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CORD BLOOD TRANSPLANTATION

Cord blood transplantation and cord blood banking

SHUNICHI KATO

Japan Cord Blood Bank Network (JCBBN)

Cord blood transplantation (CBT) has become a therapeutic option in hematological malignancies and genetic diseases not only in children but also in adults without a histocompatible sibling donor or a matched unrelated donor. More than 100,000 cord blood units have been stored, and more than 5000 CBT s have been performed through cord blood banks worldwide.

CBT has several advantages, including rapid availability without donor coordination, decreased viral transmission, reduced acute and chronic graft-versus-host disease (GVHD) and less HLA matching. CBT, however, has several limitations, including delayed neutrophil and platelet engraftment, prolonged immune recovery and increase in infectious complications.

There have been several excellent publications on clinical results of CBT from Europe, USA and Japan. Japan has been unique in the field of allogeneic stem cell transplantation because of genetic homogeneity of the population.

We report the current status of CBT and CB banking in Japan, in comparison with unrelated bone marrow transplantation (uBMT).

The first cord blood transplantation (CBT) from a sibling donor was performed in 1994 in Japan. Several local cord blood banks were born between 1995 and 1996, and the first unrelated CBT was done by the Kanagawa Cord Blood Bank in 1997.

The Japanese Government decided to establish and financially support a network of those banks in 1999. Since then more than 20,000 cord blood units have been stored for transplantation, and more than 2,000 uCBTs have been reported to JCBBN.

In the first five years (1997–2001), majority of uCBTs were done in children. However, early promising results of uCBT in adults encouraged adult patients and physicians, and the number of uCBTs in adults have been increased very rapidly in the last three years.

The committee for data collection and analysis of JCBBN has analyzed the early results in approximately 1300 transplants.

1. Childhood acute leukemia and myelodysplastic syndrome (MDS)
   The most important factor for engraftment was the number of CD34+cells/kg, and the influence of nucleated cell number was not statistically significant.
   HLA disparity did not affect the incidence and the severity of acute GVHD. The most commonly used drugs for GVHD prophylaxis were CYA+MTX and FK+MTX, and the incidence of moderate to severe acute GVHD of grade 2 or greater was not different between these two combinations.
   Event free survival (EFS, leukemia free survival with donor's engraftment) was around 50% in 1st or 2nd remission of ALL and AML, whereas EFS became less than 20% in the advanced stages.
   EFS in infantile ALL with 4;11 translocation and/or MLL gene rearrangement was 41%, and EFS in Ph positive ALL in the 1st or 2nd remission was 34%.
2. Leukemia and MDS in adults
   CD34+ cell dose was also more associated with the speed of engraftment than NCC in adults.
   EFS rates in AML and ALL in first CR were 47% and 22% at 3 year post CBT, if they were treated by conventional conditioning regimen (CST). EFS in patients with more advanced acute leukemia was around 20%.
   EFS in MDS who were treated by CST was 90% in RA and 48% in other types. The results of

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reduced intensity stem cell transplantation (RIST) in elderly patients with acute leukemia were too preliminary and longer follow-up period is needed.

3. Comparison with uBMT
   Takahashi et al. has compared the clinical outcomes of 45 uBMTs and 68 uCBTs in adult patients with hematological malignancies in a single institute. They reported lower treatment related mortality (TRM) and better DFS in uCBT than in uBMT.
   Nationwide comparisons between uBMT and uCBT, however, vary from disease to disease and from institution to institution.

These results suggest that CBT could be widely accepted for use not only in children but also in adults.

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ALLOGENIC STEM CELL TRANSPLANTATION

DLI: Where are we know?

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Allogeneic stem cell transplantation has induced lasting remissions in patients with leukaemia, lymphoma and myeloma otherwise refractory to chemo- and radiotherapy. Patients with acute and chronic graft-versus-host disease (GVHD) have a lesser risk or relapse of disease than those without GVHD. Patients given T-cell depleted grafts have a lower risk of GVHD and a higher risk of rejection of the graft and of relapse. The dilemma of either risk of GVHD or leukaemia relapse can be solved in animal models by transfusion of donor lymphocytes at a time when chimerism and tolerance is established. Here the evidence for a graft-versus-leukaemia (GVL) effect is reviewed and ways of improving the GVL effect and overcoming mechanisms of immune escape are discussed.

Donor lymphocyte transfusions (DLT) have been introduced in the treatment of leukaemia relapse after allogeneic stem cell transplantation in the late 1980's and the best results have been observed in patients with chronic myelogenous leukaemia (CML). The first patients are stil alive and in remission. In acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) results were inferior and in acute lymphatic leukaemia (ALL) results were generally poor. In multiple myeloma DLT could induce remissions, but the duration of the responses has generally been limited. There have been reports on the successful treatment of low grade lymphoma, chronic lymphocytic leukaemia and Hodgkin's disease with DLT, but in general the results are controversial. In most patients of these disease groups conditioning treatment was not myeloablative and patients received grafts of mobilized blood cells containing a high proportion of T-cells. Relapses may have already escaped immune mechanisms of T-cells when they occurred.

The hypothesis that dendritic cells of leukaemia origin are formed in CML and AML has been proven by in vitro culture of leukaemia cells in the presence of GM-CSF and interferon-alpha or IL-4. Dendritic cells had the leukaemia karyotype. Consequently treatment of recurrent CML with DLT was combined with GM-CSF and IFN-a in patients with advanced relapse or not responding to DLT alone. Responses were observed in 6 out of 9 patients treated.

In AML the combination of low dose cytosine arabinoside (LD-AraC) with mobilized blood cells and GM-CSF followed by DLT in cases without GVHD induced remission of more than 4 and 8 years. The long-term responses were limited to patients that responded to a short course of LD-AraC. The use of GM-CSF is currently studied also in other diseases as multiple myeloma and lymphoma with persistence or relapse.

Another possibility to activate T-cells in the GVL reaction is the use of bispecific antibodies. BI20 is a bispecific antibody of rat and mouse origin with one binding site to CD3 and the other to CD20, the Fc part binds to macrophages and dendritic cells inducing phagocytosis of the lymphoma cell. This antibody has been shown to induce CTL against CLL in vitro and depletes leukaemia cells in the blood. However the induction of cellular immunity has not been shown yet in vivo.

Finally unlike T cells NK cells are able to kill leukemia and lymphoma cells not expressing HLA-antigens. They are inhibited by their own HLA-antigens respectively, HLA-associated killer inhibitor receptor ligands. In the HLA-haploidentical situation they are not inhibited, if the recipients leukaemia cells do not express the KIR Ligands of the donor. We have designed a treatment protocol with unmodified mar-

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row as graft on day 0 and CD6-depleted mobilized blood cells on day 6.This way NK cells are given in addition to normal marrow cells. CD6-depleted mobilized blood cells contain NK cells and CD34-positive stem cells, no CD4-positve T cells and a minority of CD8-positive T cells. CD6-negative mobilized blood cells suppress alloreactivity in mixed lymphocyte culture. In vivo conditioning treatment could be decreased to a non-myloablative regimen and the posttransplant immunosuppression could be discontinued earlier. Chronic GVHD was less severe than in patients with HLA-identical donors. Nevertheless anti-leukaemia activity was seen in advanced and refractory stages of leukaemia. Further attempts are directed towards better control of lymphoma and CLL.

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MULTIPLE MYELOMA

Amplification and overexpression of *CKS1B* at chromosome band 1q21 is associated with reduced levels of $p27^{Kip1}$ and an aggressive clinical course in multiple myeloma

JOHN SHAUGHNESSY

Abstract
The molecular basis for aggressive transformation of multiple myeloma (MM) and other cancers is not completely understood. Global gene expression profiling on highly purified malignant plasma cells from 351 newly diagnosed patients with MM treated with autologous stem cell transplantation revealed a statistically significant over-representation of chromosome 1 genes in a group of 70 genes whose expression was linked to poor outcome. In particular, over-expression of *CKS1B*, which maps to an amplicon at 1q21 in myeloma and regulates SCF^{Skp2}-mediated ubquitination and proteolysis of the cyclin dependent kinase inhibitor $p27^{Kip1}$ was significantly over-expressed in patients with poor survival. Interphase fluorescence in-situ hybridization revealed that *CKS1B* expression was strongly correlated with DNA copy number in a subset of 197 cases ($P < 0.0001$) with both measurements. Validated in 224 patients lacking expression analysis, *CKS1B* gene amplification conferred a poor prognosis ($P < 0.0001$) and was an independent predictor of outcome in multivariate analyses ($P = 0.002$). *CKS1B* mRNA and protein expression were correlated and both were inversely correlated with $p27^{Kip1}$ protein levels. RNA interference of *CKS1B* messenger RNA in myeloma cell lines led to reduced *CKS1B* mRNA and protein, an accumulation of $p27^{Kip1}$, and profound growth inhibition. Based on these data we conclude that over-expression of *CKS1B*, mainly due to gene amplification, imparts a poor prognosis in MM, possibly as a result of enhanced degradation of $p27^{Kip1}$

Keywords: *multiple myeloma, prognosis, gene expression profiling, DNA amplification, chromosome 1q21,* CKS1B, *$p27^{Kip1}$, cell cycle regulation.*

Multiple Myeloma is a malignancy of antibody-secreting plasma cells that expand in the bone marrow causing severe osteolytic bone disease and a constellation of additional complications including hypocalcaemia, immunosuppression, anemia, and kidney failure [1]. Although outcome has been greatly improved through the use of high dose melphalan and autologous stem cell transplantations, survival is still highly variable with some patients surviving months and others greater than 10 years or more [2–4]. Aggressive disease, with increased proliferation and a higher frequency of abnormal metaphase karyotypes, elevated LDH and extra-medullary manifestations, seen in approximately 20% of newly diagnosed patients, inevitably appears in all cases, however at a variable rate of onset [4].

Recurrent, non-random genetic lesions have been identified in myeloma and these have been related to clinical course and response to therapy [5]. At the genetic level myelomas can be broadly separated into hyperdiploid and non-hyperdiploid diseases [6]. Non-hyperdiploid myelomas, typically harboring immunoglobulin-mediated translocations leading to transcriptionally activation of *CCND1, CCND3, MAF, MAFB,* or *FGFR3/MMSET* are seen in approximately 40% of cases [7–10]. The remaining 60% of myelomas are hyperdiploid with anueploidy resulting from trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21 [11–14]. Gene expression studies have revealed that virtually all myelomas, regardless of ploidy status, exhibit deregulated expression of one of the three cyclin D genes, suggesting that cyclin D activation may be an initiating genetic event in this malignancy [12]. Myelomas with translocations resulting in activation of *CCND1* are typically diploid and have a more favorable prognosis than those with translocations

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activating *MAF* or *FGFR3/MMSET* [15–18]. Deletion of chromosome 13q14, which is strongly linked to *IGH*-mediated translocations, chromosome 17p and hypodiploidy are associated with a poor prognosis [18–22]. Hyperdiploid tumors are thought to be more dependent on interactions with the bone marrow microenvironment as evidenced by higher levels of DKK1 expression, increased incidence of lytic bone lesions and their conspicuous absence in myeloma cell lines [12,23,24].

The fact that virtually all of the recurrent genetic lesions seen in myeloma are also observed in the benign plasma cell dyscrasia monoclonal gammopathy of undetermined significance (MGUS) [26–28] and that the global gene expression profiles of the two disease are indistinguishable [35], suggests that additional, as yet uncharacterized, gene mutations may be required for progression. Tandem duplications and jumping translocations of the q21 band of chromosome 1 are acquired during myeloma disease progression [29,30] and 1q amplifications have been linked to a poor prognosis in this disease [18,21,31]. No other chromosome or chromosome band undergoes the types of extensive and continual rearrangements as seen for 1q21. Deletion of 17p and acquisition of *MYC* activating translocations are also seen in end stage disease, however a definitive role of these lesions in progression has not been determined.

Global RNA expression profiling is capable of identifying DNA copy number changes in myeloma [12,21] and other cancers [31–33], and unlike array-based comparative genomic hybridization (aCGH) has the added value of pointing to genes whose expression may be altered by these changes. Furthermore, unlike aCGH, RNA profiling can identify gene-activating translocations, not resulting in gain or loss of DNA, that are frequently observed in lymphoid malignancies.

In an effort to identify genes linked to an aggressive clinical course, we applied RNA from highly purified plasma cells derived from 351 newly diagnosed patients with multiple myeloma to Affymetrix U133Plus 2.0 microarrays. Expression extremes of ~54,000 probe sets were correlated with disease-related and overall survival following 2 cycles of high-dose melphalan and autologous stem cell transplantation. Using log rank tests, 70 probe sets were identified for which expression in the fourth or first quartile was correlated with a high incidence of disease-related death. Although 10% of the genes on the microarray were derived from chromosome 1, 30% of the retained genes were derived from this chromosome ($P < 0.0001$). As rearrangements of chromosome 1 have been linked to poor prognosis in myeloma and tandem duplications and jumping translocations of the q21 band of chromosome 1 increase during myeloma progression, we focused on genes from the 1q21 in the list of 70 as candidate genes whose increased expression may be related to increased DNA copy number and leading to disease progression. Two genes, *PSMD4* and *CKS1B*, map to the 1q21 band, and of these two, *CKS1B* was most strongly associated with survival in unadjusted log rank tests (i.e. according to the list order of Table I). There were 40 deaths among 88 patients with *CKS1B* expression in quartile 4 compared to 78 among 263 patients with quartile 1–3 expression ($P < 0.0001$, false discovery rate, 2.5%). To determine if elevated expression was linked to DNA amplification we performed interphase fluorescence *in situ* hybridization analysis on 197 of the 351 cases studied by microarray. *CKS1B* amplification was evident in 46% with the percentage of cases with amplification increasing in frequency as *CKS1B* expression levels increased from quartile 1 to quartile 4 ($P < 0.0001$). These data also showed that *CKS1B* expression significantly increased as CKS1B copy numbers increased from 2 to 3 to greater than equal to 4 copies. We next investigated whether *CKS1B* amplification was associated with survival and event-free survival in a cohort of 224 patients enrolled on the same protocol prior to the initiation of gene expression profiling. *CKS1B* amplification levels were inversely correlated with both event-free survival ($P < 0.0001$, data not shown) and overall survival in this cohort ($P < 0.0001$). These effects were also observed when we combined all 421 (197+224) patients (event-free survival, $P < 0.0001$; data not shown; overall survival, $P < 0.0001$). To test whether *CKS1B* amplification was simply mirroring amplification of the entire chromosome we correlated interphase FISH data with metaphase cytogenetics. As expected, 16 of 17 (94%) cases with evidence of 1q gain by metaphase cytogenetics exhibited amplification by FISH. Importantly, *CKS1B* amplification was also observed by interphase FISH in 61 of 112 cases (54%) lacking evidence of chromosome 1q gain by metaphase cytogenetics ($P < 0.0001$). Given that the incidence of chromosome 1q21 amplification and jumping translocations increases during disease progression and all myeloma cell lines are derived from end stage disease, we evaluated *CKS1B* amplification in a panel of 22 myeloma cell lines. FISH analysis revealed 3 to 8 copies of *CKS1B* in 21 of 22 myeloma cell lines (data not shown). We next investigated whether *CKS1B* expression increased during disease progression. *CKS1B* expression at diagnosis and at relapse, evaluated in 44 paired cases, revealed increased expression in 80% ($P = 0.0001$) with the most dramatic increases occurring in those cases with Q1–Q3 expression at diagnosis. CKS1B FISH on paired baseline and relapse samples from 30 patients enrolled on the protocol showed that of twelve cases lacking amplification at diagnosis, 6 had $>=3$ copies at relapse; of 12 cases with three copies at diagnosis, six had $>=4$ copies at relapse; and of six cases with

Table Ia. Quartile 4 FDR 2.5% gene probe sets – rank correlations with 1q21 amplification index, CKS1B and PC labeling index and adjusted *P*-values for associations with overall survival

Rank (Q4)	Chromosome	Probe set	Symbol	CKS1B amplification index r[†]	CKS1B r[‡]	PCLIr[⋆]	Adjusted survival *P*-value[a]
1	8q21.13	202345_s_at	NA	0.20	0.22		0.001
2	**Xp22.2-p22.1**	**1555864_s_at**	***NA***	**0.34**	**0.47**		**0.007**
3	**5p15.33**	**204033_at**	***TRIP13***	**0.19**	**0.45**	**0.20**	**0.001**
4	1q22	206513_at	*AIM2*	0.15	0.13		0.089
5	2p24.1	1555274_a_at	*SELI*	0.28	0.31		0.001
6	21q22.3	211576_s_at	*SLC19A1*	0.17	0.23		0.007
7	3p21.3	204016_at	*LARS2*	−0.18			0.002
8	1q43	1565951_s_at	*OPN3*	0.36	0.36		0.007
9	**1q31**	**219918_s_at**	***ASPM***	**0.36**	**0.64**	**0.17**	**0.010**
10	**12q15**	**201947_s_at**	***CCT2***	**0.23**	**0.43**	**0.13**	**0.004**
11	16p13.3	213535_s_at	*UBE2I*		0.38		0.022
12	**20q13.2-q13.3**	**204092_s_at**	***STK6***	**0.31**	**0.51**	**0.19**	**0.044**
13	1p36.33-p36.21	213607_x_at	*FLJ13052*				0.150
14	Xq12-q13	208117_s_at	*FLJ12525*		0.34		0.006
15	17q25	210334_x_at	*BIRC5*	0.20	0.36	0.14	0.110
16	**3q27**	**204023_at**	***NA***	**0.29**	**0.62**	**0.16**	**0.072**
17	**1q21.2**	**201897_s_at**	***CKS1B***	**0.50**	**1.00**	**0.15**	**0.007**
18	19q13.11-q13.12	216194_s_at	*CKAP1*	0.24	0.38		0.001
19	**1q21**	**225834_at**	***MGC57827***	**0.39**	**0.66**	**0.23**	**0.140**
20	19q13.12	238952_x_at	*DKFZp779O175*		0.11		0.009
21	**17p13.3**	**200634_at**	***PFN1***	**0.30**	**0.41**		**0.002**
22	19p13.2	208931_s_at	*ILF3*	0.22	0.22		0.220
23	1q22	206332_s_at	*IFI16*	0.30	0.32	0.13	0.003
24	7p14-p13	220789_s_at	*TBRG4*		0.13	0.17	0.009
25	10p11.23	218947_s_at	*PAPD1*	0.31	0.30		0.150
26	8q24	213310_at	*EIF2C2*	0.28	0.37		0.031
27	3q12.1	224523_s_at	*MGC4308*	0.17	0.24	0.14	0.038
28	1p36.3-p36.2	201231_s_at	*ENO1*		0.23		< 0.001
29	18q12.1	217901_at	*DSG2*	0.15			0.005
30	**6q22**	**226936_at**	***NA***	**0.15**	**0.52**	**0.17**	**0.027**
31	8q24.3	58696_at	*EXOSC4*		0.20		0.330
32	**1q21-q25**	**200916_at**	***TAGLN2***	**0.47**	**0.52**		**0.120**
33	3q21	201614_s_at	*RUVBL1*	0.16	0.14		0.023
34	16q22-q24	200966_x_at	*ALDOA*	0.21	0.28		0.001
35	2p25.1	225082_at	*CPSF3*		0.39		0.073
36	1q43	242488_at	*NA*	0.18	0.27	0.14	0.090
37	3q12.3	243011_at	*MGC15606*		0.27		0.004
38	22q13.1	201105_at	*LGALS1*		0.31		0.051
39	**3p25-p24**	**224200_s_at**	***RAD18***	**0.17**	**0.41**	**0.14**	**0.040**
40	20p11	222417_s_at	*SNX5*				0.085
41	**1q21.2**	**210460_s_at**	***PSMD4***	**0.58**	**0.59**	**0.13**	**0.067**
42	**12q24.3**	**200750_s_at**	***RAN***	**0.22**	**0.40**		**0.056**
43	**1pter-q31.3**	**206364_at**	***KIF14***	**0.41**	**0.57**	**0.25**	**0.019**
44	7p15.2	201091_s_at	*CBX3*	0.14	0.20	0.16	0.150
45	**12q22**	**203432_at**	***TMPO***	**0.32**	**0.59**	**0.18**	**0.007**
46	**17q24.2**	**221970_s_at**	***DKFZP586L0724***	**0.27**	**0.47**		**0.081**
47	**11p15.3-p15.1**	**212533_at**	***WEE1***	**0.20**	**0.54**	**0.13**	**0.056**
48	3p12	213194_at	*ROBO1*				0.150
49	5q32-q33.1	244686_at	*TCOF1*				0.120
50	8q23.1	200638_s_at	*YWHAZ*	0.26	0.23		0.012
51	10q23.31	205235_s_at	*MPHOSPH1*		0.40	0.16	0.050

[†]Correlation between each gene's log-scale expression and the CKS1B amplification index (N = 197, all patients with both GEP and FISH 1q21). Blank cells correspond to correlations with P > 0.05.

[‡]Correlation between each gene's log-scale expression and CKS1B log-scale expression (N = 351, all patients with GEP). Rows with CKS1B |r| >= 0.4 are formatted bold.

⋆Correlation between each gene's log-scale expression and the PCLI (N = 305, 46 patients are missing PCLI).

[a]Multivariate proportional hazards regression of overall survival on extreme quartile expression (Q1 or Q4) for each gene, adjusted for FISH 13 80%, cytogenetic abnormalities, B2M > 4, CRP > 4, ALB < 3.5 and PCLI (N = 277, 74 patients are missing at least one measurement; see the supplemental methods for details).

Table Ib. Quartile 1 gene probe sets satisfying FDR 2.5% cutoff

Rank (Q1)	Chromosome	Probe set	Symbol	CKS1B Amplification Index r[†]	CKS1B r[‡]	PCLI r[*]	Adjusted Survival *P*-value[a]
1	9q31.3	201921_at	GNG10	−0.20	−0.30		0.600
2	1p13	227278_at	NA			−0.12	0.900
3	Xp22.3	209740_s_at	PNPLA4				0.029
4	20q11.21	227547_at	NA	−0.29	−0.28	−0.15	0.630
5	10q25.1	225582_at	KIAA1754	−0.21	−0.32		0.003
6	1p13.2	200850_s_at	AHCYL1			−0.13	0.019
7	1p13.3	213628_at	MCLC	−0.30	−0.28	−0.15	0.440
	1p22	209717_at	EVI5	−0.33	−0.29	−0.16	0.870
	1p13.3	222495_at	AD −020	−0.30	−0.24	−0.20	0.920
10	6p21.31	1557277_a_at	NA		−0.11		0.460
11	1p22.1	1554736_at	PARG1		−0.20	−0.11	0.280
12	1p22	218924_s_at	CTBS	−0.16	−0.11	−0.13	0.460
13	**9p13.2**	**226954_at**	**NA**	**−0.22**	**−0.40**		**0.090**
14	1p34	202838_at	FUCA1	−0.17	−0.23		0.066
15	13q14	230192_at	RFP2	−0.28	−0.18		0.880
16	12q13.11	48106_at	FLJ20489	−0.23	−0.23	−0.11	0.300
17	11q13.1	237964_at	NA	−0.16	−0.20		0.044
18	2p22-p21	202729_s_at	LTBP1	−0.24	−0.21		0.097
19	1p13.1	212435_at	NA	−0.21	−0.21	−0.11	0.034

[†]Correlation between each gene's log-scale expression and the CKS1B amplification index (N = 197, all patients with both GEP and FISH 1q21). Blank cells correspond to correlations with $P > 0.05$.

[‡]Correlation between each gene's log-scale expression and CKS1B log-scale expression (N = 351, all patients with GEP). Rows with CKS1B $|r| >= 0.4$ are formatted bold.

*Correlation between each gene's log-scale expression and the PCLI (N = 305, 46 patients are missing PCLI).

[a]Multivariate proportional hazards regression of overall survival on extreme quartile expression (Q1 or Q4) for each gene, adjusted for FISH 13 80%, cytogenetic abnormalities, B2M > 4, CRP > 4, ALB < 3.5 and PCLI (N = 277, 74 patients are missing at least one measurement; see the Supplemental Methods for details).

> =4 copies at diagnosis, five retained > =4 copies at relapse. Multivariate proportional hazards analyses revealed that *CKS1B* amplification, chromosome 13q14 deletion, and metaphase karyotype abnormalities all independently conferred both inferior event-free and overall survival, whereas hypo-albuminemia was the only one of three standard prognostic factors that retained adverse implications for both endpoints examined. *CKS1B* amplification was an independent predictor of outcome both as a 0–100 scale index and as a two-group category after adjustment for other variables. In these analyses, a patient group with an 0–100 scale index one unit larger than another has an estimated 0.9% higher risk of progression and 1.1% higher risk of death (i.e. an increase of approximately 1% in risk with each increase of 1 in the index). The frequency of *CKS1B* quartile 4 expression varied among previously reported genetic subgroups [5]. With respect to gene expression-based identification of those with recurrent translocations, nearly two-thirds of patients with *MAF* or *MAFB* activation, one-third each with *FGFR3*/*MMSET* and *CCND1* activation, and only 18% of those without these translocations had *CKS1B* in quartile 4 ($P < 0.0001$). When examined in the context of metaphase karyotypes, *CKS1B* quartile 4 expression was present in approximately 20% of cases with hyperdiploid or normal, i.e. uninformative, karyotypes, whereas this feature was seen in nearly 50% of patients with hypodiploid and other cytogenetic abnormalities ($P = 0.0002$). In a separate multivariate analysis that adjusted for genetic subgroups, *CKS1B* quartile 4 expression remained an independent adverse outcome predictor; the gene expression-derived translocation

Table IIa. Multivariate proportional hazards analysis[†] (n = 369)

		Event-Free Survival			Survival		
	%	HR	P	Cumulative r^2	HR	P	Cumulative r^2
CKS1B Amplification Index 0–100)		1.009	0.002	0.160	1.011	0.002	0.219
FISH Chromosome 13 Deletion	25.5	1.786	0.006	0.224	1.879	0.014	0.308
Abnormal Karyotype	35.0	1.875	0.001	0.272	2.298	< 0.001	0.393
Beta-2-microglobulin > =4 mg/L	35.8	1.478	0.046	0.305	1.396	0.170	0.422
C-reactive protein > =4 mg/L	63.4	1.533	0.028	0.320	1.586	0.055	0.448
Albumin <3.5 g/dL	16.5	1.660	0.019	0.336	1.698	0.044	0.461
Events/Deaths	127				84		

Table IIb. Multivariate proportional hazards analysis[†] (n = 369)

		Event-Free Survival			Survival		
	%	HR	P	Cumulative r^2	HR	P	Cumulative r^2
CKS1B Amplification Index > =46	32.5	1.68	0.008	0.132	2.12	0.001	0.207
FISH Chromosome 13 Deletion	25.5	1.74	0.010	0.204	1.83	0.020	0.293
Abnormal Karyotype	35.0	1.94	< 0.001	0.257	2.33	< 0.001	0.383
Beta-2-microglobulin > =4 mg/L	35.8	1.52	0.033	0.293	1.43	0.140	0.417
C-reactive protein > = 4 mg/L	63.4	1.49	0.038	0.312	1.56	0.060	0.443
Albumin <3.5 g/dL	16.5	1.69	0.016	0.331	1.73	0.035	0.455
Events/Deaths	127				84		

[†]369 of 421 patients with CKS1B amplification measurements had complete measurements for this analysis; see the supplemental methods for details.

a) Multivariate proportional hazards analysis with the continuous CKS1B amplification index. A patient group with an index one unit larger than another has an estimated 0.9% higher risk of progression and 1.1% higher risk of death (i.e. an increase of approximately 1% in risk with each increase of 1 in the index). Labeling index was not significant in either analysis ($P > 0.35$, HR <1.11, N = 325, EFS events = 116, deaths = 77, with 44 additional subjects missing the labeling index).

b) Multivariate proportional hazards analysis with a cutoff of > =46 for the CKS1B amplification index. Labeling index was not significant in either analysis ($P > 0.32$, HR <1.12, N = 325).

category as a whole conferred inferior event-free ($P = 0.034$), but not inferior overall survival $P = 0.261$); however, consistent with published data [15,21], *CCND1* spikes impacted both endpoints favorably. While not adjusted for the multiple log rank tests that identified the 70 genes, this analysis, suggests that *CKS1B* expression retains predictive power in the context of genetic risk groups. Since CKS1 is required for SCF^{Skp2}-mediated ubiquitinylation and proteasomal degradation of $p27^{Kip1}$ [42,43], we next tested the relationship between CKS1 expression and $p27^{Kip1}$ in myeloma plasma cells. Western blot analysis of protein extracts from plasma cells from 27 newly diagnosed myeloma cases and 7 myeloma cell lines for which microarray data was also available, revealed a strong correlation between *CKS1B* mRNA

Supplemental Table 1. Chromosome distribution of 2.5% FDR gene probe sets

	U133Plus 2.0		Q1		Q4		Combined		
Chromosome	Number of Genes	%	Number of Genes	%	Number of Genes	%	Number of Genes	%	P value*
1	3,659	**9.9**	9	47.4	12	23.5	21	**30.0**	< 0.0001
2	2,522	6.9	1	5.3	2	3.9	3	4.3	
3	2,116	5.8	0	0.0	7	13.7	7	10.0	
4	1,456	4.0	0	0.0	0	0.0	0	0.0	
5	1,718	4.7	0	0.0	2	3.9	2	2.9	
6	2,005	5.4	1	5.3	1	2.0	2	2.9	
7	1,798	4.9	0	0.0	2	3.9	2	2.9	
8	1,311	3.6	0	0.0	4	7.8	4	5.7	
9	1,463	4.0	2	10.5	0	0.0	2	2.9	
10	1,444	3.9	1	5.3	2	3.9	3	4.3	
11	2,069	5.6	1	5.3	1	2.0	2	2.9	
12	1,927	5.2	1	5.3	3	5.9	4	5.7	
13	730	2.0	1	5.3	0	0.0	1	1.4	
14	1,195	3.2	0	0.0	0	0.0	0	0.0	
15	1,152	3.1	0	0.0	0	0.0	0	0.0	
16	1,507	4.1	0	0.0	2	3.9	2	2.9	
17	2,115	5.7	0	0.0	3	5.9	3	4.3	
18	582	1.6	0	0.0	1	2.0	1	1.4	
19	2,222	6.0	0	0.0	3	5.9	3	4.3	
20	1,072	2.9	1	5.3	2	3.9	3	4.3	
21	468	1.3	0	0.0	1	2.0	1	1.4	
22	906	2.5	0	0.0	1	2.0	1	1.4	
X	1,273	3.5	1	5.3	2	3.9	3	4.3	
Y	80	0.2	0	0.0	0	0.0	0	0.0	
m	5	0.0	0	0.0	0	0.0	0	0.0	
	36,795		19		51		70		
Unknown	17,880								
	54,675								

*An exact test for binomial proportions was used to compare the proportion of retained probe sets mapping to chromosome 1 to the proportion for the entire array.

