polymorphism (IL-4R S503P), TNF-α -308G, and β-adrenergic receptor-2 (ADRB2 Q27E) polymorphisms and large vessel stroke. In the small vessel stroke group, VCAM T-1594C polymorphism and low density lipoprotein receptor LDLR NcoI polymorphism emerged as risk factors. The combination of TNF-α -308 GG homozygosity and the IL-4 RS503P polymorphism conferred a particularly strong risk for large vessel stroke (Odds ratio = 5.5) in this study. Another recent study [16] reported a protective role for VCAM G1238C polymorphism against symptomatic stroke (odds ratio = 0.35, P = 0.04). Steinberg et al. [17] studied 113 SCD patients with a history of stroke and 493 controls. They found that polymorphisms in four genes were associated with stroke risk: Klotho (KL), TGF-beta receptor (TGFBR3), Annexin 2 (ANXA2), and bone morphogenetic protein 6 (BMP6). The same group of investigators utilized a Bayesian network approach to analyze gene-gene interactions to develop a risk model for stroke in SCD. They analyzed 235 SNPs in 80 candidate genes in 1398 unrelated subjects with SCD. In addition to four clinical variables including α-thalassemia and Hb F, they found that SNPs in 11 genes interacted in a complex manner to modulate stroke risk. These included SNPs in BMP6, Transforming Growth Factor beta-receptors (TGFBR2 and TGFBR3), and P-Selectin (SELP). Using this model to predict stroke in a different group of SCD patients, they reported an overall predictive accuracy of 98.2% [18]. A recently completed study at our center is looking at the association of high TCD (as a risk factor for ischemic stroke) with 28 genetic polymorphisms in 25 candidate genes in 630 patients (230 high TCD, 400 normal TCD) in STOP and STOP II trials. The candidate genes include those associated with coagulation and thrombophilia (Factor V, Factor VII, Factor XIII, prothrombin, thrombomodulin, fibrinogen, PAI-1, MTHFR), endothelial cell function and inflammation (VCAM-1, ICAM-1, selectins, TNF-α, Apo A and Apo E), platelet function and activation (GpIIb/IIIa, GpIIb IX-V, GpIa/IIIa), and vascular reactivity (ACE). The analysis of polymorphisms was accomplished by a high throughput SNP genotyping method using the MALDI-TOF based Massarray™ system (Sequenom, San Diego, CA). Data analyses are currently ongoing. A summary of SNPs associated with stroke risk is summarized in Table II.

### Avascular necrosis

Studies of large patient populations such as the CSSCD (Cooperative Study of Sickle Cell Disease) have shown that α-thalassemia, age, high hematocrit, and frequent VOEs are risk factors for the development of avascular necrosis (AVN) of the femoral head in SCD [19]. We undertook a survey of the frequency of the thermolabile MTHFR (methyleneetetrahydrofolate reductase) C677T mutation in the sickle cell population, because of its association with elevated serum homocysteine levels and resultant vascular complications and in particular, its relationship to avascular necrosis. Overall, MTHFR was present in 16% of the sickle cell patients in our center (1.8% homozygote). There was a strong association of the presence of MTHFR with AVN with 35.6% of the AVN patients having the MTHFR mutation as opposed to only 12.9% of sickle cell patients without AVN (P = 0.006). Furthermore, the presence of concomitant α-thalassemia and MTHFR was found to be additive in terms of the risk of developing AVN [20]. This association however was not confirmed in the high Hb F population of Kuwaiti sickle cell patients, suggesting that different genetic factors may be operative in different populations [21]. Some other smaller studies in different patient populations have also failed to show an association between this MTHFR polymorphism and the risk of avascular necrosis in SCD [22–24]. A recently published study [25] analyzed 442 patients with AVN and 455 SCD controls from the CSSCD cohort. They studied SNPs in 66 candidate genes and found significant association of AVN with 7 SNPs in 7 genes. These are: BMP6, TGFBR2, TGFBR3, EDN1 (Endothelin-1), ERG (v-ets erythroblastosis virus E26 oncogene like), KL (Klotho), and ECE1 (Endothelin converting enzyme), and the effects of DRB1*0301, HLA DRB1*0302 genotypes were associated with increased stroke risk).
The precise mechanism(s) whereby variation in these genes causes AVN is not yet understood.

Catheter induced thrombosis and factor V R485K polymorphism

First described as a neutral polymorphism in the Factor V gene in nucleotide 1628 in exon 10 by Gandrille et al. the Arg→Val substitution at residue 485 was found at high frequency (32.4%) in sub-Saharan Africans [26]. This polymorphism was later associated with a risk of thrombosis and coronary artery disease in Asian populations and reported to lead to a mild activated protein C (APC) resistance [27]. A study of the frequency of this polymorphism in our sickle cell population showed a similar gene frequency of 0.29 (45.1% heterozygous, 6.6% homozygous). Although no association of this polymorphism was established with some complications of SCD (frequent VOEs, acute chest syndrome, leg ulcers, AVN, and priapism), interestingly the presence of this mutation was associated with an increased risk of central venous catheter associated thrombosis ($P = 0.006$, odds ratio 4.9, Table III) [28].

UGT1A1 polymorphism and bilirubin levels

The gene UGT1A1 encodes the enzyme UDP Glucuronosyl Transferase-1 that mediates the glucuronidation of bilirubin. A common polymorphism in the TATA box of this gene was found to be associated with a decreased enzyme activity and indirect hyperbilirubinemia (Gilbert’s syndrome). The sequence A(TA)6TAA is presumed the wild type while A(TA)7TAA is associated with Gilbert’s syndrome. The co-inheritance of this (TA)7 polymorphism with hereditary hemolytic anemias, such as hereditary spherocytosis, β-thalassemia, and G-6-PD deficiency results in marked hyperbilirubinemia and increased incidence of gallstones [29]. We, and others, have studied the frequency of this polymorphism in the sickle cell population and its impact on bilirubin levels and incidence of gallstones and cholecystectomy [30– 34]. In all of these studies, an association was found between the (TA)7 genotype and bilirubin levels; those with 7/7 had the highest mean bilirubin levels compared to the genotypes with 6/6 and 6/7. Our results are summarized in Table III. Furthermore, an impact of the 7/7 genotype on the results of hydroxyurea therapy was recently reported by Heeney et al. [35]. These authors reported a normalization of the bilirubin levels in sickle cell patients with the 6/6 genotype upon treatment with hydroxyurea in contrast to those with 7/7 and 6/7 genotypes whose bilirubins failed to fall to normal levels. An interesting aspect of the effect of this polymorphism was reported by Haverfield et al. in the Jamaican study. These investigators found an association between the (TA)7/ (TA)7 genotype and symptomatic gallstones except for the younger cohort. Similarly in our study, in multivariate analyses, in the younger age group (<10 years of age), Hb F levels were found to be a more important determinant of bilirubin levels then the UGT1A1 genotype. These observations indicate that the influence of genetic modifiers on a particular phenotype may vary with age in patients with SCD.

Other phenotypes

Few studies exist on genetic risk factors that may be associated with complications such as acute chest syndrome, pulmonary hypertension, leg ulcers, and priapism. Sharan et al. and reported an association of acute chest syndrome in females with a T-786C polymorphism in the endothelial NO synthase gene [36]. In one study in 45 patients with pulmonary hypertension as judged by a tricuspid regurgitant jet of >2.5 m/sec, an association was found with SNPs in BMPR2 (bone morphogenetic protein receptor 2) and ADCY6 (adenylate cyclase 6) [37]. In a study of 148 patients with SCD and history of priapism and 529 controls without priapism, it was shown that a polymorphism in the klotho (KL) gene was associated with an odds ratio of 2.6. [38]. In a study by Ashley-Koch et al. 2005, longevity (survival >50 years of age) was associated with three SNPs in the klotho gene and SNPs in the nitric oxide synthase 2 (NOS2A) and (TGFβR2) genes [39]. No studies have yet been reported showing an association of polymorphisms with a risk of sickle nephropathy.

Gene expression profiling

Gene expression profiling utilizing cDNA microarrays is a relatively novel method, particularly in terms of its applications to the study of SCD. The quality of the data obtained from microarray studies depends upon several factors: (1) a meticulous study design, parti-

<table>
<thead>
<tr>
<th>R485K</th>
<th>Thrombosis n = 10</th>
<th>Control n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>−/+</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>−/−</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

P = 0.006; Odds Ration = 4.9.

<table>
<thead>
<tr>
<th>7/7 (n = 22)</th>
<th>6/7 (n = 35)</th>
<th>6/6 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retics 10.9</td>
<td>11.4</td>
<td>11.3</td>
</tr>
<tr>
<td>Hb F 6.6</td>
<td>11.1</td>
<td>8.4</td>
</tr>
<tr>
<td>Total bilirubin 5.6</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Cholecystectomy 59%</td>
<td>51%</td>
<td>60%</td>
</tr>
</tbody>
</table>
cularly a clear and unambiguous definition of the “phenotype(s)” to be studied, (2) elimination of technical/methodological pitfalls (quantity/quality of the RNA, methods of specimen collection and processing, conditions and timing of hybridization, elimination of intra-patient variation by repeated sampling, etc.), and (3) expertise in bioinformatics in the interpretation of vast amounts of data generated. In patients with SCD, the tissues that will be amenable to gene expression profiling studies include peripheral blood (PB) cells, endothelial cells (EC) (circulating EC, as well as EC from various tissues), bone marrow (BM), and liver tissue.

Few published reports exist in this area. These include the study of gene expression profiling from the kidney tissue in transgenic sickle cell mice [40], profiling of cultured human pulmonary artery endothelial cells exposed to plasma from patients with SCD and acute chest syndrome and patients with SCD in steady state [41,42], a study of differential gene expression in PB cells from SCD patients with different crisis frequency [43], and a study of gene expression profiles form mononuclear cells in SCD patients on and off hydroxyurea (HU) therapy [44]. Jison et al. purified mononuclear cells from PB in 27 patients with SCD in steady state, 10 of which were on HU and 13 normal controls. They demonstrated differential gene expression of 112 genes involved in heme metabolism, cell cycle regulation, antioxidant and stress responses, inflammation, and angiogenesis. HU did not significantly alter mononuclear cell gene expression. Klings et al. exposed cultured human pulmonary artery endothelial cells to plasma from normal volunteers as well as to plasma from SCD patients at steady state and SCD patients with acute chest syndrome. They found that 50 genes were differentially expressed in endothelial cells upon exposure to plasma from SCD patients in steady state compared to normal plasma. These genes involved cholesterol biosynthesis, lipid transfer, cellular stress response, and extracellular matrix proteins. Upon exposure to plasma from SCD patients with acute chest, another 58 genes were differentially expressed.

We have conducted preliminary studies of gene expression with commercially available cDNA microarrays (Affymetrix U95, Affymetrix, Santa Clara, CA) from PB neutrophils and reticulocytes in SCD patients with discordant rates of painful episodes in an effort to elucidate the factors contributing to different crisis frequency. We analyzed the gene expression profiles of neutrophils from four patients with "se-

Table V. Demographic and hematologic data in mild vs. severe SCD patients (mean values).