Supplemental Table 2. Relationship between *CKS1B* gene expression quartiles and *CKS1B* amplification by interphase fluorescence in-situ hybridization in newly diagnosed myeloma

CKS1B Expression[†]	# Amplified	% Amplified
quartile 1[‡] n = 44	9	20%
quartile 2 n = 43	12	28%
quartile 3 n = 51	26	51%
quartile 4 n = 59	44	75%
total197	91	46%

[†]$P < 0.0001$. Amplification is defined as > = 20% of cells with 3 or > = 4 CKS1B signals, for validation in conjunction with Figure 2b–c, as described in the Methods. Other tables use the CKS1B amplification index and its optimal cutoff.
[‡]Quartile assignments based upon 351 patients with GEP

and protein, but no correlation between *CDKN1B (p27^{Kip1})* mRNA and protein levels. There was an inverse correlation between CKS1B and p27^{Kip1} protein levels. To confirm that CKS1B regulates p27^{Kip1} protein and possibly myeloma cell growth, we generated myeloma cell lines that constitutively express a small interfering RNA to *CKS1B*. Western blot analysis of the ARP1 myeloma cell line (containing 8 copies of *CKS1B*) transduced with lentivirus expressing iRNA to *CKS1B* or a scrambled iRNA revealed a marked reduction and increase in CKS1B and CDKN1B protein levels in CKS1B iRNA treated cells relative to control cells, respectively. A time course analysis of ARP1 cell growth also revealed a reduction in cell proliferation in CKS1B iRNA-positive cells relative to controls.

Global gene expression analysis of highly purified plasma cells from a large cohort of uniformly treated patients with myeloma identified 70 genes that were significantly correlated with early disease-related mortality. An unexpected feature of this list was that 30% of the 70 genes mapped to chromosome 1. Interestingly, all underexpressed genes from 1 were from 1p and 9% of the overexpressed genes were from chromsome 1qa feature consistent with cytogenetic and molecular cytogenetic data of frequent 1q gains and 1p losses in myeloma [18,21,28,29,44–47]. Tandem duplications and jumping translocations involving 1q21, caused by decondensation of pericentromeric heterochromatin [28,29,48], are cardinal features of progressive end-stage disease in myeloma suggesting that genes from this region may be driving myeloma progression.

DNA synthesis is mediated by the action of the cyclin E/CDK2 complex, which in turn is negatively regulated by the cyclin-dependent kinase inhibitor p27^{Kip1} [49]. The small evolutionarily conserved protein CKS1 is required for SCF^{Skp2}-mediated ubiquitination and proteasomal degradation of p27^{Kip1} [45,46], degradation of which permits DNA replication and correct progression of cells through S phase into mitosis [50]. Morris et al. recently showed that CKS proteins also interact with the proteasome to control the proteolysis of mitotic cyclins by way of regulating the transcriptional activity of *CDC20* [51], a regulatory subunit of the anaphase-promoting complex/cyclosome ubiquitin ligase [52]. Thus, CKS1 and the SCF^{Skp2}-CDKN1B-Cdk1/2 axis appear to be important for both DNA synthesis and mitosis [53]. The low p27^{Kip1} protein levels in cancer cells along with the conspicuous absence of inactivating gene mutations, has prompted speculation that hyperactivation of *CKS1B* and/or *SKP2*, may account for the low levels of p27^{Kip1} in cancer [54,55].

Recent studies have shown that loss of p27^{Kip1} is associated with shortened survival in patients with myeloma [56]. Based on the current data we propose that increased degradation of p27^{Kip1} and poor prognosis in myeloma is primarily caused by a gene-dosage related increase in *CKS1B* gene expression. In

Supplemental Table 3. Relationship of quartile 4 gene expression to amplification for genes located on bacterial artificial chromosome (BAC) used to measure 1q21 amplification

	Not Amplified		Amplified* (Amplification. Index. > = 46)				
Symbol	n/129	(%)	n/68	(%)	*P*-Value[†]	Amplification Index r[‡]	Log Rank *P*-Value[a]
PBXIP1	24	(18.6)	28	(41.2)	0.0012	0.29	0.5285
CKS1B	20	(15.5)	39	(57.4)	<0.0001	0.50	0.0002
PB591	23	(17.8)	38	(55.9)	<0.0001	0.43	0.0873
LENEP	31	(24.0)	18	(26.5)	0.8389	0.03	0.6507
ZFP67	27	(20.9)	29	(42.6)	0.0023	0.34	0.8717
FLJ32934	28	(21.7)	11	(16.2)	0.4606	−0.02	0.6207
ADAM15	23	(17.8)	29	(42.6)	0.0003	0.23	0.2808
EFNA4	26	(20.2)	23	(33.8)	0.0528	0.21	0.3212

The 0–100 scale CKS1B amplification index is a weighted sum of the proportions of clonal cells with 3 copies of CKS1B and > = 4 copies of CKS1B, defined by (.34% 3 copies + .66*% > = 4 copies)/.66
[†]For a test of the independence of amplification and 4th quartile membership (N = 197)
[‡]Correlation between each gene's expression and the 0–100 scale CKS1B amplification index
[a]Log rank test for association of Q4 membership and overall survival (N = 351, 64 deaths)

Supplemental Table 4 a. Relationship between genetic abnormalities and *CKS1B* expression in quartile 4

Abnormality		CKS1B Q4		
Category†	n/347 (%)	n	(%)	*P*-Value⋆
Expression-derived translocation				
t(11;14)	60 (17.3)	20	(33.3)	< 0.0001
t(4;14)	48 (13.8)	17	(35.4)	
t(14;16) & t(14;20)	14 (4.0)	9	(64.3)	
No Translocation Spike	225 (64.8)	41	(18.2)	
Metaphase karyotype				
Hyperdiploid	55 (15.9)	10	(18.2)	0.0002
Non-hyperdiploid	48 (13.8)	24	(50.0)	
Other Cytogenetics Abnormality	9 (2.6)	4	(44.4)	
No Cytogenetics Abnormality	235 (67.7)	49	(20.9)	
Chromosome 13 Deletion	n/334			
No	224 (67.1)	47	21.0	0.02
Yes	110 (32.9)	37	33.6	

†Translocations were determined from the expression spikes t(11;14) = CCND1, t(4:14) = FGFR3/MMSET, t(14;16) = MAF and t(14;20) = MAFB. Aneuploidy and other cytogenetic abnormalities were determined from cytogenetics, for which 4 observations were missing.
⋆Fisher's exact test of the independence of each category and CKS1B 4th quartile membership. Under the null hypothesis, Q4 contains on average 25% of patients within each level, corresponding to a proportional distribution across Q1-3 and Q4.

support of this concept we also observed that *CKS1B* over-expression and amplification commonly surfaced at relapse in patients lacking such features at diagnosis suggesting that tandem duplications and jumping translocations promote myeloma progression through amplification of the *CKS1B* gene.

CKS1B has been shown to be overexpressed in oral, gastric, colon, ovarian, and non-small cell lung carcinomas, and blastoid variant of mantle cell lymphoma [37,57–62] and its expression linked to poor survival in oral, gastric, and colorectal carcinomas [37,58,61]. Since 1q21 amplification and $p27^{Kip1}$ degradation is frequently observed in advanced malignancies, it will be important to determine whether *CKS1B* overexpression and disease progression/aggressiveness in other tumor types is linked to increased DNA copy numbers of *CKS1B*.

SKP2 overexpression has been observed in many tumor types and has been linked to poor survival in some cancers [63]. *SKP2* gene expression levels were not linked to survival in this analysis (data not shown) and we found that unlike CKS1B expression, *SKP2* expression was relatively high and not significantly different amongst plasma cells from normal healthy donors, MGUS, smoldering myeloma and myeloma (data not shown). Taken together these data suggests that CKS1B may be the rate-limiting component of the SCF^{Skp2} complex in myeloma cells.

In the current analysis we discovered that *CKS1B* overexpression was significantly linked to hypodi-

Supplemental Table 4 b. Multivariate analysis of CKS1B quartile 4 expression and cytogenetic abnormalities†

	Event-Free Survival		Survival	
	HR	P‡	HR	P‡
CKS1B Q4	1.97	0.003	2.16	0.005
Expression-derived translocation⋆				
t(11;14)	0.59	0.034	0.82	0.261
t(4;14)	1.67		1.77	
t(14;16) & t(14;20)	1.48		1.12	
Metaphase karyotype⋆⋆				
Hyperdiploid	1.75	0.006	1.84	0.013
Non-hyperdiploid	2.29		2.56	
Other Cytogenetics Abnormality	2.35		2.71	
r^2	0.218		0.223	
Events/Deaths	97		63	

†N = 347. Of 351 patients with expression data, 4 are missing cytogenetics.
‡Partial likelihood ratio test for the overall effect of the category.
⋆The *P*-value for modification of the CKS1B effect on EFS by translocation subgroup is 0.74

Supplemental Table 5. Overexpression CKS1B and CKS2 are related to ploidy.

CKSIB	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*p*-value
Hyperdiploidy (%)	66	61	27	28	2.64×10^{-7}
Hypodiploidy (%)	9	18	39	32	0.00099
Diploidy (%)	25	21	33	41	0.23
Total (N)	85	87	84	79	
CKS2	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-value
Hyperdiploidy (%)	23	42	59	62	7.74×10^{-5}
Hypodiploidy (%)	40	23	17	14	0.0096
Diploidy (%)	37	42	24	25	0.25
Total (N)	86	86	82	81	

ploidy as determined metaphase cytogenetics. We have subsequently confirmed this relationship using DNA content analysis by flow cytometry in ~300 samples (unpublished data). Using gene expression profiling to identify molecular determinants of myeloma bone disease we recently showed that *CKS2* was one of only four genes significantly overexpressed in myelomas with osteolytic lesions [23]. Subsequently we noted that osteolytic lesions predominate in myelomas with hyperdiploid gene expression signatures [12]. Indeed, overexpression of *CKS2* was linked to hyperdiploid myeloma as determined by flow cytometry (unpublished data). Several studies have suggested a link between CKS genes in DNA ploidy. Hixon et al. showed that CKS1 mediates vascular smooth muscle cell polyploidization [64] and Spruck et al. revealed that CKS2 expression is required for the first metaphase/anaphase transition of mammalian meiosis [65]. Future studies will be aimed at determining if over-expression of either of the CKS family members can alter ploidy in myeloma cells.

CKS1B gene amplification along with chromosome 13q14 deletion and abnormal metaphase cytogenetics accounted for almost 40% of the observed survival variability inthis analysis, underscoring that myeloma risk is best assessed by molecular and cellular genetic tests. Routine application of such studies, performed on a single bone marrow sample, is recommended for appropriate patient stratification in therapeutic trial design. The survival impact of new agents, such as bortezomib and thalidomide and its derivatives, will be profound if their clinical efficacy also extends to genetically defined high-risk myeloma, which to date has not been investigated. CKS1B function appears to directly or indirectly interact with ubiquitin ligases and/or the proteasome to regulate cell cycle progression [66]. New therapeutic strategies that directly target CKS1B or related pathways may represent novel, and a more specific, means of treating de novo high-risk myeloma and may prevent secondary evolution.

Cyclin D dysegulation is a common event in cancer and contributes to tumorigenesis by promoting hyperphosphorylation of the RB1 protein, activation of E2F, and transition through the early G1 to S phase of the cell cycle. We have recently reported that dysregulated expression of one of the three D-type cyclins may be initiating genetic lesions in MM [12]. Based on data presented here we propose a two-hit pathogenetic model of myelomagenesis in which activation of a D type cyclin is followed by *CKS1B* amplification leading to dysregulation of both early and late G1 to S phases of the cell cycle.

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MULTIPLE MYELOMA

Role of stem cell transplantation in myeloma

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Both autologous and allogeneic hematopoietic stem cell transplantation are used in the treatment of patients with multiple myeloma. Two randomized prospective studies have shown that autologous transplantation is superior to conventional chemotherapy at least for patients younger than 60 – 65 years of age [1,2]. Median survival has improved from 42 and 44 months to 54 and 57 months respectively. Prognostic factors have been extensively analyzed and many are similar to those with conventional chemotherapy. Favorable prognosis is seen in younger patients, patients lacking certain chromosomal aberrations (-13), those that are not heavily pretreated and those that are responsive to previous treatment. Tandem autologous transplantation appears to be superior to single transplantation, at least in patient that do not enter complete hematologic remission after the first transplantation [3]. The most favorable time for the second transplant seems to be within 6 – 12 months [4]. Attempts to reduce relapse and progression by reducing the tumor load in the autologous cell suspension through CD34+ cell selection have been unsuccessful [5,6]. Molecular remissions are rare events following autologous transplantation and patients do not seem to be cured [7].

Allogeneic transplantation may have the potential to cure a fraction of the patients [8]. Molecular remissions are more frequent than with autologous transplantation [9]. However, conventional high dose myeloablative conditioning transplants are hampered by about 30–35 % transplant-related mortality (TRM), although this mortality has been reduced in transplants performed from 1994 as compared to those performed before 1994, mainly due to earlier transplantation and more effective treatment of bacterial, fungal and viral infections [10]. Median overall survival was 50 months in later transplants. Favourable prognostic factors for myeloablative transplantation are low age, low β-2-microglobulin (β-2-m), stage I at diagnosis, responsiveness to previous treatment and only one treatment regimen before transplantation. Recently it was shown that although the female to female combination has the best outcome the relapse rate in males with a female donor is significantly lower than in males with a male donor, compensating for the higher transplant related mortality in this combination [10]. Procedural factors play a role for outcome, but documentation is poor. The most frequently used conditioning regimen for myeloablation, cyclophosphamide 60 mg $kg^{-1} \times 2$ plus 10 Gy total body irradiation with lung shielding to 9 Gy has not been surpassed by numerous other regimens reported to the EBMT registry.

A recent EBMT registry study indicates that reduced intensity nonmyelablative (NMA) regimens result in lower TRM but a higher relapse rate than with myeoablative regimens [11]. The higher relapse rate might be counteracted by a previous autologous transplantation and later donor lymphocyte transfusions. EBMT is presently running a prospective study comparing tandem autologous-NMA allogeneic transplantation to autologous transplantation alone based on the availability of an HLA matched sibling donor. Scheduled donor lymphocyte transfusions are given post transplantation dependant on response or recurrence. The safety analyses indicate 11% early TRM following the NMA transplantation.

In summary, autologous transplantation is the golden standard for the treatment of most patients with multiple myeloma. Tandem transplantation may be superior to single transplantation in younger patients that do not enter complete remission after the first transplant. Non myeloablative allogeneic transplantation reduces transplant related mortality

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compared to myeloablative transplantation but the relapse rate is higher and overall survival appears not clearly affected. It should still preferentially be performed in controlled trials. Myeloablative transplantation may be an option for selected younger patients with stage IIIA disease, preferentially for younger women that have an HLA matched female sibling donor. Patients that have been responsive to previous conventional treatment and have received only one treatment regimen before the transplant are the best candidates. However some patients that have not responded or have relapsed after autologous transplantation may be offered an allogeneic transplant if their general condition is otherwise good.

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PLATELET DISORDERS

Platelets in sepsis

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Abstract
Platelets are circulating blood cells that will normally not interact with the intact vessel wall but that may swiftly respond to vascular disruption by adhering to subendothelial structures, followed by interaction with each other, thereby forming a platelet aggregate. The activated platelet (phospholipid) membrane may form a suitable surface on which further coagulation activation may occur. These processes are part of the first line of defence of the body against bleeding but may also contribute to pathological thrombus formation in vascular disease, such as thrombus formation on top of a ruptured atherosclerotic plaque. In case of systemic inflammatory syndromes, such as the response to sepsis, disseminated intravascular platelet activation may occur, which will contribute to microvascular failure and thereby play a role in the development of organ dysfunction. In addition, in this situation platelets may be directly involved in the inflammatory response by releasing inflammatory mediators and growth factors.

Introduction

Under normal conditions 1×10^{12} platelets continuously flow along 1000 m^2 of vascular surface in the human body without adhering or aggregating. However, upon disruption of the integrity of the vessel wall, a swift and complex interaction between circulating platelets, endothelial cells and subendothelial structures occurs [1] {2071 /id}. The result of this interaction is platelet adhesion to the vessel wall and forming aggregates with each other, thereby materializing a first line of defense against blood loss. The interaction between platelets and the vessel wall is mediated by cellular receptors on the surface of platelets and endothelial cells, such as integrins and selectins, and by adhesive proteins, such as von Willebrand factor and fibrinogen.

Platelet aggregation under pathological conditions

Platelets play a pivotal role in the pathogenesis of acute atherothrombosis, which is the pathological substrate of acute vascular events, such as acute myocardial infarction and stroke. Although the mechanism by which platelets adhere to the vessel wall to achieve hemostasis is fairly well understood, the exact pathways that contribute to platelet adhesion and activation in thrombosis, e.g. upon rupture of an atherosclerotic plaque, are still unclear [2] {1966 /id}. The essential aspects of platelet activation and aggregation are for the major part the same as those in response to hemorrhage. However, in case of pathological thrombus formation there may be modifying factors, such as increased shear stress around atherosclerotic plaques and local dysfunction of endothelial cells, potentially in association with inflammatory mechanisms are probably important in pathological thrombus formation [2,3] {1966 /id; 2232 /id}. Besides playing a role in thrombus formation upon a ruptured atherosclerotic plaque, platelets also play a role in the formation of the atherosclerotic plaque itself. Platelets can release adhesive ligands, such as P-selectin and may, upon activation, provide a suitable phospholipid surface for the recruitment of mononuclear cells and lymphocytes, that contribute to the local inflammatory processes associated with atherosclerosis [4,6]{3220 /id; 3221 /id}. In addition, platelet granules contain growth factors, such as platelet-derived growth factor, which is important

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for cellular proliferation in the expanding atherosclerotic lesion.

Platelets in sepsis

Most clinicians will recognize that platelets are involved in the pathogenesis of sepsis, if only by the fact that marked thrombocytopenia is a common feature of sepsis. The incidence of thrombocytopenia (platelet count $<150 \times 10^9\ l^{-1}$) in critically ill medical patients is 35–44% [7,8] {2257 /id; 1810 /id}. A platelet count of $<100 \times 10^9\ l^{-1}$ is seen in 20–25% of patients, whereas 12–15% of patients have a platelet count $<50 \times 10^9\ l^{-1}$. Typically, the platelet count in patients with sepsis decreases during the first 4 days on the intensive care unit [9] {1209 /id}. Sepsis is a clear risk factor for thrombocytopenia in critically ill patients and the severity of sepsis correlates with the decrease in platelet count [10] {2274 /id}. The mechanism by which thrombocytopenia in sepsis occurs, however, is not completely clear. Impaired production of platelets from within the bone marrow may seem contradictory to the high levels of platelet production-stimulating pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, and high concentration of circulating thrombopoietin in patients with sepsis. These cytokines and growth factors should theoretically stimulate megakaryopoiesis in the bone marrow [11] {2275 /id}. However, in a substantial number of patients with sepsis marked hemophagocytosis may occur, consisting of active phagocytosis of megakaryocytes and other hematopoietic cells by monocytes and macrophages, hypothetically due to stimulation with high levels of macrophage colony stimulating factor (M-CSF) in sepsis [12] {2261 /id}. Platelet consumption may also play an important role in patients with sepsis, due to ongoing generation of thrombin (which is the most potent activator of platelets in vivo) [13,14] {2276 /id; 511 /id}. Of note, the involvement of platelets in the sepsis-associated coagulopathy was already identified more than 30 years ago, focusing on the interaction of platelets with endotoxin. [15] {3124 /id}. In the setting of inflammation-induced activation of coagulation, platelets can be activated directly by endotoxin [16] {2002 1895 /id} or by pro-inflammatory mediators, such as platelet activating factor [17] {2002 1896 /id}. Release of inflammatory mediators and growth factors may be another link between activation of coagulation and inflammation. Recent studies have shown that expression of P-selectin on the platelet membrane not only mediates the adherence of platelets to leukocytes and endothelial cells but also enhances the expression of tissue factor on monocytes [18]. Tissue factor expression is the triggering event in the activation of blood coagulation, resulting in the generation of thrombin, which may further activate platelets. P-selectin can be relatively easily shed from the surface of the platelet membrane and soluble P-selectin levels have indeed been shown to be increased during systemic inflammation [19].

Hence, platelets seem to occupy one of the essential cross-roads in the complex interaction between inflammation and coagulation, not only by facilitating and propagating thrombin generation but also by being an important mediator of growth factor and adhesion molecule activity. More research on the role of platelet activation in sepsis will undoubtedly be helpful in further unraveling the pathogenesis of sepsis and in the understanding of the tight cross-talk between inflammation and coagulation.

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PLATELET DISORDERS

Platelet function testing in cardiovascular diseases

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Keywords: *platelets, tests, aspirin, clopidogrel, GPIIb-IIIa antagonists*

Case Presentation 1: Mr. F. is a 60 year old man with unstable angina who takes aspirin 81 mg each day. A platelet function test demonstrates that his platelets are "resistant" to aspirin. Should his treatment be changed?

Case Presentation 2: Mr. K. is a 60 year old man with unstable angina who takes aspirin 81 mg and clopidogrel 75 mg each day. A platelet function test demonstrates that his platelets are "resistant" to clopidogrel. Should his treatment be changed?

Normal platelet function

Platelets are small cells of great importance in thrombosis, hemorrhage, and inflammation [1]. Formation of the hemostatic plug at sites of vascular injury is described in Figure 1. Platelets localize, amplify, and sustain the coagulant response at the injury site and release procoagulant platelet-derived microparticles. Platelets contain a variety of inflammatory modulators (e.g. CD40 ligand [CD40L]) that are released upon platelet activation.

Platelet function testing in cardiovascular diseases

Platelets have an increasingly well-defined, critical role in coronary artery thrombosis [2] and in other common cardiovascular diseases including stroke, peripheral vascular disease, and diabetes mellitus [1]. Although the role of platelets in thrombosis is well characterized, platelets may also have a role in the pathogenesis of the underlying atherosclerotic process [2]. Platelet function tests have been studied in cardiovascular diseases as a means to predict clinical outcomes and to monitor antiplatelet drugs. Table I summarizes these tests.

Use of platelet function tests to predict clinical outcomes

In acute coronary syndromes and after coronary stenting, flow cytometric analysis of platelet activation-dependent markers predicts major adverse cardiac events (MACE) [3]. Increased platelet surface P-selectin is also a risk factor for silent cerebral infarction in patients with atrial fibrillation [4]. However, circulating monocyte-platelet aggregates are a more sensitive marker of *in vivo* platelet activation than platelet surface P-selectin in the clinical settings of stable coronary artery disease, [5] PCI, [6] and acute myocardial infarction [6]. Furthermore, circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction [7]. Measurement of plasma CD40L in the first 12 hours after the onset of ischemic symptoms in patients with unstable angina identifies a subgroup of patients that has a much greater clinical benefit from abciximab treatment [8]. High plasma concentrations of sCD40L may be associated with increased cardiovascular risk in apparently healthy women [9]. In patients with stable angina, the PFA-100® closure time may predict the presence or absence of coronary artery stenoses at angiography, thereby potentially avoiding further diagnostic investigations [10]. PFA-100® closure time may also be predictive of the severity of myocardial damage in acute myocardial infarction [11].

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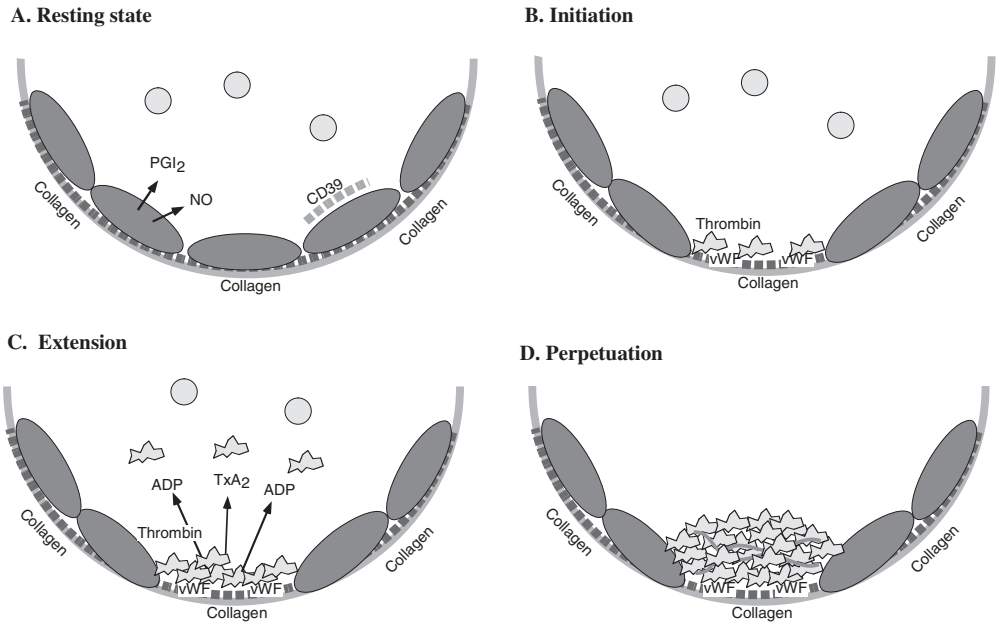

Figure 1. Steps in platelet plug formation. A) Prior to vascular injury, platelets are maintained in a resting state by endothelial inhibitory factors: prostacyclin (PGI_2), nitric oxide (NO), and CD39. B) The platelet plug is initiated by exposure of collagen and local generation of thrombin. This causes platelets to adhere via collagen and von Willebrand factor (vWF) and spread. C) The platelet plug is extended as additional platelets are activated via the release of thromboxane A_2 (TxA_2), ADP, and other platelet agonists. Platelet-to-platelet aggregation is primarily mediated by activation of platelet surface GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$) (not shown). D) A fibrin meshwork (shown in green) helps to perpetuate and stabilize the platelet aggregate. Reproduced with permission from ref. [24].

Although a number of studies have demonstrated that platelet function tests can predict MACE in cardiovascular diseases, none of these assays have yet been sufficiently studied in large clinical trials to become part of standard clinical care.

Use of platelet function tests to monitor antiplatelet drugs

Aspirin reduces the odds of a serious arterial thrombotic event in high risk patients by ~25% [12]. However, 10–20% of patients with an arterial thrombotic event who are treated with aspirin have a recurrent arterial thrombotic event during long term follow-up [12]. Failure of aspirin to prevent an arterial thrombotic event has been termed "aspirin resistance". Failure of clopidogrel to prevent an arterial thrombotic event has been termed "clopidogrel resistance". Similarly, the term "GPIIb-IIIa antagonist resistance" could be used. However, because arterial thrombosis is multifactorial, an adverse arterial thrombotic outcome in a patient may often reflect treatment failure rather than "resistance" to an antiplatelet drug. Furthermore, patient non-compliance with aspirin and/or clopidogrel is a frequent (and hard to detect) confounding problem. There is well-documented variability between patients (and normal volunteers) with regard to laboratory test responses to aspirin, [13–18] thienopyridines, [19] and GPIIb-IIIa antagonists [20]. This variability in laboratory test response has also been termed "resistance" to antiplatelet agents. The key question is: do laboratory tests of "resistance" to aspirin, clopidogrel, and/or GPIIb-IIIa antagonists predict clinical "resistance" to these drugs (i.e. MACE)? Clinically meaningful definitions of aspirin, clopidogrel, and GPIIb-IIIa "resistance" can only be based on data linking drug-dependent laboratory tests to clinical outcomes in patients. Until such links are clearly established, MACE that occur despite an antiplatelet agent should not be termed drug "resistance".

Aspirin

Aspirin irreversibly acetylates serine 530 of cyclooxygenase (COX)-1, resulting in inhibition of thromboxane A_2 release from platelets and prostacyclin from endothelial cells. Because platelets lack the synthetic machinery to generate significant amounts of new COX, aspirin-induced COX-1 inhibition lasts for the lifetime of the platelet. In contrast, endothelial cells retain their capacity to generate new COX and recover normal function shortly after exposure to aspirin. Possible mechanisms of aspirin "resistance" are listed in Table II. There is evidence that MACE in the settings of acute coronary syndromes, stroke/TIA, and peripheral arterial disease can be predicted by the following *in vitro* tests of aspirin "resistance": arachidonic acid- and ADP-induced platelet aggregation (turbidometric), ADP- and collagen-induced platelet aggregation (impedance), VerifyNow®, PFA-100®, or urinary 11-dehydro thromboxane B_2

Table I Platelet function tests in cardiovascular disease.

Basis of Test	Name of Test	Advantages	Disadvantages	Reported to Predict Clinical Outcomes	Monitoring of Aspirin*	Monitoring of Thienopyridines*	Monitoring of GPIIb/IIIa Antagonists*
In vivo cessation of blood flow by a platelet plug	Bleeding time	*In vivo* test. Physiological.	Non-specific. Insensitive. High inter-operator CV. Can leave scar.	No	No	No	No
In vitro cessation of high shear blood flow by a platelet plug	PFA-100®	Simple and rapid. Low sample volume. High shear. No sample preparation. Whole blood assay.	Dependent on von Willebrand factor and hematocrit. No instrument adjustment.	Yes [10,11,18]	Yes	Not recommended	Not recommended
Shear-induced platelet adhesion	IMPACT® (cone and plate(let) analyzer)	Simple and rapid. Point-of-care. Low sample volume. High shear. Whole blood assay.	Instrument not yet widely available.	No	Under development	Under development	Not recommended
Platelet-to-platelet aggregation	Aggregometry (turbidometric)	Historical gold standard.	Poor reproducibility. High sample volume. Sample preparation. Time consuming. Expensive.	Yes [16,19]	Yes (with arachidonic acid and ADP)	Yes (with ADP)	Yes
	Aggregometry (impedance)	Whole blood assay.	High sample volume. Sample preparation. Time consuming. Expensive.	Yes [14]	Yes (with arachidonic acid and ADP)	Yes (with ADP)	Yes
	VerifyNow®	Simple and rapid. Point-of-care. Low sample volume. No sample preparation. Whole blood assay.	No instrument adjustment.	Yes [17,20]	Yes (with arachidonic acid or propyl gallate cartridge)	Yes (with pending ADP cartridge)	Yes (with TRAP cartridge)
	Plateletworks®	Minimal sample preparation. Whole blood assay.	Not well studied.	No	No	Yes	Yes
Activation-dependent changes in platelet surface	Platelet surface P-selectin, platelet surface activated GPIIb-IIIa, leukocyte-platelet aggregates (flow cytometry)	Low sample volume. Whole blood assay.	Sample preparation. Expensive. Requires flow cytometer and experienced operator.	Yes [3,4]	Yes (with arachidonic acid)	Yes (with ADP)	Yes

Table I (*Continued*)

Basis of Test	Name of Test	Advantages	Disadvantages	Reported to Predict Clinical Outcomes	Monitoring of Aspirin*	Monitoring of Thienopyridines*	Monitoring of GPIIb/IIIa Antagonists*
Activation-dependent signaling	VASP phosphorylation state (flow cytometry)	Directly dependent on clopidogrel's target: $P2Y_{12}$. Low sample volume. Whole blood assay.	Sample preparation. Expensive. Requires flow cytometer and experienced technician.	No	No	Yes	No
Activation-dependent release from platelets	Platelet-derived microparticles (flow cytometry)	Low sample volume. Whole blood assay.	Sample preparation. Expensive. Requires flow cytometer and experienced technician.	No	No	No	No
	Serum thromboxane B_2	Directly dependent on aspirin's target: COX-1.	Indirect measure. Not platelet-specific.	No	Yes	No	No
	Urinary 11-dehydro thromboxane B_2	Directly dependent on aspirin's target: COX-1.	Indirect measure. Not platelet-specific. Dependent on renal function.	Yes [15]	Yes	No	No
	Plasma sCD40L	Majority of plasma sCD40L is platelet-derived.	Separation of plasma can result in artifactual platelet activation.	Yes [8,9]	No	No	No
	Plasma GPV	Platelet-specific.	Separation of plasma can result in artifactual platelet activation. Reflects only thrombin-mediated platelet activation.	No	No	No	No
	α granule constituents in plasma: platelet factor 4, β-thromboglobulin, soluble P-selectin	Reflect platelet secretion.	Separation of plasma can result in artifactual platelet activation. Plasma soluble P-selectin also originates from endothelial cells.	No	No	No	No

Abbreviations: COX-1, cyclooxygenase 1; PFA-100, platelet function analyzer-100; sCD40L, soluble CD40 ligand; TRAP, thrombin receptor activating peptide; VASP, vasodilator stimulated phosphoprotein.

*No published studies address the clinical effectiveness of altering therapy based on a laboratory finding of "resistance" to aspirin, clopidogrel, or GPIIb-IIIa antagonists.

For further information on these tests see also refs. [1],[25].

Table II Possible mechanisms of aspirin and clopidogrel "resistance".

Bioavailability
- Non-compliance
- Underdosing
- Poor absorption (enteric coated aspirin)
- Interference
  - ?NSAID coadministration (competes with aspirin for serine 530 of COX-1)
  - ?Atorvastatin (interferes with cytochrome P450-mediated metabolism of clopidogrel)

Platelet Function
- Incomplete suppression of thromboxane A_2 generation (aspirin)
- Accelerated platelet turnover, with the introduction into the blood stream of newly formed, drug-unaffected platelets
- Stress-induced COX-2 in platelets (aspirin)
- Increased platelet sensitivity to ADP and collagen

Single Nucleotide Polymorphisms
- Receptors: $P2Y_{12}$ H2 haplotype (clopidogrel), GPIIb-IIIa, collagen receptor, thromboxane receptor, etc
- Enzymes: COX-1, COX-2, thromboxane A_2 synthase, etc (aspirin)

Platelet Interactions With Other Blood Cells
- Endothelial cells and monocytes provide PGH_2 to platelets (bypassing COX-1) and also synthesize their own thromboxane A_2 (aspirin)

Other Factors
- Smoking, hypercholesterolemia, etc.

Rather Than "Resistance", is it:
- Aspirin or clopidogrel response variability?
- Platelet response variability?
- Treatment failure (because arterial thrombosis is multifactorial)?

(Figure 2A) [13–18]. However, in all these studies the number of MACE was low.

Thienopyridines

The thienopyridines (clopidogrel [Plavix®] and ticlopidine [Ticlid®]) inhibit ADP binding to its platelet surface $P2Y_{12}$ receptor. Possible mechanisms of clopidogrel "resistance" are listed in Table II. There is evidence from one trial that an *in vitro* test of clopidogrel "resistance" (ADP-induced platelet aggregation) predicts MACE, but the number of MACE was again low (Figure 2B) [19]. The $P2Y_{12}$ H2 haplotype is reported to be associated with peripheral artery disease [21].

GPIIb-IIIa Antagonists

GPIIb-IIIa antagonists (abciximab [ReoPro®], eptifibatide [Integrilin®], tirofiban [Aggrastat®]) inhibit fibrinogen binding to platelet surface GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$), the final common pathway of platelet aggregation. Although the term "resistance" has not been used in the literature with regard to GPIIb-IIIa antagonists, there is substantial patient-to-patient variability in the degree of inhibition of platelet

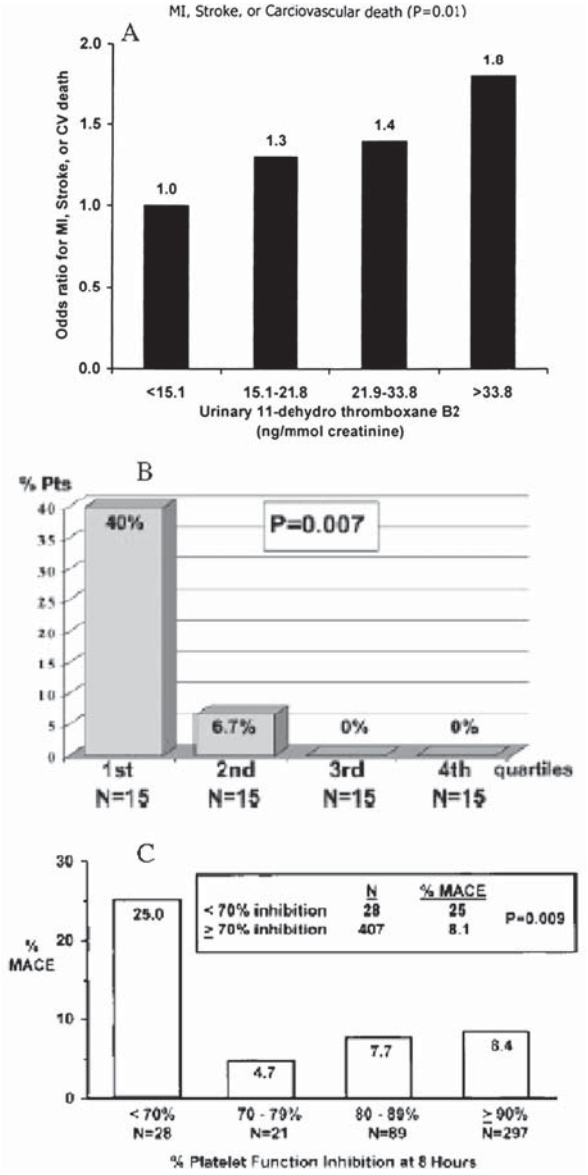

Figure 2. Evidence that *in vitro* tests of "resistance" to antiplatelet drugs predict major adverse clinical events (MACE). A) Aspirin "resistance" was determined by higher quartiles of urinary 11-dehydro thromboxane B_2. P is for trend of association. B) Clopidogrel "resistance" in study patients (Pts) was determined by quartiles of inhibition of ADP-induced platelet aggregation. C) Abciximab "resistance" was determined by VerifyNow® at 8 hours after abciximab bolus, but during infusion. Clinical follow-up was 5 years (A), 6 months (B), and 7 days (C). Reproduced with permission from refs. [15,19,20].

function by GPIIb-IIIa antagonists.[20] Furthermore, there is evidence that an *in vitro* test of abciximab "resistance" (VerifyNow®) predicts MACE (Figure 2C) [20].

Treatment of "resistance" to antiplatelet agents

Although some clinicians change treatment based on platelet function testing, [22] the correct treatment, if any, of "aspirin resistance" is unknown. Non-com-

pliance should be considered. Increasing the dose of aspirin is unlikely to be helpful [12]. Addition of a thienopyridine may be useful, with [23] or without continued aspirin therapy. However, increased antiplatelet therapy may increase the risk of bleeding and other side effects. Most importantly, no published studies address the clinical effectiveness of altering therapy based on a laboratory finding of "resistance" to aspirin, clopidogrel, or GPIIb-IIIa antagonists. In summary therefore, other than in research trials, it is not currently appropriate to test for "resistance" in patients or to change therapy based on such tests.

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DIAGNOSIS AND MANAGEMENT OF THROMBOSIS

The importance of the protein C system in the pathogenesis of venous thrombosis

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Keywords: *Thrombosis, protein C, Factor V Leiden*

Primary haemostasis and blood coagulation have evolved as important defence mechanisms against bleeding. The formation of the platelet plug is timely co-ordinated with the activation of the coagulation system, which occurs in response to the exposure of blood to extravascular tissues. The reactions of blood coagulation are carefully controlled by several anticoagulant mechanisms and under normal conditions they prevail over the procoagulant forces. The protein C system provides important control of blood coagulation by regulating the activities of factor VIIIa (FVIIIa) and factor Va (FVa), cofactors in the activation of factor X and prothrombin, respectively. The system comprises membrane-bound and circulating proteins that assemble into multi-molecular complexes on cell surfaces.

Vitamin K-dependent protein C, the key component of the system, circulates in blood as zymogen to an anticoagulant serine protease. It is efficiently activated on the surface of endothelial cells by thrombin bound to the membrane protein thrombomodulin. The endothelial protein C receptor (EPCR) further stimulates the protein C activation. Activated protein C (APC) together with its cofactor protein S inhibits coagulation by degrading FVIIIa and FVa on the surface of negatively charged phospholipid membranes. Efficient FVIIIa degradation by APC requires not only protein S but also FV, which like thrombin is a Janus-faced protein with both pro- and anticoagulant potential.

Protein S in human plasma is not only an important component of the protein C pathway but also takes part in the regulation of the complement system as it forms a high affinity complex with C4b-binding protein (C4BP), a regulator of the classical complement pathway. In human plasma, 30–40% of the protein S circulates as free protein, the remaining being bound to C4BP. Only free protein S has the ability to function as a cofactor to APC. Recently, it was found that protein S binds to the negatively charged phospholipid surface that is exposed on apoptotic cells and can mediate phagocytosis of the apoptotic cell. This observation might account for the observed lack of coagulation activation in the vicinity of apoptotic cells.

The protein C system is vitally important to keep the blood fluid. This is most clearly illustrated by the severe microvascular thrombotic disease that already in the neonatal period affects individuals with complete inherited protein C deficiency. Heterozygous deficiency is associated with approximately a 5-fold increased risk of venous thrombosis. Heterozygous protein S deficiency is affected by similar thrombosis risk as protein C deficiency. The Factor V Leiden mutation (APC resistance) is the most common gene defect associated with venous thrombosis found in 20–40% of patients with thrombosis in western countries. The Factor V Leiden mutation (G1691A) replaces Arg506 with a Gln. Mutant Factor V has full procoagulant capacity, but the protein C anticoagulant system is affected in two ways by the mutation. The first is impaired degradation of mutant Factor Va by APC because the mutation eliminates one of three APC cleavage sites in Factor Va. The second is impaired degradation of Factor VIIIa because mutant Factor V cannot be cleaved at Arg506 and is therefore a poor cofactor to APC in the degradation of Factor VIIIa. Factor V Leiden is the result of a founder effect. Heterozygous individuals have approximately a 5-fold increased risk of venous thrombosis, whereas homo-

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zygotes have around a 50-fold increased risk. The mutation is not a risk factor for arterial thrombosis.

In recent years, protein C has been shown not only to be anticoagulant but also to have anti-inflammatory and anti-apoptotic properties, which are exerted when APC binds to EPCR and proteolytic cleaves protease activated receptor 1 (PAR-1). The unique combination of anticoagulant, anti-inflammatory and anti-apoptotic properties of activated protein C (APC) has made it an attractive candidate as a therapeutic agent and administration of APC has proven beneficial in the handling of patients with severe sepsis.

Significant insights have been gained in to the structure–function relationships of large macromolecular complexes important for the activation of protein C, the regulation of tenase and prothrombinase complexes, and the cell-surface interactions with EPCR/PAR-1 resulting in anti-inflammatory and anti-apoptotic effects. However, many unanswered questions remain and some may be particularly challenging, e.g. the molecular interactions of the synergistic APC cofactor activity of Factor V and protein S in the regulation of Factor VIIIa in the tenase complex and the elucidation of the cellsurface and intracellular events associated with the anti-inflammatory and anti-apoptotic functions of the protein C system. The coming years will no doubt bring further exciting novel insights into these mechanisms.

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DIAGNOSIS AND MANAGEMENT OF THROMBOSIS

New development in anticoagulant and antithrombotic therapy: Are we on the right track?

JAWED FAREED

Anticoagulant and antithrombotic drugs have played a key role in the prophylaxis, treatment and surgical/interventional anticoagulant management of thrombotic and cardiovascular disorders. In particular, these drugs have played a crucial role in the management of venous thrombosis and pulmonary embolism. There are several newer drugs which have currently been developed for the management of venous thromboembolic disorders. These include the low molecular weight heparins (LMWHs), antithrombin agents such as the hirudin, hirulog and argatroban and indirect and direct anti-Xa drugs, represented by pentasaccharide (fondaparinux®) and BAY 59-7939, respectively. The oral heparins, anti-IIa and anti-Xa drugs are also in different phases of clinical development. Of these, one oral antithrombin agent, namely, Exanta, is approved in Europe, for qualified indications. However, the US FDA has not approved this drug for any indication. Several other agents such as the natural and recombinant anti-Xa drugs and anti-tissue factor agents are also under development. For subcutaneous indications, unfractionated heparin is gradually being replaced by LMWHs. Such LMWHs as the enoxaparin and dalteparin are commonly used for the management of venous thromboembolic disorders. However, there are eight additional commercially available LMWHs which can be used for this disorder. It is now clear that different LMWHs are clinically none interchangeable. Moreover, the generic versions of the braned product such as enoxaparin may exhibit different properties then the innovator product and therefore do not qualify for generic interchangeability. Fondaparinux® is also being developed for various subcutaneous indications. However, it exhibits lower anticoagulant effects and may not be optimal for intravenous indictaions. At a higher dosage when administered intravenously the LMWHs produce varying degrees of anticoagulation at relatively lower activated clotting times (ACT;150–200 sec). Several studies in vascular and cardiovascular interventions have shown that even at a relatively low anticoagulant level the LMWHs are as effective as unfractionated heparin at the recommended dosages which produce a relatively higher level of anticoagulation (ACT $>$ 200 secs.). Thus, these agents are currently being developed for several intervenous/hematologic indications such as bone marrow transplantation and blood cancers. It should be emphasized that different LMWHs produce different degrees of anticoagulation and should therefore be individually optimized for a specific hematologic indication. At a relatively high dosage the levels of LMWHs can be measured by using the ACT and APTT. LMWHs will find expanded indications in both the medical and surgical management of patients with hematologic and oncologic disorders. The LMWHs are also useful in the management of cancer patients. Recent trials have clearly shown that these drugs reduce the mortality outcome in cancer patients. The only approved anti-Xa drug is represented by a synthetic heparinomimetic, namely, fondaparinux®. This drug is given for the prophylaxis of post orthopedic indications. This agent is undergoing additional clinical trials in the management of several other indications. Because of the dependence on antithrombin (AT) and the sole anti-Xa effects, it has a narrow therapeutic index and its efficacy in this indication may be limited. Additional clinical trials are needed at this time to validate the clinical potential of this drug. The long lasting methylated pentasaccharide derivative, namely idraparinux, is also being optimally developed; however, there is no antidote for this agent. The antithrombin agents (hirudin, hirulog and argatroban) were initially developed for arterial indications. However, these agents are approved as a substitute anticoagulant in patients with heparin induced thrombocytopenia (HIT) and PCI. Different antithrombin agents produce their therapeutic effects by distinct mechanisms and should be considered equivalent on the basis of their antic-

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oagulant effects. Currently all of these agents are being developed for surgical and interventional use. However, since there is no available antidote at this time, the development is somewhat limited. The antithrombin agents may be useful in patients with HIT which require further clinical validation. Many other anti-Xa agents are also developed. Most of these can be given parenterally. However, the clinical data is somewhat limited. Since most of these newer anticoagulant and antithrombotic drugs are mono-therapeutic their therapeutic index is rather limited. Only in combination these agents can mimic heparins. At this time it is safe to state that heparin and its LMWH derivatives will remain the anticoagulant of choice for the management of thrombosis until these newer agents have been validated in extended clinical trials in polytherapeutic settings. Polytherapeutic approaches including the targeting of adhesion molecule and cellular receptors, modulation of inflammatory process, targeting procoagulant proteins such as the coagulation factors, TF and VWF and bifunctional antiplatelet/antiprotease drugs will be the focus of future targets in this field. Another important development in the field of antithrombotic drugs is the potential impact of generic versions of warfarin, low molecular weight heparins such as enoxaparin and dalteparin. The antithrombotic drugs are used in many of the critical indications and represent a diverse chemical, natural, hybrid agents for which the approval guidelines from the current regulatory agencies are not adequate, thus at this time the generic interchange for antithrombotic drugs is not recommended. Moreover, for such classes of drug as LMWHs and antithrombins therapeutic interchange is not recommended as well. Objective and unbiased clinical trials and group consensus are warranted for the optimal use of these agents.

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LABORATORY STANDARDIZATION IN HEMATOLOGY

Design of quality assessment system using proficiency testing in hematology and what we can gain from our application in Turkey

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Body of Abstract

Clinical laboratory errors lead to adverse effects on patient diagnosis, therapy and outcomes. Standardization plays an important role in patient care because it contributes to a decrease in the number of errors improving the harmonization of procedures and results comparability between different laboratories. For this reason standardization of haematology laboratory practice plays an important role in patient care and is an obligated practice for the total quality management (TQM), including certification and accreditation. Since the 1970s, spectrophotometer measurements for haemoglobin determination, centrifuge for PCV and diluting pipette for cell counting have been progressively substituted by automated devices performing a complete whole blood count (CBC) and WBC differential in only few seconds. Nowadays, due to the automation of haematology laboratory practice standardization is an essential requirement for improving efficiency and effectiveness as well as for ensuring a Total Quality Assurance (TQA) program. TQA should include the pre-analytical, analytical and post-analytical phases of general laboratory practice. The pre-analytical phase has to evaluate specimen processing variables such as patient identification, transcription of medical requests and quality of primary samples. The analytical phase should measure uncertainty related to bias and imprecision assure commutability of control materials, correct specimen design and handling, effective statistical treatment and communication of data. The post-analytical phase has special relevance in the haematology laboratory because it is always necessary to continuous evaluation of the reporting requirements such as correct reference ranges, comprehensive reports and interpretative comments. Here, it should never be forgotten that one of the targets of the haematology laboratory is to provide a diagnosis orientation and not only crude analytical data, histograms and graphic plots.

In the haematology laboratory, standardization should include the following topics: (a) Evaluation and selection of instrumentation and procedures. (b) Training and certification of personnel, (c) Establishment of quality control protocols, (d) Test protocols, equipment maintenance and troubleshooting records. (e) Protocols for test requisitioning and result reporting, and active review of results by laboratory director or designee and (f) Setting up policies regarding instrument maintenance and supplies.

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LABORATORY STANDARDIZATION IN HEMATOLOGY

External quality assessment scheme by private laboratories in Turkey

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Abstract
At the end of this presentation the audiences should be able to understand the goal of external quality assessment and definitions used to describe similar functions of terminology. Also they had information about overview of interlaboratory method performance studies in Turkey in Hematology.

The past decade has been a time of significant change in international health. Reform in the United Nations system aims to make organizations more responsive to the needs of Member States, and to provide a rallying point for achievement of the International development goals. To rise to this challenge will require more emphasis on effectiveness through collective action and partnerships. This, in turn, will require more dynamic, and less bureaucratic, approaches to management assuming a greater role in establishing wider national and international consensus on health policy (www.who.org);

- Strategies and standards through managing the generation and application of research, knowledge and expertise,
- Triggering more effective action to promote and improve health and to decrease inequities in health outcomes, through carefully negotiated partnerships and by making use of the catalytic action of others,
- Creating an organizational culture that encourages strategic thinking, prompt action, creative networking, innovation and accountability, and strengthens global influence.

The world is increasingly looking for greater coordination among development organizations. The global influence of the International Organization for Standardization (ISO) and the national health ministries from countries around the world, laboratories have come to embrace quality assurance as a system that combines daily quality control with interlaboratory comparison and accreditation while looking at patient test results with any error in patient outcomes. In the quest for laboratory quality, proficiency testing, accreditation and the interlaboratory quality assessment programs are essential tools to measure progress.

For achieving this goal, non-profit organizations such as IFCC, ISH, NCCLS, ECCLS or national bodies; WHO, CAP, CMS, set effective performance characteristics for diagnostic tests to ensure reliable, traceable and compareable laboratory test results (www.phppo.cdc.gov/CLIA/regs/toc.asp). Laboratory professionals take all necessary steps defined in quality assurance systems set by the above committees. These concepts include:

- Test monitoring system
  - External QC (different from profficiency testing)
  - Internal monitoring
- Proficiency testing
- Analytical system quality assessment
- Calibration verification
- Quality assessment activities
- Personal competency assessment (ISLH XIVth International Symposium. Quality Assurance Workshop. External Quality Assurance Overwiev. RM Rowan.)

The programs under the establishment of quality management system in health services have been applied with the framework of the same concepts with different terminologies that is because the programs were named differently even their operating

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Table I. Annual %CV values of CBC parameters of our programme and comparison with some others.

.	Participant number	Hb	Hct	RBC	WBC	Trombocytes
2002	56	3.31	3.32	3.04	12.40	16.70
2003	53	2.83	3.94	3.45	14.81	14.22
2004	89	3.33	3.34	3.63	11.19	14.64
2005*	94	3.50	4.20	3.67	14.23	15.68
min-max		2.22–4.02	2.58–4.57	2.72–5.08	6.73–26.52	10.49–18.23
CLIA 88		7.00	6.00	6.00	15.00	25.00
ISLH		3.00	4.00	3.00	8.00	10.00
European Countries**						
min-max		1.70–3.20		1.90–3.00	3.60–8.90	6.50–17.00

* First two distributions

** JM Jou, Hematology external quality assessment in Europe, 2004 ISLH Congress

principles and concepts are not so much different. Since the terms defining the system have been used interchangably so far, Codex Alimantarius Commission has suggested a new terminology to decrement the terminology to a unique structure (Appendix A).