<table>
<thead>
<tr>
<th></th>
<th>Mild (n = 8)</th>
<th>Severe (n = 4)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>6 M, 2 F</td>
<td>2 M, 2 F</td>
<td>0.03</td>
</tr>
<tr>
<td>Age</td>
<td>35.5</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Crisis/year</td>
<td>1.1</td>
<td>5.8</td>
<td>0.0002</td>
</tr>
<tr>
<td>Hb g dL⁻¹</td>
<td>8.9</td>
<td>8.6</td>
<td>0.6</td>
</tr>
<tr>
<td>HCT%</td>
<td>27.4</td>
<td>24.9</td>
<td>0.4</td>
</tr>
<tr>
<td>MCV fl</td>
<td>91</td>
<td>96</td>
<td>0.4</td>
</tr>
<tr>
<td>Retics%</td>
<td>10.7</td>
<td>10.8</td>
<td>0.8</td>
</tr>
<tr>
<td>WBC × 10³ l⁻¹</td>
<td>11.6</td>
<td>11.2</td>
<td>0.8</td>
</tr>
<tr>
<td>ANC (per μl)</td>
<td>6005</td>
<td>5850</td>
<td>0.9</td>
</tr>
<tr>
<td>Platelets × 10⁹ l⁻¹</td>
<td>376</td>
<td>341</td>
<td>0.6</td>
</tr>
<tr>
<td>Total Bilirubin mg dL⁻¹</td>
<td>3.8</td>
<td>6.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Hb F%</td>
<td>6.3</td>
<td>10.7</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Figure 1. Granulocyte gene expression profiling: Severe and mild patients vs. controls.
vere” disease (>3 vaso-occlusive episodes [VOE] per year), eight patients with “mild” disease (<3 VOE/year) and compared these to each other and to the gene expression profiles of neutrophils from five age and sex matched, healthy, non-sickle cell, African-American individuals. A summary of demographic features, mean hematoic values, and Hb F in “mild” and “severe” SCD patients is shown in Table V. Figure 1 depicts differences in gene expression patterns between non-sickle cell controls and mild and severe SCD patients. In general, a larger number of genes were differentially expressed between “mild” patients vs. controls, compared to that between “severe” vs. “mild” patients. Out of the differentially expressed genes (314 genes for severe vs. control, 718 genes for mild vs. control), those with greater than two fold expression were analyzed with the gene MAPP software for localization into biological pathways. Genes related to cellular proliferation, growth and maintenance, DNA repair, DNA replication, and cell cycle progression were expressed at significantly higher levels in SCD patients compared to controls. The most significant finding was the significantly higher expression of genes leading to NFkB activation and inhibition of apoptosis: IAP-1 (increased 6.7 fold and 4.7 fold in mild and severe patients respectively), IkB (decreased 0.14 fold and 0.3 fold), Apaf-1 (decreased 0.4-fold in mild), and c-jun (decreased 0.4-fold in severe); Traf-2 (TNF receptor associated factor-2; increased 3.5-fold and 2-fold); genes in the MAPK signaling pathway: ERK-2 (increased 3.5-fold and 2-fold), MAP2K3 (increased 3.5-fold and 2-fold). These data show that neutrophils in SCD patients are activated with higher expression of genes in the TNF, MAPK, and NFkB pathways consistent with an inflammatory state. Since neutrophil apoptosis is considered critical for the resolution of inflammation, delayed or inhibited apoptosis of neutrophils would further maintain this inflammatory state, even during the so-called “steady state” of the disease. Further analyses and identification of differentially expressed genes and pathways between “mild” patients vs. controls and “severe” vs. “mild” patients is in progress. A list of some of the differentially expressed genes in neutrophils from “severe” and “mild” patients is shown in Table VI. We conclude that the analyses of gene expression in neutrophils can be a useful tool in identifying pathways and genes that distinguish SCD patients from controls and in differentiating mild and severe phenotypes.

The application of the microarray technology to the study of SCD is still in its infancy. Although the preliminary data look interesting, these results need to be confirmed and validated through a large number of experiments in a large number of patients. The available data appear promising and show the feasibility of the application of this technology to SCD.

### Table VI. Differentially expressed genes in severe vs. mild SCD patients.

<table>
<thead>
<tr>
<th>Fold Difference</th>
<th>Gene ID</th>
<th>Gene Function</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.6</td>
<td>NAD Kinase</td>
<td>Signal Tx, Energy Metabolism, Detox Rx</td>
<td>0.005</td>
</tr>
<tr>
<td>9.5</td>
<td>GPR43</td>
<td>7 Transmem domain rec for signal tx for a neuroendocrine pp, Galanin</td>
<td>0.004</td>
</tr>
<tr>
<td>6.4</td>
<td>BTG family</td>
<td>Cell cycle regulation, antiproliferative</td>
<td>0.003</td>
</tr>
<tr>
<td>5.4</td>
<td>HLA-G2.2</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>4.7</td>
<td>FLJ13052</td>
<td></td>
<td>0.005</td>
</tr>
<tr>
<td>4.6</td>
<td>Similar to tumor necrosis factor</td>
<td>Inhibits TRAIL (1.2) mediated apoptosis</td>
<td>0.007</td>
</tr>
<tr>
<td>4.4</td>
<td>HLA-G1</td>
<td>Role in inhibiting natural killer cell function, tumors can escape from immunrv.</td>
<td>0.004</td>
</tr>
<tr>
<td>3.8</td>
<td>HLA-G2.1</td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>3.2</td>
<td>IFI30</td>
<td>Catalyzes disulfide bond reduction for proteolysis of the internalized proteins. Const. Expresed in Ag pre cells and induced by IFNγ in other Imp in Ag presentation</td>
<td>0.005</td>
</tr>
<tr>
<td>3.1</td>
<td>HLA-DRB1</td>
<td></td>
<td>0.005</td>
</tr>
<tr>
<td>3.0</td>
<td>KIAA0991</td>
<td>Rearranged with MLL in therapy induced AML</td>
<td>0.009</td>
</tr>
<tr>
<td>2.6</td>
<td>Helix-loop-helix zipper protein</td>
<td>NFkB activation by inhibiting the IκB proteins</td>
<td>0.004</td>
</tr>
<tr>
<td>2.4</td>
<td>Aryl Hydrocarbon receptor-interacting protein</td>
<td>Aryl hydrocarbon receptor (AHR) – HSP90 complex translocates to nucleus, HSP90 dissociates and activated AHR dimerizes with ARNT w then bind enhancer elements to regulate transcription of xenobiotic metabolic enzymes</td>
<td>0.002</td>
</tr>
<tr>
<td>2.4</td>
<td>RPL28</td>
<td>Structural mammalian ribosomal protein</td>
<td>0.002</td>
</tr>
<tr>
<td>2.3</td>
<td>A20</td>
<td>Inhibits NFκB activity and TNF mediated apoptosis. Critical for limiting inflammation by terminating TNF induced NFκB responses. TNF dramatically increases A20 expression in all ts.</td>
<td>0.004</td>
</tr>
<tr>
<td>2.1</td>
<td>DUT</td>
<td>Hydrolyzes d UTP to d UMP. Limits d UMP incorporation into DNA during replication and repair and protects from fragmentation and death</td>
<td>0.008</td>
</tr>
</tbody>
</table>

### Conclusion

The development of high throughput genotyping methods, the microarray technology, and the comple-
A candidate gene approach and study of genomewide SNPs and their association with certain complications of SCD is expected to further clarify the basis of clinical/phenotypic heterogeneity of this single gene disorder. The pros and cons of a candidate gene approach vs. genome wide association studies need to be balanced carefully. A candidate gene approach is limited by our current knowledge of the pathophysiology and by necessity may omit some of the genetic influences that may contribute to the genotype in question. It is also of utmost importance to carefully and unambiguously define the phenotype. Genome-wide studies are likely to provide some information on novel genetic influences; however, there limitation is the unrealistically large sample size that may be required. Application of methods such as the Bayesian networks may be important in providing information on gene–gene interactions.