Codex described proficiency testing as Inter Laboratory Exchange Programmes (ILEP) and described as a study in which several laboratories measure a quantity in one or more identical portions of stable materials under documented conditions. Major purpose of ILEP is to set method performance studies which include interlaboratory compatibility of results between different methods and instruments. ILEP will be used together with external QC material which gives a clue for evaluation of any new instrument or method. ILEP should include quality issues such as turnaround time, training and education of laboratory staff and appropriateness in test request and the utilization of the test results. Aim is to increase both efficiency and the operational effectiveness. The data can be used for certification and accreditation. Organizational principles of ILEP are well established and clearly presented in guidelines released from IFCC, NCCLS/NCQA in case, could be adapted to hematology from ISLH.

Unfortunately there is no external quality asurance program neither in the area of Clinical Biochemistry nor in that of Hematology in Turkey. As Duzen Laboratories Group, our first study as starting point was peer view methods performance study to build up the compatibility among the test results between the private laboratories.

Turkish Hematology Society supports our interlaboratory peer view study because of:

- Determining the present situation in Turkey,
- Extending the content and improving to an EQAS programme from proficiency testing,
- Increasing the participation from hospitals to the programme.

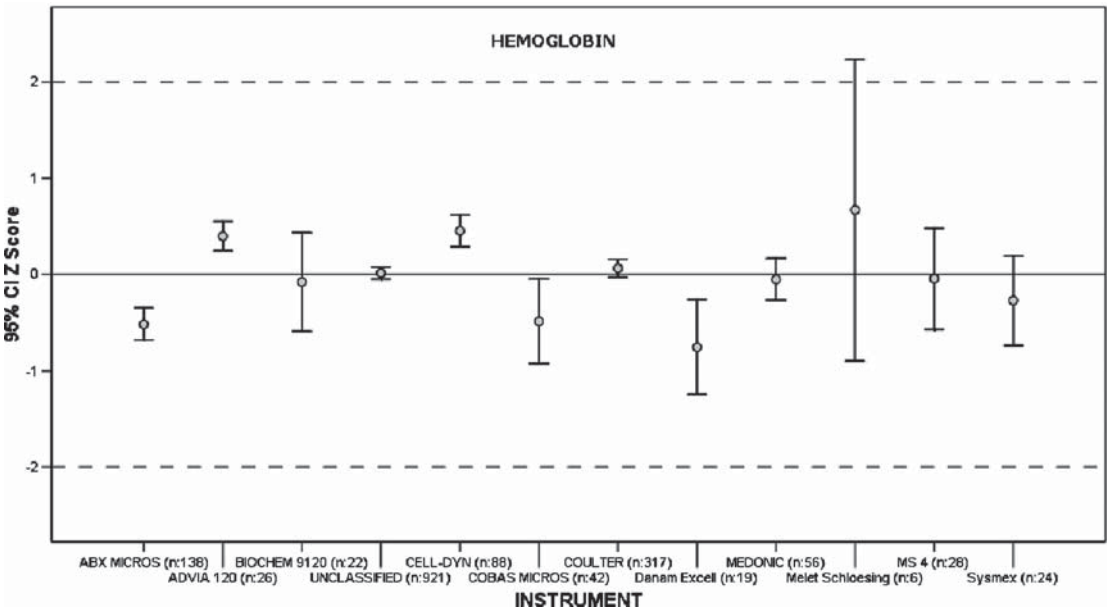

Figure 1. The distribution of variation described as SDI (z score) for Hb.

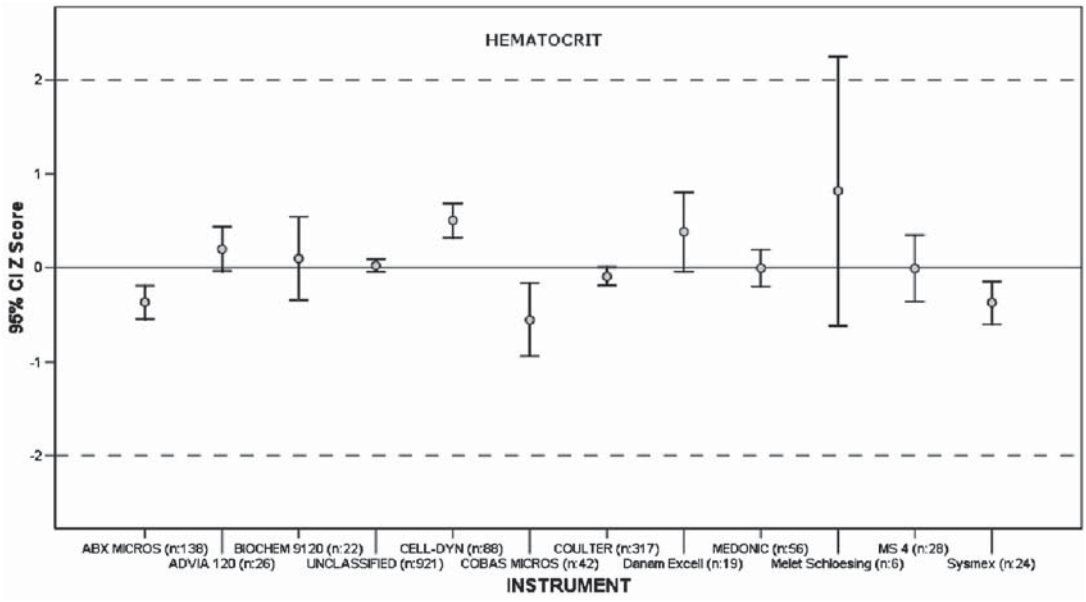

Figure 2. The distribution of variation described as SDI (z score) for Hct.

Furthermore within the last 2 years, TURKAK (Turkish Accreditation Agency) has undertaken the supervision of sample preparation, distribution and evaluation components of the program. The whole programme from sample preparation to evaluation is prepared and organized according to EQA programme given by IFCC (Fundamentals for External Quality Assessment, IFCC).

Participation to the programme is voluntary and the results are not linked to any sanctions. Results are submitted on code bases and keep confidential. Program was started with 54 laboratories and reached 110 as on June 2005 from all over Turkey. Fresh human blood is used which was taken from two volunteer healthy donors, whose known infection risk is nons existent, and the programme includes measurement of Hb, Hct, RBC, WBC and thrombocyte counting. Homogenity of the samples are controlled before distribution. Specimens are distributed 4 times in a year in temperature controlled conditions. Overnight delivery service is using for distribution and maximum time interval is 16 hours for arrival which makes possible to make the measurements in 14–18 hours. The participants send their results via Internet to our web site by using their username and passwords which are created by themselves. The anonymity of the results is provided carefully. The participants have 2 days for data entry and after 4 days they can see

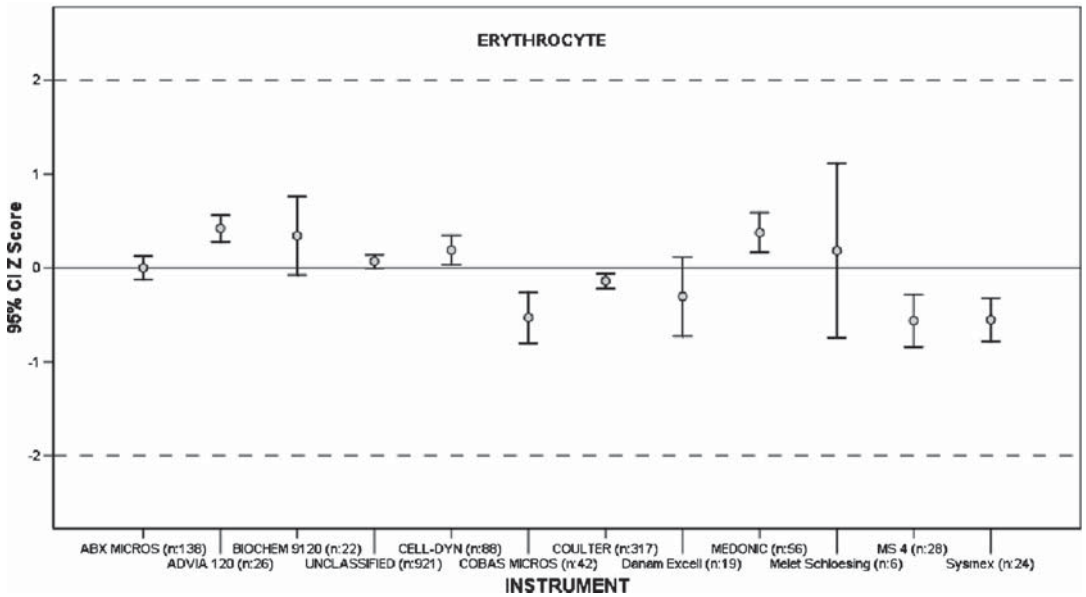

Figure 3. The distribution of variation described as SDI (z score) for RBC

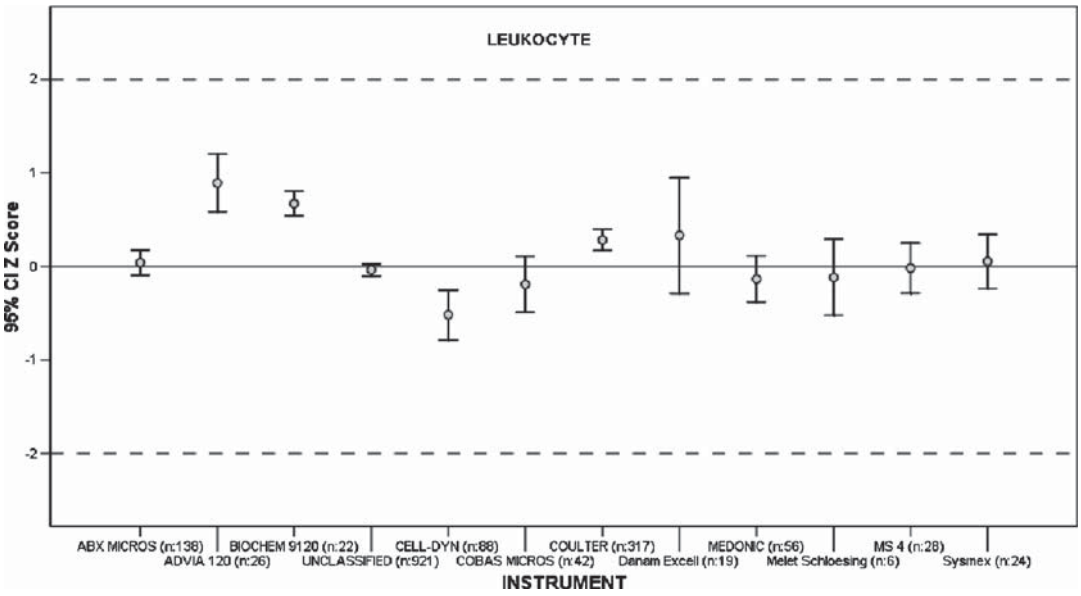

Figure 4. The distribution of variation described as SDI (z score) for WBC.

their whole reports and overall results of all participants to evaluate accumulated total results from the same web site (www.duzen.com.tr/qcr).

An inhouse software programme that covers whole aspects of EQA programmes does the evaluation. Ranges of acceptablity used in the programme have been set by IFCC and CLIA 88 criteria. Basic evaluation points in the programme are the consensus value and variation from the mean. All 4 last results in two different levels with mean, %CV, SD and SDI values are sent together with a Levey Jennings graphic to the participants in printed reports. The participants can also see their results and special evaluations about the analyzers from Internet also. They can see their results in Youden graphics in which $\pm$2SD and CLIA criteria are marked. Summary of accumulated overall results were submitted to TURKAK as well as the Turkish Hematology Society to be evaluated and discussed aiming to set a proper preparation, distribution and education system. Variations of the results for 4 years are given in Table I. Detailed report will be presented in oral presentation separately.

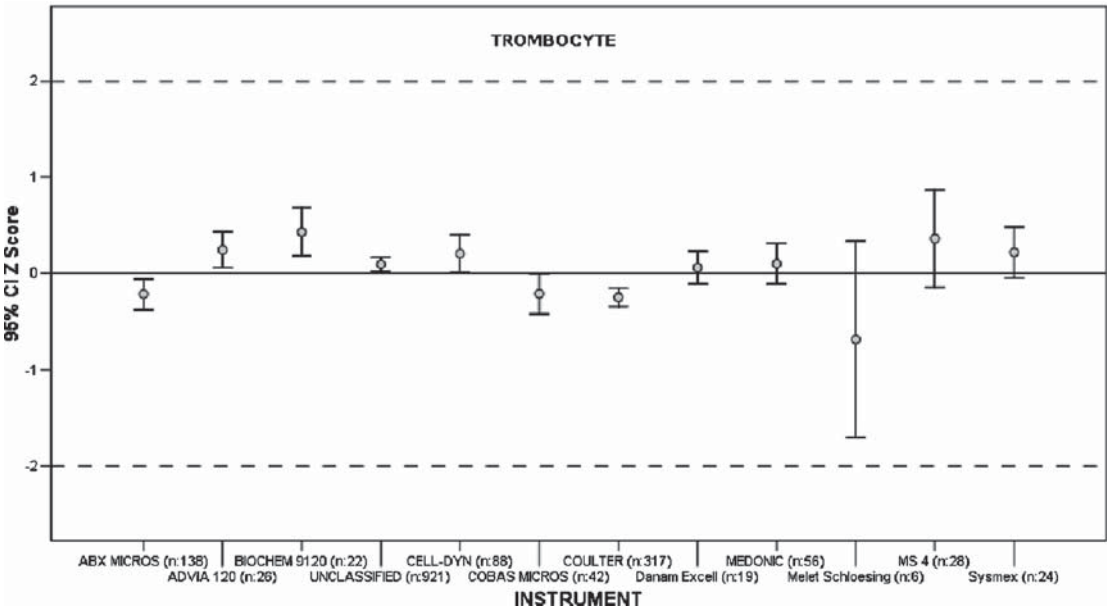

Figure 5. The distribution of variation described as SDI (z score) for trombocyte.

Table II. Within, between-subject variations and II value of CBC parameters (Biological variation: From principles to practice. CG Fraser, AACC 2005).

Analyte	Within-subject Variation (%)	Between-subject Variation (%)	Index of Individuality (II)
Hb	2.8	6.6	0.42
Hct	2.8	6.6	0.42
MCV	1.3	4.8	0.27
MCH	1.6	5.2	0.31
MCHC	1.7	2.8	0.61
RBC	3.2	6.1	0.52
WBC	10.4	27.8	0.37
Thrombocytes	9.1	21.9	0.42

Instrument based evaluation were made by using z scores (Figures 1–5). The z scores, which are in ± 2 value, are interpreted as the distribution shows acceptable variation. These findings seems like they are enough for the "first visit test" but in order to use these tests in monitoring, the CV values had to be better. Also the CV values had to decrease till to biological variations and the clinicians had to be instructed about the individuality index (II) (Table II). However CBC like tests with high II needs narrower variations to follow-up for monitoring or treatment to take a prompt action accordingly.

In order to add an extra value to our programme we make a special study in June 2005. The distributed sample is obtained from a single healthy donor and two levels of sample are prepared by taking and adding of plasma and buffy coat from one half to other. With this way we obtain 2 samples with different Hb, Hct, RBC, WBC and thrombocyte values and same RDW, MCH, MCV and MCHC values from the same person (Table III). In this study our aim was to see the variation between their results and the CV values that they obtain from their internal controls.

Table III. MCH, MCV, MCHC and RDW values that we obtain from our special study (Participant number = 28).

	MCH		MCV		MCHC		RDW	
	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
Mean	30.23	30.31	88.06	88.83	34.34	34.04	13.46	13.95
SD	0.86	0.85	2.13	2.29	1.03	1.02	1.04	1.11
CV%	2.85	2.80	2.42	2.58	3.00	2.99	7.75	7.95

After presenting our preliminary findings in Hematology meeting at 2003, some of the kit providers started applying calibrators, therefore, routine calibrator users's CV are presented. The calibrator using laboratories gives higher results as compared with others (Figure 6). We think that this is a relative result and the laboratories, which does not use calibrator gives false negative results. We will see the answer of this hypothesis with using reference material for our study in the near future.

So far we have achieved an agreement of over 95% with respect to CLIA. Since our goal is achieving further reduction in variation among the participating laboratories, to meet the ISH requirements rather than CLIA, will be the outcome on the aim of better gaining information about diagnostic accuracy (sensitivity) and limit of detection by reducing confidence limits of analyzer and calibrator. Rather than consensus value we would like to use control sample with a known value. Our aim is to use the samples that we

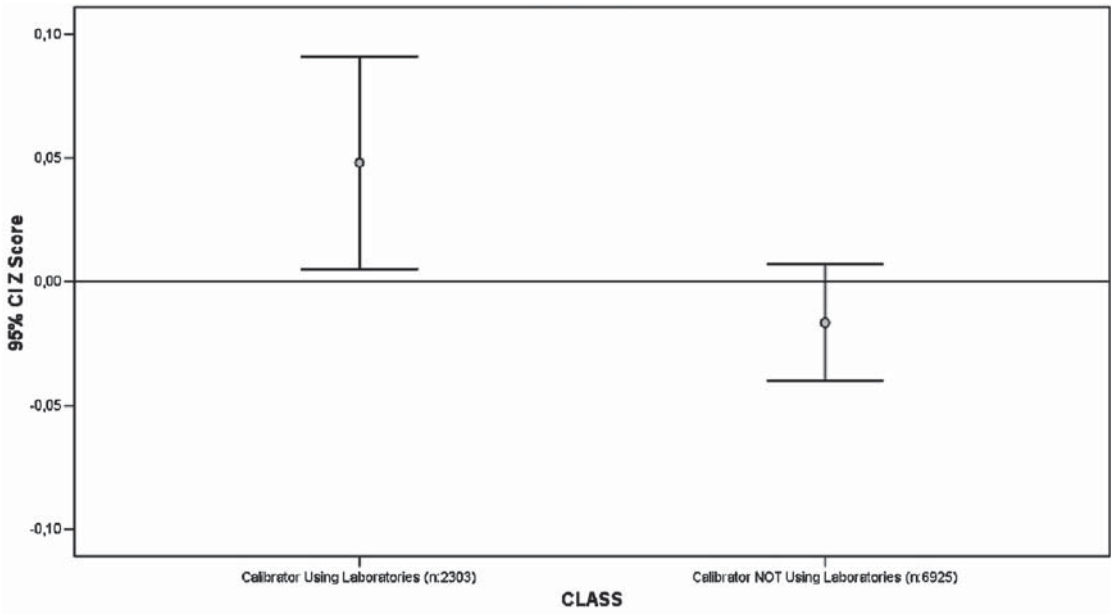

Figure 6. The distribution of variation described as SDI (z score) for calibrator using laboratories.

know their absolute values, which are prepared with reference methods. We believe this will change the discrepancies of calibrator user and reduce CV that this will be the correct way to reduce the CV values in order to use the consensus value. Our CV values do not change in years and there isn't any improvement. We know that we have to do something more but we think that this requires political enforcement of regulatory organizations and buyers of health services.

Acknowledgements

I want to express my sincere appreciation to the programme supervisors for sharing the information about "Duzen Laboratuvarlar Arasi Kalite Kontrol Programi" and assistance for manuscript to Yalçin Yildiz.

Appendix A: Harmonisation of analytical terminology in accordance with international standards, inter-agency meeting may 2004

ACCURACY

ISO Standard 78-2: Chemistry – Layouts for Standards – Part 2: Methods of Chemical Analysis (Second Edition, 1999)
Closeness of agreement between a test result and an accepted reference value.

NOTE: The term accuracy, when applied to a set of test results, involves a combination of random components and a common systematic error or bias component.

As a concept:

Codex Alimentarius Commission
The closeness of agreement between the reported result and the accepted reference value.
Note: The term accuracy, when applied to a set of test results, involves a combination of random components and a common systematic error or bias component. {ISO 3534-1} When the systematic error component must be arrived at by a process that includes random error, the random error component is increased by propagation of error considerations and is reduced by replication.

As a statistic:

Codex Alimentarius Commission
The closeness of agreement between a reported result and the accepted reference value. {ISO 3534-1}
Note: Accuracy as a statistic applies to the single reported final test result; accuracy as a concept applies to single, replicate, or averaged value.
International vocabulary for basic and general terms in metrology
Harmonised guidelines for internal quality control in analytical chemistry laboratories
The international harmonised protocol for the proficiency testing of (chemical) analytical laboratories

Closeness of the agreement between the result of a measurement and a true value of the measurand.

Note 1. Accuracy is a qualitative concept.
Note 2. The term precision should not be used for accuracy

INTERLABORATORY STUDY

Codex Alimentarius Commission
A study in which several laboratories measure a quantity in one or more "identical" portions of homogeneous, stable materials under documented conditions, the results of which are compiled into a single document.
Note: The larger the number of participating laboratories, the greater the confidence that can be placed in the resulting estimates of the statistical parameters. The IUPAC-1987 protocol (Pure & Appl. Chem., 66, 1903-1911(1994)) requires a minimum of eight laboratories for method-performance studies

INTERLABORATORY TEST COMPARISONS

International harmonised protocol for the proficiency testing of (chemical) analytical laboratories

Organisation, performance and evaluation of tests on the same items or materials on identical portions of an effectively homogeneous material, by two or more different laboratories in accordance with pre-determined conditions.

LABORATORY-PERFORMANCE (PROFICIENCY) STUDY

Codex Alimentarius Commission

An interlaboratory study that consists of one or more measurements by a group of laboratories on one or more homogeneous, stable, test samples by the method selected or used by each laboratory. The reported results are compared with those from other laboratories or with the known or assigned reference value, usually with the objective of improving laboratory performance.

Notes

1. Laboratory-performance studies can be used to support accreditation of laboratories or to audit performance. If a study is conducted by an organisation with some type of management control over the participating laboratories – organisational, accreditation, regulatory, or contractual – the method may be specified or the selection may be limited to a list of approval or equivalent methods. In such situations, a single test sample is insufficient to judge performance. It is expected that the results from 1 of every 20 tests will be outside the value for the calculated mean $\pm$ twice the standard deviation, due solely to random fluctuations.
2. Sometimes a laboratory-performance study may be used to select a method of analysis that will be used in a method-performance study. If all laboratories, or a sufficiently large subgroup, of laboratories, use the same method, the study may also be interpreted as a method-performance study, provided that the samples cover the range of concentration of the analyte.
3. Separate laboratories of a single organisation with independent facilities, instruments, and calibration materials, are treated as different laboratories.

Protocol for the design, conduct and interpretation of method-performance studies (1995)

METHOD-PERFORMANCE STUDY

Codex Alimentarius Commission

An interlaboratory study in which all laboratories follow the same written protocol and use the same test method to measure a quantity in sets of identical test samples. The reported results are used to estimate the performance characteristics of the method. Usually these characteristics are within-laboratory and among-laboratories precision, and when necessary and possible, other pertinent characteristics such as systematic error, recovery, internal quality control parameters, sensitivity, limit of determination, and applicability.

Notes

1. The materials used in such a study of analytical quantities are usually representative of materials to be analysed in actual practice with respect to matrices, amount of test component (concentration), and interfering components and effects. Usually the analyst is not aware of the actual composition of the test samples but is aware of the matrix.
2. The number of laboratories, number of test samples, number of determinations, and other details of the study are specified in the study protocol. Part of the study protocol is the procedure which provides the written directions for performing the analysis.
3. The main distinguishing feature of this type of study is the necessity to follow the same written protocol and test method exactly.
4. Several methods may be compared using the same test materials. If all laboratories use the same set of directions for each method and if the statistical analysis is conducted separately for each method, the study is a set of method-performance studies. Such a study may also be designated as a method-comparison study.

Protocol for the design, conduct and interpretation of method-performance studies (1995)

An interlabortory study in which all laboratories follow the same written protocol and use the same

test method to measure a quantity in sets of identical test items [test samples, materials]. The reported results are used to estimate the performance characteristics of the method. Usually these characteristics are within-laboratory and among-laboratories precision, and when necessary and possible, other pertinent characteristics such as systematic error, recovery, internal quality control parameters, sensitivity, limit of determination, and applicability.

PRECISION

1. ***ISO Standard 78-2: Chemistry – Layouts for Standards – Part 2: Methods of Chemical Analysis (Second Edition, 1999)***
2. ***Codex Alimentarius Commission***

The closeness of agreement between independent test results obtained under stipulated conditions {ISO 3534-1}

Notes: {ISO 3534-1}
1. Precision depends only on the distribution of random errors and does not relate to the true value or to the specified value.
2. The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results. Less precision is reflected by a larger standard deviation.
3. "Independent test results" means results obtained in a manner not influenced by any previous result on the same or similar test object. Quantitative measures of precision depend critically on the stipulated conditions. Repeatability and reproducibility conditions are particular sets of extreme conditions.

1. ***Terms and definitions used in connections with reference materials, ISO Guide 30:1992***
2. ***Harmonised guidelines for internal quality control in analytical chemistry laboratories***
3. ***The international harmonised protocol for the proficiency testing of (chemical) analytical laboratories***

Closeness of agreement between independent test results obtained under prescribed conditions.

NOTES:
1. Precision depends only on the distribution of random errors and does not relate to the accepted reference value.
2. The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results. High imprecision is reflected by a larger standard deviation.
3. 'Independent test results' means results obtained in a manner not influenced by any previous result on the same or similar material.

PROFICIENCY TESTING SCHEME

International harmonised protocol for the proficiency testing of (chemical) analytical laboratories

Methods of checking laboratory testing performance by means of interlaboratory tests
[It includes comparison of a laboratory's results at intervals with those of other laboratories, with the main object being the establishment of trueness]

QUALITY ASSURANCE

1. ***Quality assurance and quality management-vocabulary, ISO 8402:1994***
2. ***Harmonised guidelines for internal quality control in analytical chemistry laboratories***

All those planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality.

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HEMATOLOGY IN DEVELOPING COUNTRIES

Virtual microscopy: Applications to hematology education and training

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Keywords: *virtual microscopy, education, proficiency testing*

Introduction

Virtual microscopy may be defined as the simulation of microscopy, over a computer network, as opposed to real microscopy using a physical microscope. The term "microscopy" is derived from Greek roots: "mikros" (small) and "skopeo" (to view). A technical definition for the term "virtual" is a "simulation of the real thing" [1]. Synonyms for virtual microscopy include digital microscopy, digital pathology, and virtual pathology. A real microscope has four functions, which are to display, pan, zoom, and to focus an image. These are the functions that are simulated by virtual microscopy, in which "virtual slides", or large digital image files, are displayed, panned, zoomed, and focused in a virtual slide viewer on a computer screen. Recent technological advances in virtual microscopy have begun to enable its application to haematology education and training [2].

Virtual slides

A virtual slide is a giant digital image file of a selected area of a glass slide. Early developments were the concept of digital image tiles [3] and the distortion-free assemblage of digital microscopic images of histological sections [4]. A virtual slide is created by merging, or stitching digital images ("tiles") of sequential, adjacent microscopic fields, to produce a large, panoramic digital image of the glass slide. Virtual slides represent a revolutionary advance over glass slides. They are easy to store, file or retrieve, and to mark or annotate. Further, they are easy to duplicate or distribute and can be integrated into electronic patient records. They do not deteriorate over time. Of the different disciplines in pathology, haematology has among the most demanding requirements in terms of high image resolution.