Although the association studies and the results of microarray data reviewed above are far from conclusive, some interesting points and recurring themes have emerged. Many complications of SCD can be related to variation in genes in the TGF-beta super family and genes that play a role in vascular reactivity, inflammation, and apoptosis. These are emerging also from the microarray data. A study of gene expression profiling coupled with proteomics will shed further light into the functional aspects and differences between patients. This information will not only lead to a better understanding of the pathogenesis and pathophysiology of many complications of the disease, but will also likely result in the identification of novel therapeutic targets and discovery of new genes with prognostic and therapeutic implications.

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[2] Hebbel RP, Vercellotti GM. The endothelial biology of sickle cell disease. J Lab Clin Med 1997;129:288–293.


Juvenile myelomonocytic leukemia (JMML) is a rare clonal myeloproliferative disorder (MPD) of early childhood [1]. The median age at diagnosis is 2 years [1]. There is a male predominance with a male:female ratio of 2:1. Pallor, fever, infection, skin bleeding and cough are the most common presenting symptoms. Typically, there is marked hepatosplenomegaly. JMML rarely involves the central nervous system.

**Laboratory findings**

Laboratory findings include leukocytosis, anemia and thrombocytopenia. The median white count is $33 \times 10^9/L$ [1]. An absolute monocyte count greater than $1 \times 10^9/L$ is required for the diagnosis [2]. The blast cell percentage in peripheral blood (PB) rarely exceeds 20% and is most often below 2% [1]. Cytogenetic studies of JMML cells show a normal karyotype in 65% of patients, monosomy 7 in 25%, and other abnormalities in 10% [1]. Patients with monosomy 7 present with a normal or moderately elevated HbF. In patients with normal karyotype HbF is often elevated [1].

**Aberrant Ras signaling**

The Ras signaling transduction pathway is deregulated in JMML. JMML myeloid progenitors are hypersensitive to granulocyte-macrophage colony stimulating factor (GM-CSF) in vitro [3]. This observation is linked to mutations that lead to aberrant Ras signaling. Approximately 35% of the cases harbor somatic mutations in exons 3 or 13 of the PTPN11 gene [4–6]. The PTPN11 gene product, SHP-2, is a non-receptor protein tyrosine phosphatase that relays signals from growth factor receptors to Ras and other signaling molecules [7]. Ras genes encode 21-kDa signal switch molecules that regulate cell fates by cycling between inactive GDP-bound (Ras-GDP) and active GTP-bound (Ras-GTP) conformations. Somatic NRAS or KRAS2 mutations are identified in 15–25% of JMML cases [8,9]. These lesions lead to substitutions at codons G12, G13, or Q61 and impair the intrinsic Ras GTPase and confer resistance to GTPase-activating proteins. In 11% of patients with JMML the clinical diagnosis of neurofibromatosis type 1 (NF1) can be made [10]. However, it has been shown that some infants with JMML carry germ line neurofibromatosis type 1 gene (NF1) mutations although the diagnosis of NF1 had not been made clinically [11]. Neurofibromin, the gene product of the NF1 tumor suppressor gene, negatively regulates Ras by functioning as GTPase activating protein. Thus, loss of neurofibromin leads to aberrant Ras signaling. Indeed, analysis of JMML cells from patients with NF1 showed an elevated percentage of Ras in the active GTP-bound conformation [12]. Taken together, approximately 85% of all patients with JMML carry mutations in PTPN11, RAS, or NF1 (Figure I). Of note, these mutations are largely mutually exclusive suggesting that they disrupt identical pathways in myeloid cells [4,5].

**Murine models**

Mouse models support the idea that in JMML mutations in NF1, RAS, or PTPN11 are initiating rather than later cooperating events. By generating mice whose hematopoietic system was reconstituted with NF1 deficient hematopoietic stem cells it has been shown that NF1 loss, by itself, is sufficient to produce a JMML-like disorder in mice [13]. In addition, somatic inactivation of NF1 results in a fatal myeloproliferative disease with shift in hematopoiesis from the BM to spleen, an increased number of T-and B-cells and resistance to apoptosis [14]. Similarly, somatic activation of oncogenic Ras in hematopoietic cells initiates a rapidly fatal myelopro-
liferative disorder [15]. Finally, in a murine model of Noonan syndrome (see below), mice carrying an activated PTPN11 allele develop a mild myeloproliferative disease [16].

**Noonan syndrome and juvenile myelomonocytic leukemia**

Noonan syndrome is an autosomal dominant developmental disorder characterized by dysmorphic facial features, growth retardation and variable congenital heart defects. In one half of the cases the disorder is caused by heterozygous germ line PTPN11 mutations [7]. A small number of infants with Noonan syndrome develop a JMML-like disease [17]. In these young children with Noonan syndrome and JMML-like disease, the spontaneous remission of the disorder is well documented [17]. PTPN11 mutations observed in Noonan Syndrome are predicted to have generally mild gain-of-function effects. In contrast, PTPN11 mutations associated with JMML lead to stronger activation of SHP-2. Mutations in patients with Noonan syndrome who develop a JMML-like disease are predicted to have intermediate effects. Consistent with this model, in vitro and in vivo experiments on primary hematopoietic cells and cell lines show that somatic mutants confer more pronounced effects on cell growth than common mutants only found in Noonan syndrome [18,19].

**Diagnosis**

Diagnostic criteria have been proposed by the International JMML Working Group in 1998 (Table I) [2]. However, molecular studies have greatly facilitated the diagnostic approach and mutational studies have become an important tool in the diagnostic process of JMML. JMML can be mimicked by a variety of infectious agents such as cytomegalovirus, Epstein-Barr virus, human herpes virus 6 and parvovirus B19. Positive results of these viruses do not exclude the diagnosis of JMML.

**Natural course if the disease**

JMML is a rapidly fatal disorder for most children if left untreated. The median survival time without hematopoietic stem cell transplantation (HSCT) is

![Figure I. Model outlining the roles of SHP-2, RAS, and NF1 in the granulocyte-macrophage colony-stimulating factor (GM-CSF) signal transduction pathway. In juvenile myelomonocytic leukemia (JMML), molecular alterations have been demonstrated in PTPN11 (the gene encoding SHP-2), RAS and NF1 in approximately 35%, 25% and 25% of patients, respectively.](image-url)
about 1 year [1]. Low platelet count, age above 2 years at diagnosis and high HbF at diagnosis are main predictors of short survival [1]. Blastic transformation is infrequent in JMML. Most untreated patients die from respiratory failure caused by infiltration of the lungs with JMML cells.

**Antileukemic therapy**

The role of antileukemic therapy prior to HSCT is currently uncertain. The current JMML study of the Children’s Oncology Group (COG) prescribes cytoreductive therapy consisting of fludarabine and high-dose cytarabine concomitantly with 13-cis retinoic acid prior to HSCT, while most patients in Europe traditionally receive mercaptopurine or no therapy. Therapeutic strategies targeting individual components of the Ras signaling pathway include the administration of the GM-CSF analog E21R and the farnesyltransferase inhibitor R115777 (Zarnestra®) [20].

**Stem cell transplantation**

Allogeneic HSCT is currently the only curative treatment for JMML. The analysis of the EWOG-MDS/EBMT trial of 100 patients with JMML transplanted with a preparative regimen of busulfan, cyclophosphamide and melphalan shows a 5-year probability of EFS of 52% [21]. The EFS of patients transplanted from a matched family donor (MFD) and unrelated donor (URD) are not significantly different. In the current EWOG-MDS/EBMT trial, the 5-year cumulative incidence of relapse was 35% [21]. Relapse occurs early, at a median of 2 to 6 months from transplantation [21] and generally within the first year. HSCT shortly after diagnosis is recommended, because younger age at HSCT predicts improved survival [21]. In the prospective HSCT study from EWOG-MDS, multivariate analysis shows age greater than 4 years and female sex predicted poorer outcome [21]. Cytogenetic abnormalities do not confer a worse prognosis [21]. In the current HSCT study of the EWOG-MDS, splenectomy did not improve the survival after HSCT [21].

**Relapse**

Disease recurrence remains the major cause of treatment failure. Median time from HSCT to relapse is 4–6 months with only few patients relapsing more than 1 year after transplantation [21]. Graft-versus-leukemia (GVL) effect plays an important role in curing children with JMML with HSCT. Re-emerging donor cells and frank hematologic relapse have been successfully eradicated by reduction of ongoing immunosuppressive therapy. Reducing the intensity and duration of graft-versus-host disease (GVHD) prophylaxis may significantly contribute to successful leukemia control. However, donor lymphocyte infusion in JMML relapse has largely been unsuccessful [22]. Serial quantitative chimerism studies can identify patients with increasing mixed chimerism who are at high risk for relapse of JMML. Immediate withdrawal of immunosuppressive therapy is advised in these patients [23]. Despite aggressive re-emergence of the malignant clone and short interval between the first and second HSCT, a substantial proportion of children can be cured after a second HSCT [24].

**References**


Juvenile myelomonocytic leukemia


Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening disease characterized by unremitting fever, hepatosplenomegaly, cytopenias, as well as changes in coagulation and lipids. The symptoms are due to high levels of cytokines secreted by immune effector cells which display functional defects.

History and classification (Table I)
HLH was first described by Farquhar and Claireaux in 1952 as a familial disease [1]. Interestingly in their cases hemophagocytosis, which has given the disease its name, could not be found during lifetime but was prominent on autopsy. Familial hemophagocytic lymphohistiocytosis (FHLH) is an autosomal recessive disorder with an estimated frequency of 0.12/100,000 children per year [2]. Several genetic defects have been described for FHLH (see below). In addition well-characterized immune deficiency syndromes such as Chédiak-Higashi syndrome (CHS), Griscelli syndrome (GS), and x-linked proliferative syndrome (XLP) may present initially with HLH or develop HLH later [3]. Whereas in FHLH the symptoms of HLH are the primary and only manifestation, the occurrence of HLH in these immune defects is optional.

In 1979, Risdall et al. described a picture indistinguishable from FHLH in adults who received immunosuppressive treatment after organ transplantation and experienced a viral infection [4]. A few children were included and not in all patients a virus could be identified. The disease was named virus-associated hemophagocytic syndrome (VAHS). Subsequently it became evident that any infectious agent including bacteria, protozoa and fungi could trigger HLH [5,6]; thus the term infection-associated hemophagocytic syndrome (IAHS) replaced VAHS. Viruses, however, remain the most frequent triggering agents with Epstein-Barr virus as the leading organism, followed by cytomegalovirus and other herpes viruses. A fairly frequent cause for HLH is infection by leishmania, an organism for which very effective treatment exists.

IAHS occurs in children and adults and is probably more frequent than the familial form. In contrast to the first publication by Risdall most patients with IAHS reported subsequently had no known underlying immune deficiency.

It has to be emphasized that the identification of an infectious organism does not help to discriminate between FHLH and IAHS since the former is also triggered by an infection in most cases. Age is the only parameter which may be helpful to distinguish both forms; 70% of FHLH cases occur within the first year of life whereas IAHS patients are usually older. However, in the author’s experience about 10% of babies with HLH have a transient and therefore not familial form; on the other hand FHLH has occasionally been described in older children.

The picture of HLH in patients with rheumatic diseases, especially systemic onset juvenile rheumatoid arthritis, is commonly named macrophage-activation syndrome (MAHS) [7]. It has recently been suggested that this condition be included as a separate entity in the category of acquired HLH [8]. HLH can also be a complication in patients with malignant diseases especially lymphomas and some inborn errors of metabolism [9].