Virtual slides may be captured by manual tiling, robotic tiling or line scanning. Small virtual slides, e.g. a montage of 5×5 images ("tiles"), may be captured with a digital microscope and and stitched manually using imaging software e.g. Panavue® or Adobe Photoshop®. Large virtual slides, e.g. 40×40 tiles or more, may be created using automated robotic tiling workstations or virtual slide scanners. The first scanners that implemented motorized stages with robotic tiling software were introduced in 1997. These combined preset, sequential digital image capture with autofocusing, tile stitching, and output of compressed virtual slides (e.g. Bliss Slide Scanner®, Virtual Slice®). These virtual slide scanners enable an area of 1.5cm $\times$ 1.5cm of a glass slide to be captured with a 40X objective lens in a few minutes, depending on optical settings and image resolution. A number of other robotic tiling software programs (e.g. ER-Mapper®, MrSID®) originate from aerial and satellite imaging technologies. In 2000, a method of virtual slide capture using line scanning with a linear-array detector was introduced (Scanscope®). The detector is coupled to an objective lens that moves over the glass slide at constant velocity, and scans the slide in stripes or lines instead of tiles, thereby limiting image seams to one axis. Another recent innovation was the use of pulsed strobe illumination with continuous stage motion to capture virtual slides [5]. Pulsed strobe illumination provides a high photon burst that shortens CCD (charged-coupled device) integration time, thereby enabling faster motorized slide transport without blurring.

Following image capture, the digital image file may be saved in a "lossless" file format (e.g. TIFF, tagged

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image file format), in a compressed "lossy" file format (e.g. JPEG2000), or in a compressed pyramidal file format (e.g. Flashpix®, Zoomify®). File compression reduces file size, but results in loss of image quality with excessive compression ratios. The file size of a virtual slide depends upon the area of the slide captured and the resolving power of the system, which is in turn determined by the camera, optical coupling and microscope specifications. A scanned area of 1.5cm × 1.5cm is generally adequate for most haematology smears, and results in a virtual slide of several gigabytes in size, at standard magnifications. Robotic scanners allow the user to manually define the area of interest at low magnification before automated virtual slide capture. In order to reduce virtual slide scanning time and file size, a background preset of an unoccupied area of the glass slide ensures that only the area containing the specimen is scanned. Focusing can also be preset in different areas of the specimen.

Strategies in microscopy optics that can significantly reduce virtual slide file size include the capture of image fields through low magnification lenses at high resolution (pixel density), and the use of objective lenses of high numerical aperture (theroretically up to 1.0 for dry objectives and up to 1.5 for oil immersion objectives) in order to increase optical resolution. However, an increase in numerical aperture is accompanied by a reduction in the depth of field, which may result in irregular focus when capturing a large virtual slide.

The focus function ("z-axis") is dependent on the capture of images at different focal planes to form an image stack ("z-stack"). The introduction of a focusing function in virtual microscopy results in an increase in scanning time and virtual slide file size. Although most haematology smears or sections are cell monolayers, some examples of haematology specimens which require image focusing include bone marrow smears as well as red cell inclusions in blood smears.

Virtual slide display

Virtual slides may be accessed online, e.g. over the Internet or within a local area network (LAN), or offline, e.g. from fixed disks or DVD-ROM (digital versatile disks, read-only memory). A virtual slide viewer is a software that can display, pan and zoom a virtual slide on a networked PC. Desirable features are a "pixel on demand" display, a user-friendly interface, and the ability to view various file formats and to operate across different operating systems. "Pixel on demand" viewing is required to overcome the problem of sufficient bandwidth when transmitting large virtual slides over the Internet. By this method, only the image tiles (commonly 64 × 64 pixels in size) requested by the user are streamed from the server, thereby enabling very large images to be viewed at low bandwidths.

Recently, viewers with the ability to display a z-stack have been introduced. Transition between successive images captured in different focal planes can be controlled by movements of the mouse wheel.

A key function of the virtual slide viewer is navigation. In real microscopy, the pathologist is skilful at slide navigation, and is able to switch back and forth between different magnifications with ease. For virtual microscopy to gain acceptance with professional microscopists, the development of an intuitive and user-friendly software interface is essential.

Applications to education and training

With existing technology, virtual microscopy is beginning to replace the traditional microscopy classroom in medical education. Virtual microscopy undoubtedly offers significant advantages over real microscopy in education and training. A web-based virtual slide library can be permanent and enables users to revise materials "anytime, anywhere" without microscopes or glass slides. By contrast, the traditional microscopy classroom is costly to set up and to maintain, and high quality glass slides are impossible to duplicate or replace. Important advantages of virtual microscopy are that all users view the same image, and that images are easy to distribute over the WWW, or in a DVD-ROM. An effective application of virtual microscopy in haematology education is the haemopoietic system collection in the Virtual Slidebox of Histopathology [6]. In general, virtual microscopy has been well received by both students and teachers [7–9]. In one study [9], medical students found navigation with virtual microscopy easier than with real microscopy, and felt that it improved their performance. Further, virtual microscopy enhanced the instructor's ability to point out cells and lesions, and the student's overall ability to learn from the slide. Whether medical students need to learn the use of a real microscope is debatable.

A current challenge is to apply virtual microscopy to proficiency testing and surveys in pathology and haematology. Current haemato-morphology proficiency surveys by major providers such as the UK NEQAS (United Kingdom National External Quality Assurance Scheme) and the RCPA (Royal College of Pathologists of Australasia) depend upon the distribution of glass slides, whereas the College of American Pathologists distributes 35mm transparencies of images of blood smears. Specimens on glass slides can be produced in only limited numbers and are never identical, while 35mm transparencies suffer from the disadvantage of a tiny field of view. Virtual microscopy overcomes these problems and could enable haematology surveys of bone marrow aspirate

and trephine specimens. In 2003, an important lead was taken by the American Board of Pathology, with the introduction of virtual microscopy for the Primary Certification Exams in Anatomical Pathology [10]. Established morphology survey providers, such as UK NEQAS [11] and the RCPA have also taken the first steps toward virtual microscopy. One organization, LabQuality Inc [12] has created high quality virtual slides of bone marrow slides for haematology proficiency surveys.

A practical consideration in proficiency testing is that specifications of the user screen display become important. For instance, the American Board of Pathology specifies that the display should be set at a minimum of 1024×768 pixels in resolution and in True Color (32-bit) for the Primary Certification Exams [10]. It may also be necessary for pathologists and haematologists to define a minimum standard of image quality required for diagnostic applications. A move has been started by DICOM (Digital Imaging and Communications in Medicine) [14] to create a standard method for the transmission of medical images and their associated information in pathology. Some difficulties in the standardization in pathology imaging have been discussed [13].

Potential applications of virtual microscopy in haematology proficiency testing include blood film morphology, bone marrow morphology, differential cell counts, cytochemistry, immunocytochemistry and the detection of malarial parasites in blood smears. In particular, virtual microscopy could enable proficiency surveys of critical clinical parameters such as the bone marrow blast count, as well as the implementation of "locate and identify" proficiency testing exercises. The diagnostic equivalence of virtual and real microscopy has been demonstrated in a number of studies in anatomical pathology [15,16]

Conclusion

The field of virtual microscopy has gained momentum in recent years. Virtual slides represent a revolutionary advance over glass slides in terms of function and utility. With the recent introduction of focusing, virtual microscopy can now simulate all the functions of real microscopy. It is likely that virtual microscopy will become an important educational tool in the basic medical sciences. Diagnostic equivalence of virtual and real microscopy has been demonstrated in a number of studies in anatomical pathology. For virtual microscopy to gain acceptance with professional microscopists, the development of an intuitive and user-friendly software interface is essential. Virtual microscopy is a promising tool for a wide range of applications in haematology including education, training and proficiency testing.

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HEMATOLOGY IN DEVELOPING COUNTRIES

The Mexican approach to conduct allogeneic stem cell transplantation: Braking dogmata and facing the Matthew effect

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Introduction

The dogmata

Dogmata are principles, maxims or tenets; settled opinions adopted through authority instead of reason or experience. The progress and evolution of knowledge very frequently rely on the breakage of dogmata [1]. Historically, the development of allogeneic hematopoietic stem cells (HSC) transplantation has relied on high dose myeloablative chemo or radiotherapy with three main dogmatic goals: (1) to eradicate underlying disease, (2) to create bone marrow space for the incoming HSC, and (3) to suppress the recipient's immune system in preparation for the allograft so that rejection of the donor stem cell graft does not occur [2].

The broken dogmata

The evolution of knowledge has proven as wrong the first two above mentioned dogmata: In 1978, Odom et al. [3] described two patients with acute lymphoblastic leukemia who achieved a remission as a result of the development of graft versus host disease (GVHD). The concept of "graft versus leukemia" effect was then introduced. Later on, researchers from the group of the Nobel-laureate, Dr. E. Donnall Thomas in Seattle, USA, published a paper on the anti-leukemic effect of the GVHD [4]; this publication is now considered as one of the landmark papers in hematology of the twentieth century [5]. The documentation that donor-lymphocyte infusions (DLI) with no additional chemotherapy following induction of host versus graft unresponsiveness resulted in remission, thus suggesting that once given the chance by prevention of rejection, alloreactive lymphocytes can eliminate leukemia, a concept entertained by Kolb et al. [6] and Slavin et al. [7,8] was followed by focusing on durable engraftment of lymphocytes rather than myeloablation of tumor cells, resulting in the development of the non-myeloablative stem cell transplantation (NST) methods starting in Jerusalem and then in Houston [9,10]. Accordingly, it is now well known that the anti-tumor effect of the GVHD induced by HSC allografts is responsible for the control of certain malignancies, and that HSC create their own marrow space through GVHD reactions [5–17]. We have learned that certain malignancies are more susceptible than others to the graft versus tumor effect; for example: Chronic myelogenous leukemia is substantially more sensitive to this effect than acute lymphoblastic leukemia [13,14], this being probably one of the reasons of the different results obtained when allografting individuals with these diseases.

The consequences of braking dogmata

(1) Having proved that the graft versus tumor effect is the responsible for the control of certain malignancies in individuals given allogeneic HSC grafts, and that the bone marrow space does not need to be created by ablative chemo or radiotherapy, the obvious question was: It is possible to induce graft versus tumor effect by allogeneic HSC without producing a severe damage to the recipient's bone marrow, immune system and other organs? The answer to this question is yes; it is now well known that current intensive and toxic cytoreductive conditioning therapy can be replaced by nonmyeloablative immunosuppression to facilitate

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allogeneic engraftment; in *lieu* of intensive chemotherapy before transplantation, engrafted donor T cells are used to accomplish the task of eradicating the host's malignant cells [5–19]. Accordingly, it seems now clear that two immunological barriers must be overcome to successfully establish HSC allografts: One is host versus graft disease (HVGD/rejection), whereas the other is the opposite, the graft versus host reaction, which includes GVHD. Traditionally, the therapeutic regimens administered to prevent HVGD are delivered before transplant and aimed to eradicate the host's immune response, whereas the therapeutic regimens to prevent GVHD focus on the grafted donor immune cells are delivered after transplant and ideally, should only affect those donor immune cells that react with alloantigens for which the donor and host are mismatched [5–19].

(2) Widespread application of HSC transplantation had been limited by the toxicity associated with the myeloablative conditioning regimens. In attempts to achieve maximal tumor eradication, conditioning regimens had been intensified to a point at which serious nonhematopoietic organ toxicities were common and resulted in morbidity and mortality [19]. In addition, the pancytopenia induced by the high-dose regimens carries the risks of serious and even lethal infections despite the use of prophylactic broad-spectrum antibiotics; even more, the regimen-related toxicity, particularly to the liver and kidney, frequently restricts the ability to give optimal post-grafting immunosuppression therapy, which is necessary to avoid GVHD. As a result, at most transplant centers, the severity of the complications from myeloablative chemotherapy and allotransplantations had limited their use to relatively young individuals aged less than 50 or 55 years. Effective strategies to reduce the toxicity of transplantation regimens were necessary to improve overall survival rates, reduce delayed side effects and improve quality of life in long-term survivors. Stemming from the fact that former intensive and toxic cytoreductive conditioning therapy can be replaced by nonmyeloablative immunosuppression to facilitate allogeneic engraftment, it is now possible to allograft individuals aged, debilitated or with co-morbidities [5–19].

(3) Another salient point which is frequently overlooked in papers dealing with bone marrow transplantation coming from developed countries is the cost of the procedure. In our experience (vide infra), non-myeloablative stem cell transplantation (NST) is substantially cheaper than conventional ablative stem cell allografting [17,19–31]; as a result, allogeneic HSC can be offered now to more patients as a therapeutic option, this observation being critical for individuals living in developing countries. The fact that over two-thirds if the inhabitants of the world live in developing countries should be born in mind when reading these lines.

The ways of braking these dogmata

Many transplantation groups have reported encouraging results using a number of reduced-intensity or non-myeloablative conditioning regimens for patients with hematological malignancies and solid tumors [6–17]. Different approaches have been used to conduct NST: The Jerusalem approach, the Houston approach, the Bethesda approach, the Genoa approach, the Boston approach, the Seattle approach, the Dresden approach, the London approach and the Mexican approach [32,33]; all these approaches address the immunosuppressive effect more than the myeloablative effect of the conditioning regimens.

The Mexican way to brake these dogmata

In 1999, we elected to employ a regimen to conduct NST, based in those employed in Jerusalem [7], Houston [6] and Genoa [34], introducing some changes with the main goal of decreasing the cost of the procedure. The salient changes of our approach are:

Use of cheapest and available drugs. Since both intravenous melphalan and anti-thymocyte globulin are expensive and unavailable in México, we chose to use available and affordable drugs by means of the following scheme: Oral busulphan, 4 mg kg^{-1} on days -6 and -5; i.v. cyclophosphamide, 350 mg/m^2 on days -4, -3 and -2; i.v. fludarabine, 30 mg/m^2 on days -4, -3 and -2; oral cyclosporin A (CyA) 5 mg kg^{-1} was started on day -1 and i.v. methotrexate 5 mg/m^2 was delivered on days $+1$, $+3$, $+5$ and $+11$ [7,18,19].

Tailored number of apheresis sessions. We used initially three sessions of apheresis to obtain peripheral blood HSC from the donors [18], but we learned afterwards that, with the goal of obtaining between 1 and 6×10^6 viable CD34 cells kg^{-1} of recipient's body weight [12] we could cut down the number of sessions of apheresis to a median of two (range 1 to 4), thus diminishing costs of the procedures and of the disposable apheresis sets.

Elimination of prophilactic ganciclovir and intravenous IgG. Probably as a result of the reduced bone marrow damage during NST, the prompt recovery of both the hematopoiesis and immune function in this type of allografts and the use of peripheral blood, there is a very low prevalence of cytomegalovirus (CMV) disease despite a high prevalence of CMV infection in these individuals. We have faced no CMV-related deaths in patients given NST using our method [21] and have elected to eliminate the prophilactic use of both ganciclovir and intravenous IgG, thus reducing

costs; it is interesting that other NST schedules including anti-CD52 monoclonal antibody (Campath) are related to higher prevalences of CMV disease and mortality [21].

Outpatient conduction. Since the duration of both granulocytopenia and thrombocytopenia during NST is shorter than those during autologous stem cell transplants or during myeloablative chemotherapy, we elected to conduct NST on an outpatient basis provided certain conditions are fulfilled. Only patients asymptomatic, fully active, able to stay in their homes, with relatives or friends or in nearby-hotels, and with a fair educational level can be offered this program. Fundamental to the success of this approach is the availability of a 7 day-a-week clinic where medications and transfusions can be rapidly and efficiently provided [23,26,27].

Reduced number of blood products transfusions. Stemming also from the prompt recovery of the bone marrow, NST can be conducted in some instances without transfusion of blood products. In our experience, approximately one out of three individuals does not need red blood cells or platelets transfusions. The median of transfused red blood cells units is 6, range 0–19, whereas the median of platelet transfusion sessions was 2, range 0–5. Twenty percent of the patients given NST using our method do not require red blood cells nor platelet transfusions at all [25]. It is obvious that this policy results in decreases of both costs and risks derived from exposure to human blood derivatives.

Reduced donor-lymphocyte infusions. Donor lymphocyte infusions (DLI) are delivered only if the patients, on day 30, have not displayed either of the following. An evidence of partial or complete chimerism [22], GVHD or molecular remission of the malignancy. As a result of this policy, less than 10% of the patients need late DLI, thus diminishing costs as well.

Results

Using our method, we have conducted over 200 allografts in patients with different diseases: Chronic myelogenous leukemia (CML), acute myelogenous leukemia, acute lymphoblastic leukemia, myelodysplasia, thalassemia major, relapsed Hodgkin's disease, Blackfan-Diamond syndrome, adrenoleukodystrophy, aplastic anemia and solid tumors. In the whole group, the median granulocyte recovery time to $0.5 \times 10^9\,l^{-1}$ was 13 days, whereas the median platelet recovery time to $20 \times 10^9\,l^{-1}$ was 12 days. Around one third of the patients did not need red blood cell transfusions and also one third did not need platelet transfusions. In about 80% of the cases, the procedure could be completed fully on an outpatient basis. Follow-up times range between 30 and 1500 days. Fifteen patients failed to engraft and recovered endogenous hemopoiesis; half of them developed acute GVHD, whereas 33% developed chronic GVHD. The median post-transplant survival (SV) has not been reached, whereas the 1500 day overall SV is 58%. The 100-day mortality was 18% and the transplant-related mortality was 24%. The best results of our program have been obtained in CML, whereas the worse in acute lymphoblastic leukemia; these differences may be related with the susceptibility of the malignancy to the graft versus tumor effect. It is now clear that certain neoplasias such as CML are very sensitive to this effect whereas ALL and other malignancies are less susceptible to this immune effect. In the total group of patients, the median cost of each NST procedure was 18 000 US dollars [20–27], a figure which contrasts with that informed from the US for conventional bone marrow transplantation, which is 300 000 US dollars [2]. As a result and an example, it is now clear that, using our method, it is cheaper to allograft an individual with CML than to provide treatment with imatinib mesilate for one year.

Within the group of patients with chronic myelogenous leukemia (CML), 21 have been published [23]: Eleven were grafted in chronic phase, six in blast phase and four in accelerated phase; the median age of the patients was 43 years, with a range of 20 to 61; ten individuals were above 45 years old. The median post-transplant survival of the patients is above 750 days, whereas the 750-day survival is 60%. Four of the six patients grafted in blast phase have died. Twelve patients (57%) developed acute GVHD and 12/17 (70%) individuals developed grade I-II chronic GVHD. All the patients engrafted and achieved hematological remissions; in 15 individuals a molecular remission could be recorded. We have also grafted 21 children [21]; the median age of this group was 13 years. The median post-transplant overall survival of the children is above 1350 days, whereas the 34-month survival is 55%; 4/21 patients (19%) developed acute GVHD and 2/15 (13%) developed chronic GVHD. The 34-month survival of children with non-malignant diseases was 83%, whereas the 25-month survival of those with malignant disorders was 44% ($P < 0.01$) (Figure 3). The NST methods which we have chosen allows also allografting of umbilical blood cells [30,31].

In acute myelogenous leukemia (AML), we have grafted individuals who could have received conventional grafts: 25 allografts were prospectively given to 24 patients with AML, eligible for conventional allografting; two individuals had secondary forms of AML. The median age of the patients was 35 years, with a range of 12 to 56. All patients engrafted; median time to achieve an absolute neutrophil count

$>0.5 \times 10^9\ l^{-1}$ was 12 days (range 0–26), whereas the median time to a platelet count $>20 \times 10^9\ l^{-1}$ was 13 days (range 0–26). Patients developed mixed chimerism 15 to 100 (median 30) days after the allograft. The follow-up periods range between 33 and 2670 days (median 450). The median post-transplant overall survival of the patients has not been reached and is above 89 months, whereas the 683 days both overall and progression-free survival is 66%. In 14 grafts (56%) acute GVHD ensued; in 12 cases grades I-II and in 2 cases grade IV which was fatal in both. In 9/19 patients (47%) limited chronic GVHD developed. In 22 cases (88%), the procedure could be completed fully on an outpatient basis. The 100-day and the transplant-related mortality were both 8% [32].

Patients with aplastic anemia have also been allografted using the "Mexican" approach. In a multicenter study in México, we grafted 23 aplastic anemia patients, who had no response to previous conventional pharmacologic treatment. The patients were followed for an average of 25 months. By a median of day +11 an ANC $>0.5 \times 10^9 l^{-1}$ was reached; and by day, +12 the platelet count had reached $>20{,}000 \times 10^9\ l^{-1}$. Acute grade I-II GVHD ocurred in 4 patients, whereas limited chronic GVHD presented in 6 cases. Twenty one patients (91.3%) achieved engraftment. Two patients failed to engraft and 4 developed late rejection, two of these individuals died, 2 survive with high transfusion requirements, whereas 2 received a second peripheral blood stem cell infusion and achieved sustained engraftment. Twenty one (91%) of the 23 patients are alive, whereas 19 of 21 (90%) remain in complete remission. The average cost was about 15 000 thousand US dollars for this kind of reduced intensity allotransplant [33].

Recently, the effectiveness of the Mexican Approach to conduct NST was tested outside México. In a multicenter study in several Latin American countries grouped under the LACOHG (Latin American Cooperative Onco Hematology Group), the "Mexican" method has been also used to allograft individuals with both CML and AML. Twenty four patients with Ph1 (+) chronic myelogenous leukemia (CML) in first chronic phase were prospectively allografted in four Latin American countries: México, Brasil, Colombia and Venezuela, using HLA-identical siblings as donors. Median age of the patients was 41 years (range 10 to 71); there were 8 females; median time from diagnosis to the allograft was 344 days (range 46–10, 280 days). Patients received a median of $4.4 \times 10^6\ kg^{-1}$ CD34 (+) cells. Median time to achieve above $0.5 \times 10^9\ l^{-1}$ granulocytes was 12 days, range 0–41, whereas median time to achieve above $20 \times 10^9\ l^{-1}$ platelets was also 12 days, range 0–45. Twenty two patients are alive 81 to 830 (median 497) days after the NST. The 830-day survival is 92%, whereas median survival has not been reached, being above 830 days. Eleven patients (46%) developed acute GVHD (5 cases grade I and 6 grade II) whereas 7 of 23 (30%) developed chronic GVHD. Two patients died 43 and 210 days after the NST, one as a result of sepsis and the other one of chronic GVHD. The 100-day mortality was 4%, whereas the transplant-related mortality was 8% [34]. The results obtained in this multicenter study are better than those which we described previously [23], because only CML patients in chronic phase were included in this second Latin American study.

We have also shown that the "Mexican" approach to conduct NST can be offered to patients with either an HLA identical (6/6) or compatible donor (5/6) [35]: in 58 allografts in individuals with various malignant and non-malignant hematological diseases using sibling donors, we compared allografts done from HLA identical (n = 40) or compatible (n = 18) siblings, respectively, the overall median survival was found to be 33 versus 8 months ($P < 0.01$), the 52-month survival was 47 versus 38% ($P > .2$), the prevalence of acute graft versus host-disease (GVHD) 57 versus 38%, that of chronic GVHD 25 versus 11% and the relapse rate 45 versus 55%. The two patients who failed to engraft were both 5/6 matches. Probably stemming from the low number of patients, and despite a trend toward worse results in patients allografted from HLA compatible (5/6) siblings, most differences in outcome were not significant [35].

The consequences of braking dogmata

NST has been one of the most exciting developments in the treatment of hematologic malignancies in the last five years [19]; however NST should not be envisioned as an "easy way" to conduct allogeneic bone marrow transplantation [36–40]. Worldwide, NST is still a therapeutic modality that has been reserved for certain individuals: Aged, debilitated or afflicted by other diseases. In some centers in México and in other developing countries, NST has been adopted as the conventional method to conduct bone marrow transplantation mainly because of its affordabilty. Consideration of costs should not be overlooked in any part of the world, but they are particularly critical in developing countries [15,41–46]: Eighty percent of children with cancer worldwide die of the illness because lifesaving treatments, such as HSC transplantation, are not available in under-developed countries [41–46]. In some developing countries, the cost of the "Mexican approach to conduct NST has been shown to be 15–20 times lower than that of a conventional allograft in developed countries.

The Matthew effect

A verse in the biblical book of Matthew reads: "*Unto every one that hath shall be given. . . . . but from him that hath not shall be taken away even that which he hath*" supports the origin of the concept of "the Matthew effect", described in a classic paper in Science by Robert K. Merton [47]; he noticed that in science, credit for a discovery or knowledge tends to go to the most famous researcher associated with it rather than to the most deserving one [48,49]. C.N. R. Rao notes that "the Matthew effect" is not uncommon even for work done in advanced countries, but hurts a person in a developing country much more because he does research with great difficulty; sometimes it takes many years to complete the work and then get no credit is very disappointing and frustrating [48]. The "Mexican approach" to conduct bone marrow transplantation has not escaped the "Matthew effect": the method, which has been used in several countries, and is endowed with several advantages over other procedures to conduct NST, is frequently overlooked in reviews or papers dealing with the topic [36].

Conclusions

Most patients who have been allografted in México and other developing countries using the "Mexican approach" to conduct NST could not have afforded the cost of a conventional or more expensive stem cell transplant. Prospective studies will define if NST will eventually replace conventional stem cell grafting [45,46,52]; however, very frequently in developing countries, the decision for a given patient is not between offering either a conventional bone marrow transplant or a NST; the decision has to be made between NST or no other effective treatment. Because of its cost, NST could be considered as an early treatment option in countries where limited resources currently prevent usual allogeneic bone marrow transplantation; role-definition and appropriate timing for this therapeutic approach in patients are required. We are learning which malignancies are more susceptible to the graft versus tumor effect, one of the main effects of NST in addition to the replacement of the bone marrow cells, and as a consequence, we are also learning in which malignancies NST is more useful. The "Mexican approach" to conduct NST has been shown to be effective for allografting individuals with malignant and non-malignant conditions. Despite the fact that most studies with reduced intensity conditioning have a relatively short follow up, there is information which indicates that the procedure is related with a lower prevalence and severity of GVHD [53], and with a similar efficacy as that of conventional allografting. Since this method is more feasible and affordable for patients and physicians in developing countries, the number of allografts in these places has increased substantially, as well as the publications related to bone marrow transplantation stemming from places where this therapeutic maneuver was considered as unaffordable previous to the development of this technology [46].

Despite the fact that allografting with reduced intensity conditioning may be related with several disadvantages such as mixed chimerism and relapse of the malignancy, braking several dogmata related to bone marrow allografting has resulted not only in the progress of knowledge, but also in the accessibility of many patients to sophisticated therapeutic actions, in some cases, the only true curative option for these individuals. Braking dogmata has been proved to be worthwhile in the case of HSC transplantation.

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HEMATOLOGY IN DEVELOPING COUNTRIES

Hematology education in a problem-based curriculum

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The 20th century has witnessed a major shift from traditional discipline-based curriculum to an integrated curriculum starting in 1950s. In 1970s a new era has begun with the introduction of problem-based learning in medical education. In accordance with the recommendations of the World Federation of Medical Education, undergraduate medical education should be student-centred, problem-based, integrated, community-based, with electives available and systematic. This approach is widely known as SPICES, an acronym derived from the above mentioned principles.

Problem-based learning (PBL) is being used in more and more medical schools around the world. However not all of these schools use PBL as the major learning method. PBL is a strong, sound and effective learning method as long as it is used as a major method. In PBL the students are presented a patient scenario to work on and during this process they discover their knowledge gaps, determine their learning objectives, find relevant sources and learn these. Clinical cases are used as triggers to initiate the learning process. Students work in small groups with a tutor (facilitator) and there are 6–10 students in each group. Tutors do not serve as knowledge sources but a person to facilitate and motivate learning, by asking relevant questions. Usually a case is discussed in 2 or 3 PBL sessions, each lasting about 2 hours. There are 1–4 days between sessions and students read and learn between these sessions; when they convene again they present what they have learnt, to each other. The content and the sequencing of case scenarios are designed according to the level of students. Both basic science and clinical science issues and all relevant disciplines are addressed in case scenarios. Thus the students form the habit of holistic approach to patient problems. In the first years of medical school basic science learning objectives are emphasized, whereas in the following years clinical science issues comprise the majority of learning objectives. Some academicians, especially clinicians, tend to think that PBL is an ideal way to teach clinical science issues and that most clinicians do use PBL during ward rounds; however this is not true because case discussions during ward rounds and other clinical activities is not PBL but problem solving exercises or problem oriented discusssions. Students almost always have prior knowledge of the subject that is discussed and they apply their knowledge during these activities. In PBL, on the other hand, students are exposed to patient problems and are asked to learn what is necessary to solve the problem; therefore they do not have a prior knowledge and the necessary knowledge is gained *during* the PBL process. Actually PBL is used mostly to let the students learn basic science concepts, rather than clinical knowledge.