Genetics and pathophysiology
HLH arises on the basis of various genetic or acquired immune deficiencies. Only recently several genetic defects have been described in FHLH: Mutations in the perforin gene [10], the UNC13D gene [11], and the syntaxin 11 gene [12]. Whereas in our experience mutations in one of these three genes can be found in 80% of the Turkish patients, only 30% of the German
patients harbored a perforin or UNC13D mutation. Mutations in syntaxin 11 so far have only been found in patients from Turkey. The genes implicated in the pathogenesis of Chédiak-Higashi syndrome (the LYST gene), Griscelli syndrome (The RAB27A gene) and x-linked proliferative syndrome (the SH2D1A gene) have also been identified. In a substantial number of patients thought to have genetic disease based on a positive family history or a relapsing course, the gene defect is as yet unknown.

The clinical picture of HLH is due to hyperinflammation caused by hypersecretion of inflammatory cytokines by activated T-lymphocytes and histiocytes infiltrating all organs. In spite of the excessive activation and expansion of cytotoxic cells, patients with HLH have severe impairment of cytotoxic function of natural killer cells and cytotoxic T cells [13]. All known defects in HLH are involved in the function of cytolytic granules; granule trafficking (LYST), docking at the membrane (RAB27A), granule priming (UNC13D), deficient granule content (PFR), impaired lymphocyte activation (SH2D1A) and probably impaired interaction between dendritic and killer cells (syntaxin 11). Defective cytolytic activity seems to be the common denominator which predisposes to HLH.

The mechanisms leading to impaired cytotoxic function in apparently immune competent patients with acquired HLH are less clear. Interference by cytokines or virus-encoded proteins are possible mechanisms. The prevalence of EBV associated HLH in Asia suggests a specific genetic susceptibility.

Clinical symptoms and laboratory findings (Table II)

The disease typically starts after a free interval weeks to months after birth. In rare cases already newborns become symptomatic. The initial symptoms at first are compatible with a normal infection. The first sign is high fever frequently associated with signs of an upper respiratory or gastrointestinal infection. Nearly all patients have an enlarged liver and/or spleen. Enlarged lymph nodes, transient rashes and neurological symptoms such as an opisthotonic posture, seizures or cerebral nerve palsies are less frequent [14].

Table II. Symptoms and laboratory findings at first presentation and at diagnosis*

<table>
<thead>
<tr>
<th>Symptom</th>
<th>At first presentation % of patients</th>
<th>At diagnosis % of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>78</td>
<td>100</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>78</td>
<td>98</td>
</tr>
<tr>
<td>Bicytopenia</td>
<td>56</td>
<td>98</td>
</tr>
<tr>
<td>Ferritin</td>
<td>23</td>
<td>65</td>
</tr>
<tr>
<td>Triglycerides &gt;3 mmol l⁻¹</td>
<td>52</td>
<td>76</td>
</tr>
<tr>
<td>Hemophagocytosis</td>
<td>36</td>
<td>88</td>
</tr>
<tr>
<td>NK cell activity negative or decreased sCD25 = &gt;2400 U ml⁻¹</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Ferritin</td>
<td>57</td>
<td>70</td>
</tr>
<tr>
<td>LDH = &gt;500 UI l⁻¹</td>
<td>55</td>
<td>51</td>
</tr>
<tr>
<td>ALT = &gt;100 UI l⁻¹</td>
<td>30</td>
<td>38</td>
</tr>
<tr>
<td>Bilirubin &gt;34 μmol l⁻¹</td>
<td>30</td>
<td>32</td>
</tr>
<tr>
<td>CSF cells = &gt;5 μl l⁻¹</td>
<td>32</td>
<td>39</td>
</tr>
<tr>
<td>CSF protein = 0.5 gl l⁻¹</td>
<td>32</td>
<td>46</td>
</tr>
</tbody>
</table>

* (Janka et al. 2005) sCD25 = α chain of the soluble interleukin 2 receptor; LDH = lactate dehydrogenase; ALT = alanine aminotransferase; CSF = cerebrospinal fluid. In some instances (sCD25, LDH) percentage of patients with high values was lower at diagnosis due to unspecific therapies.

Typical laboratory values are thrombocytopenia and anemia, and less frequently neutropenia. Pancytopenia is progressive in untreated patients. Characteristic biochemical findings are high triglycerides, a low fibrinogen or a global coagulation disorder, and high ferritin. Less frequent are high transaminases, bilirubin and LDH, low total protein and hyponatremia [9]. A bone marrow examination is mandatory but only a minority of patients have hemophagocytosis at presentation. A bone marrow biopsy is usually even less sensitive. Characteristically the bone marrow is cellular in spite of profound peripheral pancytopenia. Erythropoesis is usually increased and may exhibit dysplastic changes. The cerebrospinal fluid shows a moderately increased cell count and/or protein content in more than 50% of the cases even in the absence of neurological symptoms.

A valuable disease marker is a high level of the α chain of the soluble interleukin 2 receptor (sCD25). Impaired natural killer (NK) cell activity is a hallmark of the disease. It is found in FHLH, CHS, GS and XLP as well as in acquired HLH. In the latter, impaired NK cell activity may be due in part to reduced numbers of NK cells and is usually reversible.

Diagnostic criteria and problems

The revised diagnostic criteria of the Histiocyte Society are shown in Table III.

At initial presentation the diagnosis may be difficult because of several reasons:

- an infectious organism is identified, a normal infection suspected and the severe symptoms of HLH are overlooked
Clinical and laboratory criteria (5/8 criteria)

- Familial disease/known genetic defect
- Hemophagocytosis in bone marrow, CSF or lymphnodes
- Decreased or absent NK-cell activity
- sCD25 $> 2400 \text{ U ml}^{-1}$
- Cytopenia $> 2$ cell lines
- Hypertriglyceridemia and/or hypofibrinogenemia
- Ferritin $> 500 \text{ ng ml}^{-1}$
- Splenomegaly
- Fever
- Hypertransaminasemia and/or elevated protein, elevated transaminases and bilirubin, LDH $> 1000 \text{ U}^{-1}$

Supportive evidence are cerebral symptoms with moderate pleocytosis and/or elevated protein, elevated transaminases and bilirubin, LDH $> 1000 \text{ U}^{-1}$

* Janka and Schneider 2004; † For method see Schneider et al. 2002.

### Therapy and prognosis

Untreated familial disease is fatal in all cases. Also acquired IAHS has a high fatality rate of 50% in children [16]. If a treatable organism is found appropriate therapy should be given but with the possible exception of leishmaniasis antiinfectious therapy is usually not sufficient to control HLH. The immediate aim of treatment is to suppress hypercytokinemia that is responsible for the life-threatening symptoms. In familial cases this has to be followed by stem cell transplantation (SCT) as the only curative disease. Since many patients do not have a family history or a proven genetic defect a surrogate marker for genetic disease is persistent disease activity or relapses on or off treatment. In patients without family history and complete resolution of all symptoms elective cessation of therapy is recommended to prevent an unnecessary SCT for transient, acquired HLH. This is not without risk since a relapse may be accompanied by severe symptoms. Thus these patients have to be closely monitored to restart therapy in time.

Standard treatment for HLH is a combination of corticosteroids, cyclosporin A and etoposide. All patients with known familial disease, suspected genetic disease due to age below 1 year and patients with life-threatening symptoms such as coagulopathy, profound cytopenia or neurological disease should receive therapy according to the present HLH 2004 protocol (available at website: www.histio.org/society/protocols). Etoposide may be life-saving especially in patients with EBV-associated HLH [17]. There are a few patients with acquired HLH and mild symptoms in whom corticosteroids and immunoglobulins may be sufficient. However, disease may progress rapidly and this risk outweighs the possible side effects of etoposide. For children with MAS treatment with high doses of corticosteroids and/or cyclosporin A is recommended.

Reactivation during treatment is a frequent problem, either systemically or in the CNS. Immunosuppressive treatment should be reinforced and SCT be performed as early as possible. Intrathecal therapy is recommended in CNS relapses in view of the deleterious long-term effects of uncontrolled CNS disease.

Most children respond to treatment within 1–4 weeks. There is no established salvage therapy for non-responders. Antithymocyte globulin which was shown to be effective as first line treatment [18] is rarely effective when the HLH protocol fails. Single patients have responded to daclizumab or alemtuzumab.

The results of the HLH 94 study have recently been published [19]. The overall survival rate in 113 children was 55% at 3 years. Patients with a transplant from a matched sibling donor (MSD) or matched unrelated donor had the same results.

### Conclusion

HLH is a well described clinical entity characterized by fever, hepatosplenomegaly, cytopenias and various laboratory changes. Hyperinflammation is due to genetic or acquired cytotoxic defects of immune effector cells. HLH is still frequently overlooked. Treatment with chemoimmunotherapy in due time controls the disease in most patients and facilitates SCT for genetic cases with a high cure rate.
References


PEDIATRIC HEMATOLOGY

Fanconi anemia: Current management

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Abstract

Fanconi anemia (FA) is an autosomal recessive chromosomal instability disorder, characterized by congenital anomalies, defective hematopoiesis and a high risk of developing acute myeloid leukemia and certain solid tumors. All racial and ethnic groups are at risk, and at least 11 complementation groups have been identified and the genes defective in eight of these have been identified (FANCA, C, D2, E, F, G, L and BRCA2). FA-A is the most common complementation group, accounting for approximately 65% of all affected individuals. The gold-standard screening test for FA is based on the characteristic hypersensitivity of FA cells to the crosslinking agents, such as mitomicin C or diepoxybutane. Recent progress has been made in identifying the genes bearing pathogenetically relevant mutations, but slower progress has been made in defining the precise functions of the proteins in normal cells, in part because that the proteins are multifunctional. Molecular studies have established that a common pathway exist, both between the FA proteins and other proteins involved in DNA repair such as NBS1, ATM, BRCA1 and BRCA2. Stem cell transplantation (SCT) is the only option for establishing normal hematopoiesis. To reduce undue toxicities due to inherent hypersensitivity, nonmyeloablative conditioning for transplants has been advocated. This review summarizes the general clinical and hematologic features and the current management of FA.

Fanconi anemia (FA) is the commonest type of inherited bone marrow failure syndrome with the birth incidence of around three per million. The inheritance pattern is autosomal recessive with the estimated heterozygote frequency being one in 300 in Europe and the US.

Clinical features

The most frequent characteristic birth defects in FA include skin hyperpigmentation and/or café au lait spots (55%), short stature (51%), abnormal thumbs and radii (43%), abnormal head (26%), eyes (23%), kidneys (21%) and ears (21%). Low birth weight (<2,500 g) and developmental disability are found in 11% of patients, respectively. However, 25% or more of known FA patients have few or none of these features.

The most important clinical features of FA are hematological and these are responsible for the greatest morbidity and mortality in homozygotes. At birth, the blood count is usually normal and macrocytosis is often the first detected abnormalities. This is followed by thrombocytopenia and anemia, and pancytopenia typically presents between the ages of 5 and 10 years, with the median age of onset being 7 years. Moreover, patients with FA may even present with myelodysplastic syndrome (MDS), or acute myeloid leukemia (AML). The relative risk of AML is 800-fold, and the median age in reported cases is 14 years.

Also, patients with FA are at a particularly high risk of developing specific solid tumors at unusually young ages, including head, neck, esophagus, and gynecological squamous cell carcinomas, as well as liver tumors. The relative risk of developing total cancers is 48 in FA patients in comparison with general population, with the highest relative risk of 4,300 for vulva and/or anus tumor.

Therefore, FA should be considered in any young adult with any of the following subtle but characteristic physical anomalies, hematologic cytopenias, unexplained macrocytosis, MDS/AML, or squamous cell cancer even in the absence of severe pancytopenia or a positive family history.

FA diagnosis

In the proper clinical context the gold-standard screening test for FA is based on the characteristic
hypersensitivity of FA cells to the crosslinking agents (mitomycin C [MMC], diepoxybutane [DEB], cisplatin). Culture of replicative cells (usually phytohemagglutinin [PHA]-stimulated peripheral blood lymphocytes or skin fibroblasts) in the presence of low doses of either MMC, or DEB followed by examination of metaphase spreads for evidence of chromosomal breaks and radials chromosomes can establish the diagnosis of FA. Data are reported as aberration per cell, as well as percentage of cells with aberrations, usually for 20 to 100 cells. The percentage of cells with aberration may be useful, because patients with hematopoietic somatic mosaicism may have only a few cells with breaks.