In medical school curricula where PBL is the major method, two different approaches are used. One is organ-system based approach, where the modules and cases are organised according to organ systems. In the other approach cases are presented in a mixed order; however there must be a logic of sequence of cases, such as age groups (children, the elderly, etc.) or sypmtoms (such as pain, fever, pallor, etc). The disadvantage of the first approach is that when the students are presented a patient problem they tend to think only the processes or diseases about the system of the relevant module and do not think about other organ-systems.

Hematology is a discipline where basic science knowledge is a prerequisite to understand clinical disorders. On the other hand basic science issues such as molecular structure and disorders are closely linked with clinical issues in hematology. Thus, hematology cases are among the most ideal ones to understand basic molecular and cellular mechanisms and show

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the effects of these changes. Hematological cases can be used starting from the first year of medical education and are used to learn basic science concepts in addition to hematological signs, symptoms and diseases.

In schools with PBL curricula organized according to organ-systems, hematological system is generally addressed in multi-system modules, such as "dermatological-hematological-musculoskeletal", in addition to ones such as "molecules, cells and tissues", "pathobiology and host defence".

The traditional discipline-based curriculum of Ankara University Faculty of Medicine has been changed to a student-centred, problem-based curriculum in 2002. The first 3 years are organised in modules with PBL is the major learning method. Lectures, laboratory practicals, clinical skills training and field work are among the other education methods. The first year programme has been designed to give the students a basic knowledge of the human body organization from molecules to cells, tissues and organs. The second year modules have been designed according to age or gender (children, adults, women, the elderly). The third year curriculum has modules with emphasis on major symptoms (fever, pain, masses and swellings, skin manifestations, etc). Hematological patient scenarios have been used in all 3 years to serve various learning objectives; hematological findings (blood counts) have been used in almost every case.

The hematological cases used are as follows:

Year	Case	Major learning objectives
1	Sickle cell anemia	DNA structure, protein synthesis, hematopoiesis, blood cells
3	Hodgkin disease- immune deficiency- febrile neutropenia	The role of leucocytes in host defence and clinical consequences
3	Acute myeloid leukemia	Malignancy, hemostasis, DIC, tumor lysis syndrome
3	G6PD deficiency	Enzyme pathways and clinical correlation; bilirubin metabolism and jaundice;

Apart from these cases hematological manifestations and laboratory data are discussed in many other cases. For example, iron metabolism and deficiency is addressed in a giardiasis case along with malabsorption; hemostasis is discussed in a stroke case; hematological manifestations of systemic disease are seen in SLE scenario. Therefore hematology is learnt not only as a separate discipline but also as a system that is affected by various physiologic and pathologic processes.

Looking from the other way basic hematology topics are addressed as follows:

Topic	Year	PBL Case	Additional Methods
Blood cells and hematopoiesis	1	Sickle cell anemia	Lecture, laboratory
Protein and hemoglobin synthesis and results of disordered synthesis	1	Sickle cell anemia	Lecture, laboratory
Anemias	2	Giardiasis	Lecture, laboratory
	3	Hemolytic anemia (G6PD deficiency)	
	3	Leukemia	
Leukocytes and disorders	2	Measles	Lecture, laboratory
	3	Hodgkin disease +febrile neutropenia	
Platelets and hemostasis	1	Stroke	Lecture, laboratory
	3	Leukemia	
	3	Myocardial infarction	

This PBL-based, integrated and spiral curriculum lets the students learn the topics of every discipline in a clinical context. Thus, basic science topics are learnt in a clinically relevant way. Different cases can be used to address same objectives and even PBL cases can be changed in time.

PBL is a powerful learning tool and can be used as the major method in preclinical medical education. Although there are some medical schools that have "pure PBL" tracks most PBL schools use a "hybrid model", to support PBL with lectures and laboratory practicals. An important point here is that PBL objectives should not overlap with lecture objectives to let the students get utmost benefit from PBL sessions. With a good curriculum design no hematology topic remains omitted. The most important principle is that every basic science topic should be included in the curriculum and that every organ-system should be reviewed during preclinical phase of medical education. Thus, a blueprint of the curriculum matrix should be prepared when a PBL-based curriculum is being designed.

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PEDIATRIC HEMATOLOGY

Chromosome copy number and leukemia–Lessons from Down's syndrome

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Cancer is a genetic disease of the somatic cell. Virtually all cancers, including leukemias, harbor multiple acquired genetic abnormalities. These abnormalities are commonly manifested by growth chromosomal abnormalities. These abnormalities can be either structural or numerical. Aneuploidy, change in the normal chromosomal copy number, is one of the frequent abnormalities in cancer. For example high hyperdiploid acute lymphoblastic leukemia (ALL) is the most common leukemia observed in young children. The leukemic cells contain extra copies of multiple chromosomes. The chromosomes involved are non-random. Chromosomes 21, X, and 6 are almost always involved, as are chromosomes 4,10,17 and occasionally 18. This specific leukemia syndrome suggest a causative role for the chromosomal numerical aberrations. Yet it the mechanisms by which acquired trisomies contribute to leukemia is unknown. Children with Down Syndrome (DS) have a 20-fold risk and about 600 risk for acute metakaryocytic leukemia. This strong association between germline trisomy 21 and leukemia strongly suggest that trisomy 21 is leukemogenic. Elucidation of the pathogenesis of the leukemias of DS is likely to reveal the mechanisms by which aneuploidy contribute to leukemia and cancer.

Approximately 10% of children with DS are born with a megakaryocytosis syndrome commonly called "transient myeloproliferative disorder" (TMD) or "transient abnormal myelopoiesis" (TAM) or "transient leukemia". As suggested by the different names the disorder is usually transient and resolves spontaneously within up to several months. The term "transient leukemia" is occasionally used because the blasts in this transient disorder are mostly of clonal megakaryoblastic origin. The biological mechanism of the spontaneous resolution is unclear. About 1–2% of DS patient will develop, however, full blown malignant acute megakaryoblastic leukemia (AMKL) during their first four years of life that will not regress without chemotherapy. In fact, the risk of AMKL is about 600 times higher in children with DS. The factor(s) underlying the transformation from "benign" TMD into "malignant" AMKL are largely unknown.

Thus the megakaryocytic malignancies of DS provide a "natural genetic model" of multistep lineage specific leukemogenesis. Both the congenital disorder and the full blown AMKL are characterized by differentiation arrest of the megakaryocytic lineage. The marrow and the liver of infants with TMD contain a large number of dysplastic micromegakaryocytes. Identical cells are observed in the early stages of the AMKL of DS. Both disorders are also characterized by thrombocytopenia, indicative of poor platelets formation by the dysplastic megakaryocytes.

The peculiar association between DS and childhood megakaryoblastic disorders has led to intensive search for gene or genes on chromosome 21 than may cause the differentiation arrest and initiate the leukemia. A surprising twitch in this story came with the discovery that a gene on ***chromosome X***, GATA1, was mutated in the megakaryoblasts from all the patients with DS and either TMD or AMKL. The mutations were also found in fetal liver of aborted DS fetuses. The mutations are acquired as they are not found in remission samples, and are specific to the megakaryoblastic disorders associated with trisomy 21. No GATA1 mutations were found in other AMKLs, in sporadic acute myeloid leukemia (AML) or in the acute lymphoblastic leukemia (ALL) associated with DS. Thus a clear model for multistep

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leukemogenesis in DS emerges: In a relatively high proportion of DS patients, acquired mutations in GATA1 are selected in-utero and are probably responsible for the differentiation arrest and the initiation of clonal proliferation of immature megakaryoblasts. These mutations are necessary but insufficient for the development of the full blown AMKL that affect some of these patients during early childhood.

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

Two isoforms of GATA1 are usually detected: a full length GATA1 translated from the first ATG on exon 2, and a shorter form (GATA1s) that is initiated from an ATG on exon 3. GATA1s is lacking the amino-terminal transactivation domain and is therefore less active than full length GATA1. The normal function of GATA1s is unknown. Presumably the balance between these two products serves a regulatory function in normal megakaryocytic development. All the acquired mutations in the megakaryoblastic disorders of DS result in elimination of the full length GATA1 and the preservation of GATA1s. GATA1s is less active in promoting megakaryocytic differentiation and, therefore, less mature, abnormal, megakaryoblastic precursors accumulate.

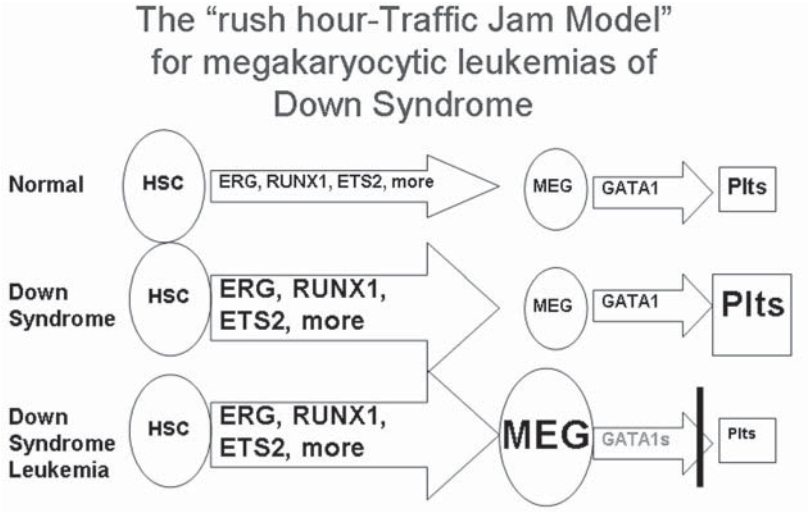
The collaboration between gene(s) on chromosome 21 and mutated GATA1 in megakaryocytic malignancies of DS is unique in its intrauterine occurrence and in its putative initiating role of a common and generally reversible clonal hematopoietic proliferation syndrome. At least three fascinating questions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

a. Why all the mutations selected results in the formation of the short isoform of GATA1? Does this isoform have a dominant pro-leukemogenic effect
b. Why GATA1 mutations and the megakaryoblastic proliferation occur only in-utero? In DS germline trisomy 21 exists in all hematopoietic precursors throughout life. GATA1 dependent megakaryopoiesis in the bone marrow also continues throughout postnatal life. So why does the selection of mutations in GATA1 in DS patients occur only in the fetal liver?
c. What is the gene or genes on chromosome 21 that, when existing in one additional copy, select for the cells carrying the GATA1s mutation?

A clue to the answer of the first two questions comes from a recent study from the laboratory of Stuart Orkin. Knock-in of the mutated GATA1s into GATA1 null mice resulted in normal adult megakaryopoieis. However, examination of the fetal liver revealed abundant proliferation of megakaryocytic progenitors. This study suggests that the GATA1s isoform directly enhances the proliferation of embryonic but not postnatal megakaryocytic progenitors. Another possible explanation of the unique in-utero selection of the mutated GATA1 cells in DS is that certain conditions in the feral liver, but not in the bone marrow, of DS fetuses enhance the proliferation and survival of these progenitors. Indeed, very little is known about the regulation of the physiological fetal liver hematopoiesis. It remains to be seen if certain genes on chromosome 21 play a specific role in fetal liver hematopoiesis.

The collaboration between gene(s) on chromosome 21 and mutated GATA1 in megakaryocytic malignancies of DS is unique in its intrauterine occurrence and in its putative initiating role of a common and generally reversible clonal hematopoietic proliferation syndrome. What is/are the gene/genes on chromosome 21 that promote(s) proliferation and provide(s) survival advantage to cells that acquired mutations in GATA1, a gene on chromosome X? The strongest candidate has been RUNX1 (also known as AML1 or CBFA2). RUNX1 is a transcription factor that is required for normal hematopoiesis. It is commonly mutated and involved in various translocations in both myeloid and lymphoid leukemias. Inherited mutations in RUNX1 causing haploinsufficiency with low level of expression in hematopoietic stem cells cause a syndrome of familial thrombocytopenia and increased susceptibility to leukemia. However, RUNX1 abnormalities have generally not been detected in AMKL and, except for a single case report, mutations in RUNX1 have not been found in AMKL associated with DS. A recent study from the Goldfarb's laboratory has demonstrated that RUNX1 directly interacts with GATA1 and that this interaction is important for megakaryopoiesis (Elagib et al. Blood 2003;101: 4333–41). Surprisingly, GATA1 transactivation domain was shown to be critical for this interaction. Thus one possibility is that the elimination of that domain in GATA1s provides "two hits in one event" – the preservation of a hypoactive isoform of GATA1 that block differentiation and the formation of abnormal complexes of RUNX1, lacking GATA1, that, plausibly, promote survival and proliferation of these cells. This speculation has to be experimentally tested, as is the question of how an excess of one allele of RUNX1 in trisomy 21 or a potential dysregulation of RUNX1 expression, predispose for this unique collaboration with mutated GATA1.

We have recently demonstrated the potential involvement of ERG, an ets transcription factor on



Abbreviations: HSC = hematopoietic stem cell; MEG = megakaryocytic progenitors, Plts = platelets.

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chromosome 21q (Rainis et al., Cancer Research 2005, in press). ERG is a protooncogene that is rarely involved in AMKL caused by the ERG-TLS translocations. We have shown that it is expressed in CD34 cells, in normal megakaryocytes and platelets, in megakaryocytic leukemias (whether or not associated with DS) but not in normal or malignant erythoblasts. ERG was induced upon megakaryocytic differentiation of eryhtroleukemia cells. Forced expression of ERG in the eryhtorleukemia cell line caused a phenotypic shift from the erythroid into the megakaryocytic lineage. Thus ERG seems to be a positive regulator of normal and malignant megakaryopoiesis.

I propose the "**rush hour traffic jam model**" for the occurrence of megakaryocytic leukemias in DS (see Figure). Extra copies of several genes on chromosome 21 (including ERG, RUNX1, probably ETS2 and possibly some others) create a positive pressure towards megakaryopoiesis, similarly to the traffic pressure towards downtown during rush hour. Supporting this suggested enhanced megakaryopoiesis is the observation that normal DS infants have significantly higher platelets counts during the first six months of life (Kivivuori Clin Genet 1996;46:15–19). The GATA1s mutation is similar to a "traffic accident" – it prevents megakaryopoiesis from reaching the target – platelet formation. The consequence is a pile-up of megakaryocytic precursors. In contrast to the car traffic-jam, only megakaryocytic progenitors with the GATA1s accumulate. This clonal accumulation results in the congenital leukemic phenotype.

What are the general implications of DS megakaryocytic leukemias? First, it is a prime example of collaborating pro-proliferation and differentiation arresting mutations in leukemia. Second, it may provide a general model for the role of aneuploidy in leukemia. Unique to whole chromosome aneuploidy is the parallel amplification of multiple genes which may act cooperatively in the same leukemogenic pathway. Finally, deciphering the regulatory factors working towards the selection of GATA1s progenitors during fetal liver hematopoiesis in DS may be relevant to sporadic childhood leukemia since recent studies have clarified that most, if not all, childhood leukemia arise during fetal hematopoiesis.

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PEDIATRIC HEMATOLOGY

Etiopathogenesis of pediatric thrombosis

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Introduction

Thrombosis is a relatively rare event during childhood, affecting 0.7/100000 children, with the highest frequency found in newborn infants and adolescents [1,2]. The etiology of thrombosis is influenced by age, neonate versus non-neonate, as well as site, arterial versus venous. Thrombosis during childhood is almost always multifactorial, with both genetic and environmental factors important in the onset and progression of thrombosis [3,4]. This paper will review the etiology and pathogenesis of thrombosis in pediatric patients.

Infants and children beyond the neonatal period

The majority of infants and children present with thrombosis while hospitalized for a significant underlying medical condition. Idiopathic thrombosis is very rare in childhood; most symptomatic children manifest two or more risk factors, as displayed on Table I.

Venous thrombi beyond the neonatal period

Venous thrombosis is more common in children than arterial occlusions. Inflammation related to infection, surgery, trauma, malignancy or connective tissue disorders is present in most children with venous thrombosis [3,4]. Blood disorders contributing to endothelial damage and stasis including sickle cell anemia, polycythemia and leukemia are rare but important risk factors for thrombosis.

Indwelling catheters are involved in approximately 30 to 60% of cases of venous thrombosis [3,5]. Catheter-related thrombi develop in children who require long-term support with venous access devices for chronic disorders such as cystic fibrosis, hemophilia, sickle cell anemia or short gut syndrome. However, in the setting of intense inflammation, such as sepsis or trauma, short-term femoral catheters placed for resuscitation often result in venous occlusion.

The term thrombophilia denotes a category of blood conditions that are associated with an increased propensity to develop thrombi. Thrombophilia may be genetic or acquired and both types are commonly determined in children with thrombosis [6,7]. Antithrombin deficiency is associated with a number of disorders including nephrotic syndrome, protein losing enteropathy and chemotherapy with L-asparaginase. Protein C deficiency is acquired by consumption during severe bacterial infections such as meningococcal sepsis and meningitis. Auto-immune protein S deficiency has been described following viral infections such as varicella [8,9].

Antiphospholipid antibodies (APA) include the lupus anticoagulant (LA), anticardiolipin antibody and anti-β2GPI antibodies. In young children APA usually occur following infection and are transient. APA in adolescents more commonly are persistent. By definition, detection of APA six weeks or more following initial determination constitutes the APA Syndrome (APAS). However following infections with organisms such as varicella or streptococcus, APA have been determined to resolve over several months, without apparent long-term risk of thrombus recurrence [9]. APAS may be secondary to systemic lupus erythematosus or other collagen vascular disease. However APAS in pediatric patients is usually primary, or idiopathic. Patients with APAS are at high risk for recurrent thrombosis following discontinuation of anticoagulation and most must be treated with warfarin for an indefinite time [10].

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Table I. Underlying conditions predisposing to thrombosis in infants, children and adolescents

Inflammatory States: Infections, Surgery, Malignancy, Massive Trauma
Inflammatory Disorders: Systemic Lupus Erythematosus, Rheumatoid Arthritis, Inflammatory Bowel Disease, Diabetes Mellitus
Antiphospholipid Antibodies: Lupus Anticoagulant, Anticardiolipin Antibody, Anti-B2GP1 antibody
Acquired Deficiencies of Coagulation Regulatory Proteins: Nephrotic Syndrome, Protein Losing Enteropathy, Sepsis, Respiratory Distress Syndrome (Preterm Infants), Specific Factor Autoantibodies
Drugs: Estrogens, Corticosteroids, L-Asparaginase
Primary Hematologic Disorders: Polycythemia/Hyperviscosity, Essential Thrombocythemia, Leukemia with a high white blood cell count, Sickle Cell Anemia, Immune Hemolytic Anemia

The LA can be detected in approximately 25% of children at the time of thrombosis diagnosis [11]. Children with acute thrombosis who manifest the LA are at increased risk for pulmonary embolism [10]. The LA promotes thrombosis by mediating increased expression of tissue factor and increased activation of factor X.

A list of genetic thrombophilia traits is shown on Table II. Genetic thrombophilic risk factors include conserved point mutations like factor V Leiden and prothrombin 20210. Factor V Leiden is resistant to physiologic down-regulation by activated protein C following activation of factor V by thrombin, while the prothrombin 20210 mutation results in a 15 to 30% increase in plasma concentration of the procoagulant protein prothrombin. Other conserved polymorphisms are associated with increased plasma concentrations of PAI-1 and homocysteine, or increased platelet interactions through membrane glycoproteins.

Certain genetic mutations promote hypercoagulability through decreases in concentration or function of various coagulation proteins including antithrombin, protein C, protein S, plasminogen or fibrinogen. Hundreds of mutations have been described in genes encoding coagulation proteins and these non-conserved mutations tend to convey more severe thrombotic morbidity. Other genetic thrombophilic risk factors such as hyperhomocysteinemia are modified by environmental influences from diet, exercise level and smoking. The Perinatal and Pediatric Subcommittee of the International Society of Thrombosis and Hemostasis has recommended that children with thrombosis be evaluated for thrombophilia [12].

Table II. Genetic causes of hypercoagulability

Conserved Coagulation Protein Mutations: Factor V Leiden, Prothrombin 20210, MTHFR, PAI-1
Coagulation Protein Deficiency/Dysfunction: Non-conserved Mutations in Antithrombin, Protein C, Protein S, Heparin Cofactor II, Fibrinogen, Plasminogen
Other: Elevated Lipoprotein (a), Homocysteine, Factor VIII, Sickle Cell Anemia

The inheritance of single thrombophilic genes increases the risk of symptomatic thrombosis during childhood, but does not appear to alter thrombus outcome or recurrence [13]. The concurrent inheritance of multiple prothrombotic traits increases the risk of both primary and recurrent thrombosis [14]. Thrombosis in children with genetic thrombophilia may be prevented by use of prophylactic anticoagulation around high risk procedures, such as surgery or immobilization. Children with thrombophilia do not generally require indefinite anticoagulation unless they experience thrombus recurrence or manifest evidence of excessive thrombin generation in a steady state, for example, with a persistently elevated D-dimer in the absence of infection or inflammation [15].

Small vessel thrombosis occurs in the syndromes of purpura fulminans (PF) as well as thrombotic thrombocytopenia purpura (TTP)/hemolytic uremic syndrome (HUS). Recurrent episodes of TTP/HUS beginning with onset during infancy have been described in children with genetic deficiencies of the metalloproteinase, ADAMST-13, the complement protein, H Factor, or the H Factor membrane cofactor, CD46. Similar chronic clinical syndromes with later age of onset have been associated with autoimmune decreases in these same proteins. The disorders are characterized by microangiopathic hemolytic anemia, thrombocytopenia and renal, liver and central nervous system dysfunction.

Special issues of venous thrombosis in adolescents

Therapies such as estrogens alter concentrations and functions of several coagulation proteins and shift the balance of coagulation toward thrombosis.

Factor V Leiden, protein S deficiency and antithrombin deficiency are examples of hormonally responsive coagulopathies. Affected females often present with deep venous thrombosis (DVT) within a few weeks to months after beginning oral contraceptives or hormone replacement therapy, or during pregnancy. In addition to the direct effects of estrogens, physiologic changes associated with puberty promote thrombogenesis through increased red cell mass and viscosity. The presentation of severe genetic thrombophilia has been delayed until puberty in some affected children [16].

A vascular anatomic variant named the May-Thurner anomaly often presents with left lower extremity DVT in adolescents. In this condition, the right iliac artery constricts the left iliac vein. Treatment with thrombolysis or mechanical thrombectomy and left iliac vein stenting has been employed in addition to anticoagulation.

Sequellae of venous thrombosis in pediatric patients

The syndrome of limb pain sufficient to limit activity in association with limb swelling, visible collateral formation and/or skin changes characterizes the post thrombotic syndrome (PTS). PTS has been reported in 10 to 60% of children following an episode of DVT [17–19]. Occlusive clot, elevated factor VIII and D-dimer both at DVT presentation as well as three to six months following the acute event, adolescent age and excessive body mass index (BMI) have been associated with the development of PTS [15,20,21]. Early restoration of venous blood flow, use of compressive stockings and reduction of inflammation may reduce the incidence or severity of PTS.

Arterial thrombi in non-neonates

Arterial thrombosis is usually caused by endothelial trauma. Most arterial thrombosis occurring beyond the neonatal period is related to the use of arterial catheters, chiefly cardiac catheterization. The rare syndrome called the Catastrophic APAS is characterized by unprovoked arterial and venous thrombosis and should be suspected in all children with peripheral arterial thrombi that are not catheter-related.

Stroke affects 8/100,000 children per year [22]. Eighty percent of strokes affect infants and children beyond the neonatal period and seventy-five percent of pediatric strokes are arterial. Arterial ischemic stroke (AIS) in the region of the brain perfused by the middle cerebral artery is the most common site of unprovoked arterial thrombo/embolism in children. Up to a third of children manifest a positive anticardiolipin antibody at the time of presentation with AIS [23]. A history of preceding upper respiratory infection is also common in children presenting with stroke. A patent foramen ovale may be responsible for recurrent embolic AIS. Valvular vegetations from APAS may also cause embolic stroke. Contrast (or bubble) echocardiography can exclude both of these possibilities. Genetic thrombophilia may present with arterial stroke, especially in infants [23]. A complete blood count should be performed on all children with stroke to exclude hematologic abnormalities including sickle cell anemia, thrombocytosis, polycythemia and leukemia.

Neonatal thrombosis

Overall, the rate of thrombosis in the neonatal period is much higher that that later in infancy [6]. Studies performed forty years ago demonstrated that neonatal clotting is accelerated in rate and degree as reflected on whole blood tests such as the thromboelastogram while plasma clot formation is delayed accounting for physiologic prolongations determined on the prothrombin time (PT) and activated partial thromboplastin time (PTT) [24]. Recent research has determined that increased thrombin generation in neonatal plasma is caused by decreased levels of tissue factor pathway inhibitor and antithrombin, while circulating soluble tissue factor is increased in preterm plasma [25,26]. In addition, physiologically increased hematocrit appears to predispose the newborn infant to thrombosis. Maternal diabetes is associated with an increased risk of neonatal thrombosis, and this risk may be mediated through further decreases in physiologically low levels of regulatory proteins, protein C and antithrombin. Both the plasma concentration and the multimeric size of the von Willebrand factor are increased in neonatal plasma. Adhesion of neonatal platelets, mediated by platelet GPIb and von Willebrand factor, is brisk and both the template bleeding time, as well as the closure time of the platelet function analyzer (PFA-100), are shorter in the neonate than in healthy children or adults, and contribute to the propensity for arterial thrombosis.

Neonatal venous thrombi

Indwelling venous catheters are present in a large proportion of cases, especially in thrombosis associated with bacteremia or clinical sepsis syndrome. The most common sites of unprovoked venous thrombosis in the neonate are renal vein thrombosis (RVT) and central nervous system sinovenous thrombosis (CSVT) [27]. Severe genetic thrombophilia usually presents in the neonatal period. Homozygous or compound heterozygous deficiencies of protein C or protein S present with disseminated intravascular coagulation, purpura fulminans or large vessel thrombosis.

Neonatal arterial thrombi

Arterial thrombi are usually associated with indwelling catheters, especially umbilical artery catheters and cardiac catheterization. Perinatal stroke appears to have a unique etiopathogenesis. In utero, the foramen ovale is patent. Thrombi originating from the placenta may enter the right heart through the umbilical vein and pass through the patent foramen ovale. The most direct vascular path from the ascending aorta is through the left carotid artery into the middle cerebral artery. Approximately seventy percent of neonatal strokes affect the left middle cerebral artery, causing right hemiparesis.

Special issues in neonatal thrombosis

Despite the large number of known genetic and acquired risk factors for thrombosis, a cause cannot be determined for approximately half of the newborn infants affected by stroke or venous thrombosis. The

risk for progression or recurrence of idiopathic perinatal thrombosis appears to be low.

Summary

Although uncommon, thrombosis is an important clinical problem in pediatric patients. True idiopathic thrombosis is extremely rare in children. Therefore, an underlying cause should always be sought. Thrombosis in children is almost always multifactorial, involving genetic and acquired thrombophilia, vascular damage and underlying inflammation. Genetic thrombophilia is frequently found in neonates as well as older children and adolescents with thrombosis. A laboratory evaluation for thrombophilia is almost always warranted in symptomatic children to determine the contributors to thrombogenesis, to prevent recurrent episodes and to allow genetic counseling for family members. Newborn infants and adolescents have unique age-related factors in the etiology and pathogenesis of thrombosis that should be considered. Children appear to suffer rates of PTS at least as high as that found in adults. Further research is needed regarding etiology and pathogenesis of thrombosis in children for prevention of primary and recurrent thrombotic events, optimal therapy for thrombosis in neonates and children, and prevention or limitation of PTS in affected patients.

Acknowledgements

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IRON METABOLISM

Iron overload and chelation

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Abstract
Iron is one of the most common elements in nature. As a transition metal it is very efficient in electron transport and redox reactions. The proteins and enzymes in which iron is an essential component play a key role in respiration, energy production, detoxification of harmful oxygen species and cell replication. Despite the abundance of iron in nature, the solubility of its stable ferric form is extremely low. Hence, living organisms were compelled to develop efficient mechanisms for iron transport and storage.

Keywords: *Iron chelation, thalassemia, deferoxamine, deferiprone, ICL670, NTBI*

Iron homeostasis

In recent years a number of key mechanisms have been described which are responsible for adaptation to changing environmental conditions[1]. Production of the iron storage protein ferritin and the transferrin receptor (TfR) protein is reciprocally regulated by a translational mechanism in which the iron regulatory protein (IRP) is reversibly bound to the iron response elements (IRE) of their respective mRNAs.

A similar iron-dependent translational mechanism may be responsible for the production of divalent metal transporter I (DMTI) responsible for the uptake of ferrous iron from the brush border of duodenal enterocytes and ferroportin (IREGI) responsible for the export of ferrous iron through the basolateral membrane of the same cells. The brush border ferric reductase, converts ferric to ferrous iron for use by DMTI, and Hephaestin, a transmembrane-bound ferroxidase, converts ferrous to ferric iron, creating a concentration gradient of ferrous iron across the cell membrane facilitating iron egress. At low iron conditions the translation of TfR, DMT1 and ferroportin is enhanced, with the opposite occurring at high iron conditions.

In addition, a new protein, Hepcidin, has been described recently and is probably the most important regulator of iron homeostasis [2]. Hepcidin functions as an inhibitor of iron absorption and of release from macrophages. Its production is increased by iron overload and inflammation and is suppressed by iron deficiency. Thus, in iron deficiency powerful compensatory mechanisms involving increased activity of iron transport proteins and inhibition of Hepcidin are activated in order to restore normal iron balance. However, these mechanisms are only partly effective, and iron deficiency anemia (IDA) is one of the most common problems in clinical hematology.

Genetics of hereditary hemochromatosis

The HFE protein combines with β_2-microglobulin for presentation on the cell surface. The central role of HFE mutations in the pathogenesis of HH is clearly illustrated by the development of severe hemochromatosis in HFE knockout mice [3]. It is quite likely, that the HFE protein does not regulate directly iron absorption and relase from macrophages, but indirectly through the control of hepcidin production.

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Both hepcidin and HEF are expressed mainly in hepatic parenchymal cells. Serum hepcidin in HH is inappropriately low, and may be responsible for increased intestinal iron absorption and the lack of reticuloendothelial iron accumulation characteristic of HH. It is also quite likely that inappropriately low hepcidin production may be the final common pathway of TfR 2 (a transmembrane protein homologous with TfR) mutations and other hereditary iron overload syndromes characterized by the HH phenotype. Mutations of hepcidin, TfR 2, ferroportin, L-ferritin IRE, or atransferrinemia may all result in the hereditary hemochromatosis phenotype or inappropriately high serum ferritin. However in Western populations, HH is attributed in over 80% of subjects to homozygous C282Y mutation or compound heterozygosity for the C282Y and H63D mutations of HFE.

Transfusional siderosis

The subtle balance of normal iron homeostasis is grossly overwhelmed by the abnormal erythropoiesis associated with thalassemia major. As a result of ineffective erythropoiesis, plasma iron turnover increases 10 to 15-fold [4], resulting in the outpouring of catabolic iron which exceeds the iron-carrying capacity of transferrin. This results in the emergence of toxic non-transferrin-bound iron (NTBI) which is directly involved in the production of harmful oxygen derivatives and damage to vital tissues such as the heart, liver and endocrine organs [5]. NTBI is efficiently chelated by deferoxamine (DFO) and other iron chelators, preventing peroxidative damage and even reversing existing damage. The long-term efficacy of DFO has been extensively documented in large multicenter trials.

Iron chelation therapy

Effect of deferoxamine

The introduction of DFO for iron chelation therapy of transfusional siderosis has changed the life expectancy and life quality of patients with thalassemia major. Its long-term efficacy has been extensively documented in large multicenter trials in Italy and elsewhere.[6] In a recent report on thalassemic patients treated by DFO at a single institution, survival at 40 years was 83% and in compliant patients born after 1975 survival at 25 years was 100% [7]. The cohort-of-birth related improvement in survival was reflected in an inverse, mirror-like decrease in cardiac mortality, supporting the assumption that prevention of cardiac mortality is the most important beneficial effect of DFO therapy.

The strongest direct evidence supporting the beneficial effect of DFO on hemosiderotic heart disease is the reversal of established myocardiopathy in some far advanced cases. In former years, the course of established myocardial disease in transfusional hemosiderosis was uniformly fatal. More recent experience indicates that such patients may still be salvaged by intensified chelating treatment. Employing continuous 24-hour i.v. DFO infusion via indwelling catheters, Davis and Porter achieved reversal of cardiac arrhythmias and congestive heart failure [8]. The actuarial survival of their 17 high-risk thalassemic patients (15 with established cardiac disease) following intensification of iron chelation was 61% at 13 years and none of the compliant patients died. Reversal of cardiac arrhythmia, previously unresponsive to medical treatment was achieved in 6 of 6 patients. This occurred in some cases within a few days of starting treatment and therefore cannot be attributed to normalization of iron stores but to the depletion of a putative limited toxic labile iron pool. Miskin et al. described 8 thalassemic patients with poor compliance and symptomatic heart disease attributed to iron overload, in whom standard s.c. therapy was replaced by drug administration via an indwelling central venous line for 8–10 hours daily [9]. Following intensification of iron chelation treatment, reversal of all cardiac abnormalities has been achieved.

Unfortunately, compliance with the rigorous requirements of daily subcutaneous infusions is a serious limiting factor and in non-compliant patients life expectancy is no different from that in the pre-DFO era. This is the rationale behind the intensive efforts to identify alternative, orally effective iron chelators.

These efforts have led to the development of several important compounds including deferiprone (L1, DFP) and the bishydroxyphenyl thiazole ICL670.

Effect of deferiprone

Treatment with the oral chelator deferiprone (DFP) should be considered in patients unable to use deferoxamine or patients with an unsatisfactory response to deferoxamine as judged by liver iron and serum ferritin measurements. At a DFP dose of $75\,mgkg^{-1}day^{-1}$, iron stores may decrease in some patients, remain stable in others and increase in some others [10]. Thus, careful monitoring of iron stores, preferably by measurement of tissue iron and of cardiac function is important during treatment with DFP as it is with DFO.

A few long-term, prospective trials are now available comparing the ability of chelation therapy with either deferoxamine or deferiprone to prevent heart disease In a recent study, 54 DFP-treated patients were compared with 75 DFO-treated patients retrospectively for cardiac complications and survival [11]. Although this was a non-randomized study, the age, duration of chelation therapy, mean serum ferritin

and percentage of initial cardiac dysfunction in the two groups were comparable. By the end of the 6-year follow-up period 3 patients died, all in the DFO group, despite attempted rescue by intensified i.v. therapy. Worsening of pre-existing cardiac disease or new onset of cardiac abnormalities was observed in 4% of the deferiprone group compared with 20% of the DFO-treated patients.

Anderson et al. [12] have shown significantly higher $T2^*$ values, presumed to reflect lower cardiac iron concentrations in patients treated long term with DFP than in patients treated with DFO. The authors concluded that conventional treatment with DFO did not prevent excess cardiac iron accumulation in more than half the patients with thalassaemia major and that oral DFP was more effective at removing cardiac iron. This was a retrospective non-randomized study and, although great efforts were made for proper matching of the two groups, only 15 patients were treated by DFP whereas the 30 DFO controls had to be selected from a large group of 160 patients receiving DFO. The important points raised by this report should be further studied by prospective randomized trials involving sufficient numbers of patients.

Effect of ICL670

ICL670 is a new once-daily tridentate oral chelator requiring two molecules to form a stable complex with each iron atom. ICL670 promotes iron excretion mainly in the bile [13]. A large international, multicenter trial has been conducted in 586 thalassemic patients randomized to receive ICL670 once daily at doses of 5, 10, 20 or 30 $mgkg^{-1}$, or subcutaneous DFO at doses of 20–60 $mgkg^{-1}day^{-1}$ for 5 days/week. The effect of ICL670 on liver iron concentration was dose-dependent. Doses of 20 and 30 mg /kg induced stable or falling liver iron concentrations whereas doses of 5 and 10 $mgkg^{-1}$ were too low to induce a negative iron balance. ICL670 was generally well tolerated. These data indicate that ICL670 is well-tolerated, and effective by once-daily oral administration for the treatment of chronic iron overload in beta thalassemia patients receiving regular blood transfusions [14].

Combined chelation

Improved chelating efficiency and improved compliance with combined deferiprone and DFO regimen has been first reported by Wonke et al. [15] in thalassemic patients. Deferiprone was given daily, and DFO 5 days per week. This has resulted in a decrease in serum ferritin in all 13 patients previously failing to respond to standard deferiprone treatment. The effect of combined DFO and DFP on UIE appeared to be additive, and no toxic side-effects have been observed. Combined therapy reduces serum ferritin in patients who had previously failed to achieve a satisfactory response to DFP alone. This approach to chelation therapy may be an attractive option for patients who are unable to comply with DFO infusions on more than a few days a week and who have an inadequate reduction of iron stores with DFP alone. To date, this combination therapy has shown no unanticipated side effects when given for periods of a year or more.

It is to be hoped, that better understanding of the pathophysiology of iron toxicity and the mechanism of iron chelation may promote the development of improved strategies of iron chelation therapy.

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IRON METABOLISM

The Egyptian experience with oral iron chelators

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Abstract
As no physiological mechanism exist for excreting transfusional iron overload in thalassemia, chelation therapy is the mandatory way to remove iron to prevent end organ damage and prolong survival. Desferoxamine (DFO) has been the major iron chelating agent used extensively worldwide for more than three decades for treatment of transfusional iron overload. However compliance has been a major obstacle in achieving an optimal therapeutic results. During the last 20 years the search for an affective oral iron chelators alternatives to Sc. DFO has been intensive. Different compounds have been studied, most of them although effective in animals have shown unacceptable toxicity with the exception of Deferiprone (L_1) and ICL670.

Keywords: *Thalassemia, Egypt, iron chelators*

Experience on Deferiprone (L_1)

Since January 2002, 108 transfusion dependant thalassemia patients (mean age 13.7 years) have been recruited to a Randomized Open – Label Phase III study with L1 and or DFO. This to evaluate the efficiency and safety of L_1 single and combined with DFO by measuring the levels of serum ferritin (SF), Urine iron execration (UIE) and liver iron content (LIC) at base line, after 3 mo. (n = 44) and 12 mo. (n = 66). Secondary end point included liver iron score (LIS), liver and heart function, compliance, tolerance and toxicity of the treatment regimens. Patients were classified into three arms; Arm A received L_1 + DFO, Arm B and Arm C received L_1 or DFO as monotherapy respectively. Drug regimen: for L_1 daily dose of 60 to 75 mg/kg Bw for Arm A & B. The dose of DFO was 23 to 50 mg/kg Bw for 2 days in Arm A and for 5 days in Arm C. Patients evaluation included complete blood count weekly for the first two months of treatment, there after fortnightly. In All Arms SF decreased significantly after 3 and 12 months of treatment in 100% in Arm A & C and in 86% in Arm B ($P = 0.001$ & 0.02 & 0.04 respectively). The highest UIE was in week one and 12 in all arms. A synergistic or even additive effect could be observed in Arm A as the UIE was clearly higher during the days of combination treatment if compared with the UIE when L_1 is given solely. A significant decrease in the LIC was observed in all Arms, however the LIS was more Significant in Arm A ($P = 0.009$ vs. $P = 0.67$). A significant improvement in the diastolic function of the heart was observed in all arms after treatment. The liver function test (ALT) increased after 3 months of treatment in 46% in Arm A, 73.3% in Arm B and 50% in Arm C. Most of the patients with increasing ALT were hepatitis C positive cases. Arthropathy was more marked in patients in Arm B (38%) and neutropenic episodes were observed in two cases one in Arm A and another in Arm B. Agranulocytosis (ANC $< 200/mm^3$) was detected in one case in Arm B, for whom we stopped the treatment.

Experience on ICL 670

It is a tridentate oral Iron Chelator given once daily. Forty three patients with a median age of 12 years were recruited sequentially since May 2004 for an open label multicenter trial on efficiency and safety of long term treatment with ICL 670 in beta thalassemia with transfusional iron overload. Patients received the treatment in a daily dose of 20 mg /Kg Bw based on their LIC at base line. Preliminary results revealed appreciable lowering of serum ferritin in most of patients (> 67%). All patients tolerate the drug satisfactory with few treatable side effects in the form of vomiting and skin rashes. From theses studies it seems that the combined therapy L_1 + DFO is the most effective in lowering the SF, LIC and increasing

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the UIE. Close monitoring of liver enzymes and neutrophilic count is recommended for patients receiving L_1, especially in hepatitis C positive cases. Preliminary results of ICL670 were convenient being well tolerated and effective once daily oral iron chelator.

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ACUTE LYMPHOCYTIC LEUKEMIA

Impact of molecular profiling and cytogenetics in acute lymphoblastic leukemia

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Primary genetic abnormalities of leukemic cells have important prognostic significance. In B-cell precursor acute lymphoblastic leukemia (ALL), hyperdiploidy (more than 50 chromosomes) and *TEL-AML1* fusion, which account for 25% and 23% of childhood cases but only 7% and 2% of adult cases, respectively, are associated with a favorable prognosis. Hypodiploidy (less than 44 chromosomes), found in less than 2% of pediatric or adult cases, confers an inferior outcome; the rare cases with low hypodiploidy (33 to 39 chromosomes) and near-haploidy (23 to 29 chromosomes) have a particularly poor prognosis. The t(4;11) with *MLL-AF4* fusion which occur in 40% to 50% of infant cases, 2% of childhood and 5% to 6% of adult cases, and Philadelphia chromosome with *BCR-ABL* fusion whose incidence increases with age from 3% in children to 20% in adults to over 50% in those above 50 years of age, signify a poor prognosis. Interestingly, there is a marked influence of age on the prognosis of these genetic subtypes. In Philadelphia chromosome-positive ALL, children 1 to 9 years old with a low presenting leukocyte count have a better prognosis than adolescents, while adults have a dismal outcome. Among cases with *MLL-AF4* fusion, infants fare considerably worse than older children, and adults have a particularly poor outcome. The basis for these differences may be related some combination of secondary genetic events, the development stage of the target cell undergoing malignant transformation, and the pharmacokinetic characteristics of the patients. In T-cell ALL, the presence of t(11;19) with the *MLL-ENL* fusion and the overexpression of *HOX11* confer a good prognosis. Recent study demonstrates activating mutations of *NOTCH1* gene in more than half of all T-cell ALL cases; its prognostic significance has yet to be determined. Gene-expression profiles can differentiate lineage and molecular genetic subtypes of ALL, and identify treatment-specific changes in gene expression in leukemic cells. Recent studies show that gene-expression profiling can also identify, within a given subtype of leukemia, previously unrecognized genes whose expression may have prognostic significance. Although gene-expression profile has not yet been used for risk classification, the findings already have led to efforts in identifying proteins that might serve as therapeutics targets or biologic markers. Host factors exert a crucial influence on the effectiveness of treatment. Genetic polymorphisms in drug-metabolizing enzymes, transporters, receptors, and drug targets result in wide inter-patient differences in drug disposition (absorption, distribution, metabolism, and excretion) and pharmacological effects. Patients who have homozygous or heterozygous deficiency of thiopurine methyltransferase, the enzyme that catalyses the S-methylation (inactivation) of mercaptopurine, have more rapid and profound reduction in leukemic cell burden after treatment with mercaptopurine in the early treatment phase and tend to have better event-free survival than those with wild-type enzyme activity, probably because they had, in effect, received a higher dose intensity of mercaptopurine. Glutathione S-transferases catalyze the inactivation of many antileukemic agents and their metabolites and protect the genome against oxidative stress. The null genotypes of these enzymes have been associated with a lower risk of relapse, perhaps because of the reduction in inactivation of cytotoxic chemotherapy. A tandem-repeat polymorphism within the enhancer region of the thymidylate synthase, one of the major targets of methotrexate, has been linked to increased

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risk of relapse. However, the importance of individual genetic polymorphism is also treatment-dependent. For example, the use of higher dose of methotrexate may overcome the relative drug resistance of the higher activity of thymidylate synthase. Conceivably, multiple genetic polymorphisms interact to influence antileukemic outcome, and pharmacogenetics will provide a tool to improve individualization of therapy.

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HEMATOMORPHOLOGY

Morphology in the diagnosis of red cell disorders

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Abstract
Despite the advances in automated blood cell counting, the blood film retains a crucial role in the diagnosis of red cell disorders. It is particularly important in haemolytic anaemias and in the differential diagnosis of macrocytic anaemia. However, all cases of anaemia in which the diagnosis is not immediately obvious require a blood film. Blood film examination sometimes provides a definitive diagnosis but more often suggests a differential diagnosis that indicates which further tests are most appropriate. The blood film has the advantage of speed; this is clinically important in any severe anaemia but particularly in acute haemolytic anaemia, thrombotic thrombocytopenic purpura and megaloblastic anaemia. Polycythaemic as well as anaemic patients require blood film examination.

Modern automated blood counting instruments produce a great deal of data about characteristics of red cells. In addition to the red blood cell count (RBC), haemoglobin concentration (Hb), haematocrit (Hct), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) they may measure the red cell distribution width (RDW) and, less often, the haemoglobin distribution width (HDW). The RDW quantitates the range of sizes of individual red cells and thus correlates with anisocytosis. The HDW quantitates the range of haemoglobin concentrations of individual cells and thus correlates with anisochromasia. Some instruments can indicate that there are increased numbers of hypochromic or hyperchromic cells and can show if these are large or small. Some can indicate the presence of red cell fragments and many can detect the presence, and possibly the number, of nucleated red blood cells (NRBC). Most can now quantitate reticulocytes, although this will not necessarily be done on every sample. Most instruments provide histograms or red cell size; some also provide two-dimensional plots of size against haemoglobin concentration so that the laboratory scientist can recognize characteristic patterns. Nevertheless, despite the wealth of information available, there is still much to be learnt from careful examination of a blood film [1].

Blood film examination can validate automated instrument results and confirm or exclude factitious results. An increased MCV should always be confirmed by blood film examination; elevation may be factitious, as a result of the presence of a cold agglutinin. Red cell indices that appear improbable should also always lead to blood film examination. Factitious results leading to unlikely red cell indices can be the result of hyperlipidaemia or parenteral nutrition with lipid emulsions, turbidity as the result of a very high white cell count, the presence of a paraprotein, the presence of a cryoglobulin, accidental heating or freezing of a sample or continuing in vitro lysis in *Clostridum perfringens* septicaemia, ageing of a sample and contamination by subcutaneous fat [2]. The blood film generally indicates the nature of the problem responsible for the aberrant results. A platelet count that is low, high or otherwise unexpected should always be validated on a blood film. This will sometimes lead to detection of a red cell abnormality. A high platelet count may be the result of hyposplenism, easily detected on a blood film, or may be a factitious elevation resulting from the presence of numerous schistocytes or microspherocytes. A blood film will not only validate a low platelet count but may show red cell changes indicating the likely cause, e.g. liver disease, megaloblastic anaemia or a myelodysplastic syndrome.

In addition to validating and possibly explaining abnormalities shown in a full blood count, assessment of morphological features can also yield information that no automated instrument can yet give and can

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permit integration of morphological and numerical data in the light of the age, gender and clinical setting. Blood film examination is often useful in haemolytic anaemias, haemoglobinopathies and thalassaemias, microcytic anaemias, macrocytic anaemias and, less often, normocytic anaemias.

In the haemolytic anaemias, the blood film may provide a definitive diagnosis or a differential diagnosis. Certain conditions, e.g. hereditary elliptocytosis, South-East Asian ovalocytosis and hereditary pyropoikilocytosis, are so morphologically distinctive that the blood film permits an almost certain diagnosis. The film in South-East Asian ovalocytosis, for example, shows a unique combination of macro-ovalocytes, which appear to be almost double the size of the other cells, and stomatocytes, including stomatocytes with two stomas or Y-shaped or V-shaped stomas. Other blood film abnormalities are less distinctive but indicate a differential diagnosis. The distinction between spherocytes and irregularly contracted cells is very important since the diagnostic significance is quite different. Spherocytes most often indicate either hereditary spherocytosis or autoimmune haemolytic anaemia. However, in a neonate they may indicate alloimmune haemolytic anaemia due to transplacental passage of maternal antibodies and, in other circumstances, they may be indicative of a delayed haemolytic transfusion reaction or alloimmune haemolytic anaemia resulting from administration of anti-D or incompatible plasma. Microspherocytes, sometimes designated spheroschistocytes, have a somewhat different significance, indicating that red cell fragmentation has occurred. They may be present, together with other schistocytes, in microangiopathic haemolytic anaemias and mechanical haemolytic anaemias; in this context, the blood film is of critical importance in the speedy diagnosis of thrombotic thrombocytopenic purpura. In patients with severe burns microspherocytes are present together with microdiscocytes and budding red cells. In hereditary pyropoikilocytosis, microspherocytes are present, together with a striking range of other poikilocytes. Irregularly contracted cells resemble spherocytes in that they lack central pallor but can be distinguished from them by their irregular outline. They have a different significance from either spherocytes or microspherocytes. Irregularly contracted cells usually indicate either oxidant damage or instability of haemoglobin. They are characteristic of acute haemolysis in glucose-6-phosphate (G6PD) deficiency, following administration of oxidant drugs or chemicals (e.g. dapsone) to individuals with normal red cell enzymes and in certain haemoglobinopathies (see below). They are a feature of Zieve's syndrome (haemolytic anaemia associated with acute alcoholic fatty liver and hyperlipidaemia). In G6PD deficiency and following oxidant exposure irregularly contracted cells are often accompanied by keratocytes ('bite cells'), hemighost cells ('blister cells') and even ghost cells. Sometimes there are protrusions from the surface that, on a Heinz body preparation, are found to represent Heinz bodies. Detection of the characteristic features of oxidant-induced damage is very important in suggesting the diagnosis of G6PD deficiency since a G6PD assay is not always abnormal during acute haemolysis [3]. Rarely irregularly contracted cells are the result of release of copper from the damaged liver in the late stages of Wilson's disease. Other morphological clues to the presence and nature of a haemolytic anaemia include stomatocytosis, acanthocytosis, red cell agglutination, basophilic stippling or the presence of intracellular organisms. Stomatocytosis is usually the result of liver disease, often alcoholic liver disease. Less often it is the result of one of a range of rare inherited haemolytic anaemias. The importance of blood film examination is demonstrated by the recent recognition of a number of cases of phytosterolaemia as a result of the observation of stomatocytes in the blood film [4]; the authors suggested that all patients found to have hypercholesterolaemia should have a blood film examined in order that such cases should be recognized. Acanthocytes are characteristic of haemolytic anaemia resulting from liver failure and are also seen in a range of rare inherited haemolytic anaemias. Red cell agglutinates are characteristic of the presence of a cold agglutinin and of paroxysmal cold haemoglobinuria but are not usually seen in warm autoimmune haemolytic anaemia. Basophilic stippling is a rather non-specific abnormality but nevertheless can be an important clue to a diagnosis of lead poisoning or pyrimidine 5′ nucleotidase deficiency. Microorganisms relevant to haemolytic anaemia can be either parasites (in malaria or babesiosis) or bacteria (as in Oroya fever and rare cases of Whipple's disease). In some cases of haemolytic anaemia there are no distinctive features. Diagnostic possibilities then include non-spherocytic haemolytic anaemia, paroxysmal nocturnal haemoglobinuria and Wilson's disease.

The blood film in haemoglobinopathies and thalassaemias often shows target cells and sometimes irregularly contracted cells. In sickle cell disease there may be sickle cells, boat-shaped (or oat-shaped) cells, target cells and other features of hyposplenism, irregularly contracted cells, linear red cell fragments, distinctive sickle cell-haemoglobin C poikilocytes (when haemoglobin C is present) [5,6] and 'Napoleon hat cells' (when haemoglobin S-Oman is present) [7]. Sickle cell anaemia and sickle cell-haemoglobin C disease can usually be distinguished on a blood film, while awaiting more definitive tests, by consideration of the Hb and the morphological features [5,6]. Haemoglobin C disease has a very characteristic film with a mixture of target cells and irregularly contracted cells; sometimes the film of sickle cell-haemo-

globin C disease is similar but, in an emergency (e.g. pre-operatively), a sickle solubility tests permits the distinction to be made. Irregularly contracted cells are also characteristic of unstable haemoglobins and to a lesser extent haemoglobin E homozygosity and heterozygosity; they may be present in small numbers in β thalassaemia heterozygosity. A diagnosis of α^0 and β thalassaemia trait is more reliably suggested by the red cell indices than by blood film morphology; the blood film sometimes shows only microcytosis whilst in other patients there is also poikilocytosis, basophilic stippling and the presence of target cells. Among the α thalassaemias, basophilic stippling is particularly suggestive of haemoglobin Constant Spring. Haemoglobin H disease can usually be suspected from the blood film and the blood count, including the reticulocyte count. The blood film shows marked hypochromia and microcytosis and poikilocytosis is usually quite marked. The reticulocyte count is increased and, in contrast to α and β thalassaemia heterozygosity, the MCHC is usually clearly reduced.

The differential diagnosis in microcytic anaemia is sometimes aided by blood film examination. Useful features include the presence or absence of anisochromasia or a dimorphic blood film, the presence of basophilic stippling or Pappenheimer bodies and the presence of increased rouleaux or the features of hyposplenism. Anisochromasia is typical of untreated iron deficiency anaemia whereas a dimorphic blood film suggests either iron deficiency on treatment or a sideroblastic anaemia; congenital sideroblastic anaemia is usually associated with microcytosis whereas acquired sideroblastic anaemia is most often, but not always, associated with a mixture of hypochromic microcytic cells and normochromic macrocytic cells. Basophilic stippling suggests either thalassaemia trait or lead poisoning; the latter is quite rare in most countries but its recognition is very important to the patient. Iron deficiency anaemia and severe anaemia of chronic disease are not always distinguishable on the blood film but the presence of rouleaux, increased background staining or reactive changes in white cells favours the latter diagnosis. Hyposplenism in a patient with hypochromia and microcytosis suggests, in the absence of a history of splenectomy, that the patient has coeliac disease.

The blood film is often of value in the differential diagnosis of macrocytic anaemia. In megaloblastic anaemia anisocytosis, poikilocytosis, oval macrocytes and hypersegmented neutrophils are helpful features. In comparison, in liver disease or alcohol excess macrocytes are usually round, hypersegmented neutrophils are not present and there may be target cells or stomatocytes. If the patient has not had a splenectomy, features of hyposplenism in a patient with a macrocytic anaemia raise the possibility of megaloblastic anaemia as a result of coeliac disease; the blood film may be dramatically abnormal with very numerous Howell–Jolly bodies. When macrocytosis is the result of a myelodysplastic syndrome, the blood film may show a population of hypochromic microcytes and Pappenheimer bodies or there may be dysplastic features in other lineages; a high platelet count in a patient with macrocytic anaemia raises the possibility of the 5q-syndrome. Unexplained macrocytosis is sometimes a feature of multiple myeloma and in this case there is likely to be increased rouleaux formation and increased background staining to suggest the diagnosis. If macrocytosis is the result of a haemolytic anaemia there will be polychromasia and an increased reticulocyte count, with or without more specific features. Congenital dyserythropoietic anaemia is a rare cause of macrocytic anaemia, characterized by marked anisocytosis and poikilocytosis. A rather more common cause is the administration of drugs that lead to macrocytosis; the laboratory scientist or haematologist should be alert to the possibility that a patients may be taking antiretroviral drugs, such as zidovudine, without this fact being included in the clinical details given on the request form.