Flow cytometry can detect the proportion of cells that are arrested at G2/M cell cycle after culture with a clastogen such as nitrogen mustard. It has advantage of examining thousands of cells and is less labor-intensive and subjective, but it is usually done in a specialized laboratory.

Complementation analysis requires patient lymphocytes, EBV-lymphoblasts, or fibroblasts to culture with cells or retroviruses which introduce known normal FANC genes into the patient’s cells. This test is limited to the availability of cells or cloned DNA from known FA genotypes, and is performed in a very limited number of research laboratories. Mutated genes can also be identified by denaturing high performance liquid chromatography (DHCLP) heteroduplex analysis.

**Genetics and molecular pathogenesis**

At least eleven complementation groups are known to date. Genes for 8 groups have been characterized (FANCA, C, D2, E, F, G, L and BRCA2). FANCA is the most common complementation group, in which more than 200 mutations have been documented. Identification of the 8 subtypes facilitated the cloning of the FA genes. The first gene, FANCC on chromosome 9q22.3, was discovered in 1992. The breast cancer susceptibility gene, BRCA2, has been identified as a FA gene (formerly known as FANCD1); biallelic mutations in BRCA2 have been observed in FA subtype B and D1 cells, suggesting that BRCA2 is the FANC gene corresponding to both of these complementation groups.

Knock-out mouse models of FA provide insight into the role of individual mutations. Knock-out of FANCA and FANCG and two different knock-outs of FANCC have been generated in mice. The phenotype of these mutant mice is identical and consists of cellular sensitivity to DNA cross-linking agents, abnormal G2-M progression of the cell cycle similar to Fanconi patients, and hypoplasia of gonads.

Cells and cell lines from Fanconi patients are phenotypically similar, regardless of the complementation group that they represent. The hypothesis that Fanconi proteins A, C, D2, E, F, and G function in a common cellular pathway was substantiated by data showing that proteins A, C, E, E, F, and G form a constitutive nuclear protein complex. Activation of this protein complex by DNA damage or cell cycle progression results in the conversion of the downstream FANCD2 protein from an unubiquitinated isoform for from to a monoubiquitinated isoform. Monoubiquitination does not occur if the protein complex A to G is not intact, and therefore Fanconi cells from A, C, E, F, and G patients do not show monoubiquitinated FANCD2. In normal cells after monoubiquitination, FANCD2 localizes to nuclear foci where it co-localizes with other DNA-repair proteins such as BRCA1. Although BRCA2 may also be genetically linked to the Fanconi pathway, its exact role is unclear.

A genotype-phenotype study examined the consequences of mutations of FANCC in patients. Kaplan-Meier analysis showed that IVS4 or exon 14 mutations define poor-risk subgroups clinically, they are associated with earlier onset of hematologic abnormalities and poorer survival compared with patients with other exon I mutation and with the non-FANCC population. This was confirmed in a 20-year follow-up perspective by the International Fanconi Anemia Registry (IFAR). Another report describing the association of complementation group and mutation type with clinical outcome showed that Fanconi patients with mutations in the FANCG gene and those homozygous for null mutations in FAANCA are also high risk groups with a poor hematologic outcome.

**Management**

The projected median survival of patients with FA is approximately 30 years but survival is extraordinary variable. The most life-threatening early event is bone marrow failure. Matched sibling donor (MSD) SCT is now accepted as the best therapy available to cure the FA patient of marrow aplasia and to prevent progression to myelodysplasia or leukemia. An otherwise healthy patient with FA and significant pancytopenia (ANC <1,000/mm^3, hemoglobin ≤8 g/dL or a platelet count ≤40–50,000/mm^3) with an available HLA-MSD is an excellent candidate for hematopoietic SCT. Initial efforts to transplant FA patients using standard preparative regimens and graft-versus-host disease (GVHD) prophylaxis were plagued by two serious and often lethal problems: severe toxicity from chemotherapy and exaggerated GVHD. Bone marrow transplantation (BMT) protocols were subsequently modified for FA, and the outcomes improved substantially.

The data on HLA-identical MSD SCT performed on 151 FA patients from 42 institutions were summarized in 1995 with the 2-year survival rate of 66%.
Using cyclophosphamide (total dose, 20 mg/kg) and thoracoabdominal irradiation (5 Gy) for conditioning and cyclosporine A for GVHD prophylaxis, 50 FA patients transplanted form MSD had a 5-year disease-free survival of about 75%. Another protocol for 16 FA patients with MSD used cyclophosphamide alone (total dose 100 mg/kg). The actuarial survival rate at 37 months was 88%. Fludarabine, a purine antimetabolite with potent immunosuppressive properties, was successfully incorporated into a conditioning protocol without radiation. The University of Minnesota is currently using T-cell depleted bone marrow (with Isolex CD34 positive selection) or cord blood. The incidence of acute and chronic GVHD after cord blood transplantation has been comparable to BMT, although engraftment was slower with cord blood. The incidence of acute and chronic GVHD was reduced with cord blood grafts, even in cases of HLA-mismatched transplants. At this time, however, there are no data to indicate whether one stem cell source (marrow vs peripheral blood vs umbilical cord blood) is better than another. At this time, most centers have reserved the use of umbilical cord blood for those patients who cannot identify a suitable marrow donor.

For patients who are not candidates for transplantation, androgen therapy sometimes induces meaningful responses in pancytopenic patients. This treatment is known to affect liver function adversely. Thus, several transplant centers recommend androgens not be given to any FA patients unless no suitable donor is available. Use of cytokines such as G-CSF or erythropoietin has been beneficial in some patients, provided the marrow shows no evidence of a clone or dysplasia.

Knowledge of the complementation group or mutation may permit the potential use of gene therapy and pre-implantation genetic diagnosis and embryo selection both to rule out FA and rule in an HLA match.

For patients with stable disease, annual surveillance exams and bone marrow aspiration (with cytogenetic studies) and biopsy are suggested. For patients with complex cytogenetic abnormalities or MDS, closer follow-up is warranted.

Suggested Readings