There are many causes of a normocytic normochromic anaemia, including renal failure and the early stages of both anaemia of chronic disease and iron deficiency. The blood film is often not very helpful but multiple myeloma can usually be suspected, because of the presence of rouleaux and background staining, and if there is a leucoerythroblastic film either idiopathic myelofibrosis or bone marrow infiltration should be suspected.

Blood film examination is relevant to patients with polycythaemia as well as patients with anaemia. The presence of an absolute increase in the basophil count (not reliably detected by automated counters) or giant platelets favours a diagnosis of polycythaemia vera rather than relative or secondary polycythaemia.

Conclusion

All cases of anaemia in which the diagnosis is not immediately obvious require a blood film. This is particularly important if the anaemia is severe and if rapid diagnosis is needed. The blood film should be interpreted in the light of the clinical history and the results of a blood count.

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HEMATOMORPHOLOGY

Hematomorphology

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Case presentations

Case 1: Sea-blue histiocyte syndrome

A 26-year-old female presented in the third trimester of her first pregnancy with mild splenomegaly and marked thrombocytopenia. Serum bilirubin was elevated (Total: 1.9 mg dl^{-1}, Direct: 1.0 mg dl^{-1}). ALT and AST were elevated to $\sim$3 times the ULN and S. Alk. Phosphatase was 1.5 the ULN. Viral hepatitis markers and ANA were negative. CBC was normal except for thrombocytopenia (Platelets: 42×10^9 l^{-1}). BM was cellular and contained a large number of sea-blue histiocytes (Slides 1A, 1B). The parents of the patient were not related. She had a brother whose CBC was normal and a sister who was found to have mild thrombocytopenia. Both refused further investigations.

Case 2: Niemann-Pick's disease

A 19-month-old boy presented with moderate pancytopenia and marked hepatosplenomegaly. His parents were first cousins. Other siblings were similarly affected. Foamy histiocytes were seen in BM aspirate and liver biopsy smears (slides 2A and 2B respectively).

Case 3: Chediak-Higashi syndrome

A 14-month-old girl with history of frequent and severe pyogenic infections was referred for evaluation of a febrile episode. She was the first child of a consanguineous marriage (parents were first paternal cousins). Physical examination revealed pallor, partial albinism, photophobia and marked hepatoslenomegaly. CBC showed pancytopenia. Large cytoplasmic granules were seen in all types of white blood cells and their precursors (Slides 3A, 3B, 3C).

Case 4: Visceral Leishmaniasis

A 7-year-old girl presented with prolonged fever, lassitude and weight loss. She was referred for evaluation of possible malignancy. Physical examination revealed moderate hepatosplenomegaly. CBC showed mild pancytopenia and a normal differential WBC count. Leishmania amoastigotes [Leishman-Donovan (LD) bodies] were seen within bone marrow macrophages. Some free parasites were also noted (Slide 4A). Serum protein electrophoresis demonstrated a marked increase in gammaglobulins, simulating a monoclonal band on the densitometric tracing (Slide 4B).

Case 5: ALL and Down's syndrome

A 3-year-old boy with constitutional trisomy 21 was referred for evaluation of anemia and a high leukocyte count. CBC: Hb 8g dl^{-1}, platelets 15×10^9 l^{-1}, leukocytes 67×10^9 l^{-1} with 95% blasts (Slide 5A). Past medical history was negative for transient myeloproliferation. Immunophenotyping: CD19+, CD 10+, TDT+. Bone marrow karyotyping revealed no additional chromosomal abnormalities (Slide 5B).

Case 6: ALL and Sickle Cell Trait

A 6-year-old boy presented with signs and symptoms suggestive of acute leukemia. CBC: Hb 9 g dl^{-1}, Platelets 15×10^9 l^{-1}, Leukocytes 108×10^9 l^{-1} with 55% blasts (Slide A). Hb electrophoresis revealed heterozygosity for HbS (Slide 6B). Sickle cells seen in this case are due to spurious hypoxemia resulting from in vitro cellular utilization of oxygen. Immunophenotyping: The blasts were CD19+, CD10+ and TDT+. BM cytogenetic studies revealed a normal male karyotype.

Case 7: Neonatal ALL

A 5-day-old girl was found to have marked leukocytosis and hepatosplenomegaly. CBC: Hb 13 g dl^{-1},

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Platelets 11 $\times 10^9$ l^{-1}, WBC: 75 $\times 10^9$ l^{-1} with 95% blasts (Slide 7). Immunophenotyping revealed a pro-B-ALL pattern with aberrant expression of CD13 and CD7.

Case 8: Mature B-ALL

A 7-year-old boy was admitted to hospital for observation because of vomiting and colicky abdominal pain. Physical examination revealed mild hepatosplenomegaly. Abdominal US demonstrated enlargement of several intra-abdominal lymph nodes in addition to hepatic and splenic enlargement. CBC on admission: Hb 12.6 g dl^{-1}, Platelets 161 $\times$ 10^9 l^{-1}, WBC 26 $\times 10^9$ l^{-1} with 13% blasts displaying deep cytoplasmic basophilia and prominent vacuolation. BM: Extensive infiltration with blast cells resembling Burkitt lymphoma cells (Slide 8A). Immunophenotyping: Mature B-ALL pattern (CD19+, CD10 −, sIg+).

Case 9: AML-M2

A 67-year-old female was referred for further evaluation of severe anemia and progressive jaundice. The patient was taking oral iron supplements for the past 3 years. Physical examination revealed pallor, jaundice and marked splenomegaly. The liver was markedly enlarged and tender on palpation. CBC: Hb 4.7 g dl^{-1}, MCV 72, MCH 21, RDW (CV) 18.5%, Platelet count 29 $\times 10^9$ l^{-1}, WBC 8.7 $\times 10^9$ l^{-1}, Diff count: granulocyte predominance with a left shift and 23% blasts. Few NRBCs were seen. Prothrombin time 15.1 seconds (INR 1.35), APTT 28 seconds (N: 25–40). Serum iron 188 μg dl^{-1} (N: 56–160), TIBC 193 μg dl^{-1} (N: 250–400), T. Saturation 97.7%, S. ferritin 1314 ng ml^{-1} (N: 12–159). Other Lab. Data: Total bilirubin 4.7 mg dl^{-1}, direct bilirubin 2.3 mg dl^{-1}, LDH 2338 U/l (N: 230–460), Alt & AST ~3 times ULN, γGT ~20 times ULN, total serum protein 6.1 g dl^{-1}, serum albumin 3.3 g dl^{-1}, BUN, creatinine, uric acid, glucose and electrolytes were normal. Viral hepatitis markers were negative. BM: Markedly hypercellular, Blasts 78% of ANC (Slide 9A), MPO reaction was positive in 80% of blast cells. The Perl's stain revealed markedly increased iron stores (Slide 9B) and absence of pathological sideroblasts. Immunophenotyping: CD34+, HLA-DR+, CD13 −, CD33+, CD15+.

Karyotyping: An abnormal clone with t(8;21) and a subclone with the same translocation and trisomy 8. 46,XX,t(8;21)(q22;q22)[7]/47,idem,+8[13].

Case 10: AML-M2Baso

A 5-year-old girl was referred for further evaluation of anemia. Physical examination was unremarkable except for moderate pallor. CBC: Hb 7 g dl^{-1}, Platelets 22 $\times 10^9$ l^{-1}, WBC 26 $\times 10^9$ l^{-1}. Diff count: N 35%, L 15%, Mono 4%, Eos 1%, Baso 4%, Meta 5%, Myelo 6%, Blasts 30%. BM: Hypercellular, Blast 80% of ANC. Basophils increased: 15% of NEC (Slide 10). Immunophenotyping: CD34+, HLA-DR+, CD13+, CD33+, cMPO+. Karyotyping: t(6;9).

Case 11: AML-M2Eo

A 21-year-old male complained of weakness and fatigue of a few weeks' duration. Physical examination revealed pallor and ecchymoses. CBC: Hb 5.8 g dl^{-1}, platelets 8 $\times 10^9$ l^{-1}, WBC 23 $\times 10^9$ l^{-1}. Diff count: N 22%, L 17%, Mono 5%, Eos 5%, Baso 3%, meta 5%, Myelo 7%, Blasts 36%. BM: Hypercellular; erythroid precursors and megakaryocytes were markedly reduced. Numerous eosinophils and eosinophil myelocytes, some with abnormal basophilic granules were noted. Basophils were also increased. Blast forms constituted 40% of ANC (Slide 11). Immunophenotyping: CD34+, HLA-DR+, CD13+, CD33+, cMPO+. Karyotyping: Trisomy 8.

Case 12: AML-M3

A 39-year-old female at 16 weeks of gestation presented with persistent headache and generalized bruising. CBC revealed pancytopenia (Hb 8 g dl^{-1}, Platelets 3 $\times 10^9$ l^{-1}, WBC 2.9 $\times 10^9$ l^{-1}). Diff count: 7% hypergranular promyelocytes. Laboratory evidence of disseminated intravascular coagulation (DIC) was present. The bone marrow aspirate was hypercellular. More than 95% of cells were abnormal promyelocytes (Slide 12A), showing strong MPO activity (Slide 12B). Immunophenotyping: CD13+, CD33+, CD9+, CD2+, cMPO+. CD34 −, HLA-DR −, CD14 −, CD15 −, CD11b −, CD68 −. Karyotyping: 46,XX,t(15;17)(q22;q11) (Slide 12C).

Case 13: AML-M4Eo

A 52-year-old female presented with a one month history of weakness and malaise. Physical examination was unremarkable except for pallor.

CBC: Hb 7.1, MCV 103 fl, Platelets 55 $\times 10^9$ l^{-1}, WBC 16.2 $\times 10^9$ l^{-1}, Diff count: N 10%, L 18%, Mono 40%, Eos 0%, Baso 0%, Meta 2%, Myelo 4%, Promono 5%, Blasts 21%. M: Hypercellular. Blast forms: 50% (mixed populations of myeloblasts and monoblasts). Erythroid precursors and megakaryocytes: markedly reduced. Monocytes and promonocytes: 12%. Eosinophils and eosinophil myelocytes: 15% (Slides 13). Immunophenotyping identified a monocytic component (49%) of the gated blasts expressing C11c, CD14, CD13, CD33 and CD15 and a myeloid component expressing CD34, HLA-Dr and cMPO.

Karyotyping: 46,XX;inv(16)(p13q22).

Case 14: AML-M5a

A 55-year-old female presented with a two week history of low grade fever and fatigue. Physical examination revealed gingival hypertrophy, generalized lymphadenopathy and mild hepatosplenomegaly. CBC: Hb 6.6 g dl^{-1}, MCV 89 fl, Platelets 57×10^9 l^{-1}, WBC 163.7×10^9 l^{-1}, Diff count N 5%, L 5%, Mono 11%, Eo 0%, Baso 0%, Meta 2%, Blast cells 77%. BM: more than 90% of nucleated elements were monoblasts with voluminous cytoplasm and round to oval nuclei showing fine chromatin pattern (Slide 14). Myeloperoxidase activity was seen in 5% of these blasts and 90% were positive for α-naphthyl butyrate esterase. Immunophenotyping: CD 34+, HLA-DR+, CD13+, CD14(My4)+, CD14(Mo2)+, CD15+, CD33+.

Case 15: CML in accelerated phase

A 65-year-old female, known case of Ph positive CML developed resistance to therapy with increasing WBC and platelet counts. Occasional intact megakaryocytes were seen in the peripheral blood (Slide 15A). BM: Markedly hypercellular. The left shift in myelopoiesis was consistent with chronic phase CML. A repeat karyotype revealed a Ph positive clone with variant Ph including chromosomes 2, 9 and 22 (Slide 15B).

Case 16: A MPD with pancytopenia and marked BM eosinophilia

A 30-year-old female presented with pancytopenia and marked hepatosplenomegaly. CBC: Hb 8.7 g dl^{-1}, MCV 81 fl, Platelets 88×10^9 l^{-1}, WBC 1.9×10^9 l^{-1}, Diff count: N 31%, L 62%, Mo 7%, Eo 0%, Baso 0%. BM: Hypercellular. Eosinophils and their precursors: markedly increased, some showing basophilic granules. Blast forms were $<5\%$ (Slide 16). Karyotyping: t(4;12).

Case 17: 5q- syndrome

A 70-year-old male was referred for evaluation of severe anemia. Physical examination revealed pallor, grade III/IV systolic ejection murmur and mild hepatosplenomegaly. CBC: Hb 4.3 g dl^{-1}, MCV 100 fl, platelets 270×10^9 l^{-1}, WBC 10.7×10^9 l^{-1}, DIFF count: predominance of neutrophils. Some pseudo Pelger-Huet forms were noted. BM: Cellular with depressed normoblastic erythropoiesis. It was remarkable for marked megakaryocytic dysplasia with many hypolobulated and mononuclear forms (Slides 17a, 17b). Karyotyping: Isolated del (5)q (q15q33).

Case 18: CMML

A 66-year-old male was referred for further evaluation of anemia. Physical examination revealed mild splenomegaly. CBC: Hb 7.9 g dl^{-1}, MCV 85 fl, Platelets 83×10^9 l^{-1}, WBC 22.4×10^9 l^{-1}, Diff count: N 22%, L 21%, Mo 11%, Eo 4%, Baso 0%, Meta 5%, Myelo 8%, Promyelo 3%, Promono 11%, Blasts 15%, NRBCs 2/100WBC. Significant dysplasia of granulocytes and monocytes was noted (Slide 18A). BM: Hypercellular with trilineage dysplasia. M/E ratio 12:1. Erythroid precursors and megakeryocytes are decreased. Blast cells and monocytic elements form 8 and 7% of ANC respectively (Slide 18B). BM karyotyping revealed an abnormal clone with rearranged 3q. 46,XY,inv(3q).

Case 19: Infectious mononucleosis

A 3-year-old boy presented with fever and sore throat for several days. Physical examination revealed posterior cervical lymphadenopathy, slightly enlarged tender liver and mild splenomegaly. CBC: 11.8 g dl^{-1}, WBC 9.8×10^9 l^{-1}, Platelets 130×10^9 l^{-1}. Diff count: N 16%, L 50%, Mono 4%, Eo 1%, Baso 0%, Atypical lymphocytes 29% (Slide 19). High titers of IgM and IgG antibodies to EBV viral capsid antigen were detected.

Case 20: Chronic Lymphocytic Leukemia (CLL)

A 25-year-old male was found to have marked lymphocytosis in a pre-employment laboratory workup. Physical examination was unremarkable. CBC: Hb 14.1 g dl^{-1}, MCV 90 fl, Platelets 169×10^9 l^{-1}, WBC 34.1×10^9 l^{-1}, Diff count: N 10%, L 89%, Mono 1%, Eo 0%, Baso 0%, smear cells+. Lymphocytes were predominantly of small, mature-appearing forms (Slide 20). Immunophenotyping: HLA-DR+, CD19+, CD5+, CD20+, CD23+, λ light chain restriction, FMC7 −, CD10 −, CD22 −, CD38 −. BM aspirate: lymphocytes amounted to 50% of nucleated cellular elements.

Case 21: B-Cell prolymphocytic leukemia (B-PLL)

An 80-year-old female was referred for evaluation of marked leukocytosis. She was asymptomatic. Physical examination revealed marked splenomegaly. No hepatomegaly or lymphadenopathy was detected. CBC: Hb 10.4 g dl^{-1}, MCV 81, WBC 75×10^9 l^{-1}, Platelets 89×10^9 l^{-1}. Diff count: N 3%, L 10%, Mo 0%, Eo 0%, Baso 0%, Prolymphocytes 87% (Slide 21). Immunophenotyping: CD19+, CD20+,

CD22+, CD79b+, FMC7+, SmIgIgM+ (bright, λ light chain restriction), CD10−, CD5−, CD23−.

Case 22: Splenic Lymphoma with circulating villous lymphocytes

A 55-year-old man, known case of chronic hepatitis due to HCV infection was found to have mild lymphocytosis with some lymphocytes showing cytoplasmic villi. This finding was overlooked and the patient presented 4 years later complaining of weakness and loss of weight. Physical examination revealed marked splenomegaly and mild hepatomegaly. No lymphadenopathy was detected. CBC: Hb 11.8 g dl^{-1}, MCV 77, Platelets 142×10^9 l^{-1}, WBC 30.9×10^9 l^{-1}, Differential WBC count revealed 74% lymphocytes; several villous forms were noted (Slide 22). Serum protein electrophoresis showed no monoclonal gammopathy. Immunophenotyping: CD19+, CD20+, CD 22+, CD79b+, FMC7+ ,sIgM+ (bright, λ light chain restriction), CD5−, CD11c−, CD23−, CD25−, CD103−.

Case 23: Hairy Cell Leukemia (HCL)

The patient is a 55-year-old male. Routine workup prior to an ophthalmic operation revealed moderate splenomegaly and pancytopenia. Hb 8.6 g dl^{-1}, MCV 88 fl, Platelets 60×10^9 l^{-1}, WBC 5×10^9 l^{-1}, Diff count revealed 60% atypical lymphocytes whose abundant cytoplasm has an uneven border with numerous irregular projections (Slide 23). Immunophenotyping: CD11c+, CD19+, CD22+, CD25+, CD79b+, FMC7+, CD103+, sIg+ (bright, κ light chain restriction), CD5−, CD23−. BM aspiration yielded a "blood tap". BM biopsy revealed a decrease in all normal hematopoietic elements and infiltration by abnormal lymphoid cells. Increased and focally pericellular reticulin fibrosis was noted.

Case 24: Adult T Cell Leukemia Lymphoma (ATLL)

A 70-year-old man presented with fever, malaise and weight loss of several weeks' duration. Physical examination revealed generalized lymphadenopathy and mild hepatosplenomegaly. CBC: Hb 10 g dl^{-1}, Platelets 85×10^9 l^{-1}, WBC 23×10^9 l^{-1}, Diff count: N 15%, 85% atypical lymphocytes with highly convoluted nuclei (Slide 24). Immunophenotyping: CD2+, CD3+, CD4+, CD5+, CD25+, HLA-DR+, CD7−, CD8−, TdT−. Serum calcium level was elevated (14.5 mg/dl). Serologic test for HTLV-I was not available.

Case 25: Sezary syndrome

A 68-year-old man had pruritic skin lesions and malaise increasing over several months. Physical examination revealed diffuse erythema and scaling of the skin, bilateral axillary and inguinal lymphadenopathy and mild hepatosplenomegaly.

CBC: Hb 13 g dl^{-1}, Platelets 160×10^9 l^{-1}, WBC 7×10^9 l^{-1} with 70% atypical lymphocytes showing cerebriform nuclei (Slide 25). Immunophenotyping: CD2+, CD3+, CD4+, CD5+, CD7−, CD8−, CD25−, HLA-DR−,TdT−.

Case 26: Plasma cell leukemia

A 45-year-old male presented with backache of few weeks' duration. Physical examination was unremarkable. X-ray examination revealed generalized osteoporosis of the spinal and pelvic bones. CBC: Hb 10 g dl^{-1}, Platelets 90×10^9 l^{-1}, WBC 49×10^9 l^{-1}, Diff count revealed a leukoerythroblastic reaction with 50% lymphycytes showing predominance of plasmacytoid forms. Thirty percent plasma cells with heterogenous and mostly abnormal morphology were noted (Slide 26A). Serum Calcium, BUN and Creatinine were elevated (11.2, 50 and 2.2 mg dl^{-1} respectively. Total serum protein was 6.0 g dl^{-1}, with decreased γ-globulin fraction (0.03 g dl^{-1}, N: 0.7–1.5). Serum albumin, α1-, α2- and β-globulin levels were normal. Urine protein was markedly elevated; electrophoresis revealed a small albumin fraction and a monoclonal spike in the region (Slide 26B). Immunoelectrophoresis identified the monoclonal protein as immunoglobulin λ light chains (Slide 26C). BM aspirate revealed extensive infiltration by myeloma cells (Slide 26D). Immunophenotyping: Myeloma cells were CD38+, cIgλ+, CD10−, CD19−, CD20−, CD22−.

Case 27: Plasmacytoma of lung and multiple myeloma in a child

A 6-year-old boy was referred for evaluation of fever and cough of 4 weeks' duration. Investigations revealed a left hilar mass. Biopsy demonstrated pleomorphic monoclonal plasma cells positive for λ-light chains and IgA. CBC findings were normal except for a mild normocytic anemia for age. Examination of BM aspirate revealed 20% flaming myeloma cells (Slide 287). Serum protein elelectrophoresis showed a monoclonal band (2.5 g dl^{-1}) which was identified by immunofixation as IgA-λ. Lambda light chains were detected in urine.

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NEW FRONTIERS IN COAGULATION

Thrombophilia and pregnancy

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Keywords: *Thrombophilia, pregnancy, factor V, heparin*

Inherited and acquired thrombophilia are the main cause of thrombosis in pregnant women. A growing number of reports over the last years have suggested that these disorders are also associated with an increased incidence of vascular pathologies resulting in poor gestational outcome. This review covers recent data concerning thrombophilia and vascular placental pathology, and discusses available therapeutic modalities to prevent placental vascular thrombosis and maximize successful gestational outcome.

Venous thromboembolism

Pregnancy is a hypercoagulable state. Thrombophilic risk factor are found in the majority (50–70%) of women with gestational venous thromboembolism (VTE). The prevalence of VTE during gestation and puerperium is increased in women with inherited thrombophilic states such as antithrombin, protein C and protein S deficiencies [1,2]. Recent data suggest that the Factor V Leiden mutation and the Factor II G20210A allele variation (Factor II mutation) are important risk factors for VTE in pregnant women. Likewise, the Factor II mutation is 3–5 times more prevalent in gestational VTE (10–20%) than in normal Caucasian pregnancy populations (2–5%). However, most carriers of Factor V Leiden mutation or Factor II G20210A will not develop clinical symptoms during gestation [3]. Antiphospholipid antibodies can be found in 10–20% of gestational VTE cases.

The risk for a first episode of gestational venous thromboembolism (VTE) is about 1/200 and 1/300 for Factor II mutation and Factor V Leiden mutation, respectively [3]. However, the risk for recurrent gestational VTE in women who had experienced VTE in the past is about 10 to 20-fold higher and is in the range of 5% (range 2–13%) and is particularly high in women who harbor thrombophilia and where previous event was during pregnancy or hormonal therapy. In view of its high prevalence thrombophilia should be searched for in women with gestational VTE.

Recurrent fetal loss

Recurrent fetal loss (RFL) defined as three or more pregnancy loss is a well-established finding in certain acquired thrombophilic disorders, such as antiphospholipid syndrome [4] and essential thrombocythemia [5].

A case-control study in 60 women with the inherited thrombophilias, antithrombin, protein C and protein S deficiencies documented an increased risk for RFL [6]. Of 188 pregnancies in women with thrombophilia, 42 (22%) resulted in pregnancy loss compared to 23/202 (11%) in controls: odds ratio (OR) 2.0; 95% confidence interval (CI) 1.2–3.3 [6]. In addition, a high incidence of gestational abnormalities was reported in 15 women with dysfibrinogenemia associated with thrombosis. Of 64 pregnancies, 39% ended by miscarriage and 9% by intrauterine fetal death [7].

A number of recent case-control studies have evaluated the prevalence of the Factor V Leiden mutation in women with RFL. Despite differences in ethnic Caucasian subpopulations and selection criteria for RFL, three studies documented signifi-

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cantly increased prevalence of Factor V Leiden mutation in women with RFL.

In women with RFL of unknown cause, following exclusion of chromosomal abnormalities, infections, anatomic alterations, and endocrinologic dysfunction, studies by Grandone et al. [8] and by our group [9] have suggested that evaluation for the Factor V Leiden mutation is highly warranted since a significant percentage of women with RFL are found to be carriers of the mutation. Nevertheless, it should be emphasized that other reports did not document an association between the Factor V Leiden mutation and RFL [10]. The risk for RFL is greater in homozygous carriers than in heterozygous carriers of the Factor V Leiden mutation [11].

Of interest, activated protein C (APC) -resistance in the absence of the Factor V Leiden mutation has also been associated with pregnancy loss [12,13]. A potential explanation for the association between RFL and APC-resistance is that the APC-sensitivity ratio falls progressively throughout normal pregnancy either in correlation with changes in Factor VIII, Factor V and protein S levels [14], or without such a correlation [15].

Women with thrombophilia have an increased percentage of losses at later stages of gestation. For example, second-trimester losses or intrauterine fetal death accounted for 57 of 158 fetal losses (36%) in 37 women with thrombophilia compared to only 23/135 (17%) in women with RFL without thrombophilia ($P = 0.0004$) [16]. Activated protein C resistance and the Factor V Leiden mutation are more common in women with second-trimester pregnancy loss [16] and in women with post-embryonic first-trimester losses [17].

Combinations of thrombophilic states may further increase the risk for RPL. For example, coexistence of the Factor V Leiden and homocysteinuria [18] or a combination of the Factor V Leiden with familial antiphospholipid syndrome [19] was reported to result in thrombosis and recurrent fetal loss. It is therefore not surprising that the European Prospective Cohort on Thrombophilia (EPCOT) study documented the highest odds ratio for stillbirth (OR = 14.3, 95% CI 2.4–86) in patients with combined thrombophilic defects [20]. In our recent study involving 76 women with RFL, 6 (8%) had a combination of thrombophilic polymorphisms compared to 1/106 (0.9%) of controls ($P < 0.02$) [9]. Factor II mutation and homozygosity for the variant methylene tetrahydrofolate reductase C677T allele variation both contribute to RPL when presenting in combination with other thrombophilic defects.

Placental vascular complications

Pre-eclampsia affect 3–5% of all pregnancies. Whether pre-eclampsia is associated with thrombophilia is currently debatable. However, the body of evidence and meta-analysis suggest that thrombophilia and particularly Factor V Leiden, Factor II mutation and hyperhomocystenemia are associated with severe early onset pre-eclampsia [21]. Similar debate is carried out in regard to intrauterine growth restriction (IUGR). It is suggested that thrombophilia is associated with severe but not with mild IUGR [22,23]. A small number of studies suggest an association of placental abruption with thrombophilia [21].

Without therapeutic intervention, less than 20% of gestations in women with thrombophilia and RFL result in live birth [9]. This is similar to rates reported in women with the antiphospholipid syndrome who experience RFL. Mechanisms responsible for the association of inherited thrombophilia with RFL have not been elucidated. Pathological studies of placentas obtained from gestations terminated by fetal loss have revealed thrombotic changes and infarcts. These can be observed in the maternal vessels in a large proportion of placentas of women with stillbirth [24].

Therapeutic regimens

Women with previous VTE who harbor thrombophilia should receive LMWH prophylaxis during gestation. Women with thrombophilia without previous thrombotic event and without placental vascular complications, are advised to receive post-partum prophylaxis. In women with severe thrombophilia such as homozygous Factor V Leiden combined thrombophilia, antenatal prophylaxis may be warranted. Up to 65% of vascular gestational abnormalities can be accounted for by genetic thrombophilias [25], the implication is to screen for these mutations in all women with vascular gestational abnormalities. Furthermore, this high prevalence of genetic thrombophilias, which is similar to the findings in women with pregnancy-related venous thromboembolism [26], and the findings of thrombotic changes in the placentae of the majority of women with thrombophilia and stillbirth [24], suggest that antithrombotic drugs may have potential therapeutic benefit in women with gestational vascular complications.

The potential advantages of low molecular weight heparin (LMWH) over unfractionated heparin are higher antithrombotic ratio (meaning less bleeding for better antithrombotic effect), longer half-life with a potential need for only one injection per day, smaller injected volume, and less heparin-induced thrombocytopenia. A recent collaborative study has demonstrated the safety of using LMWH during 486 gestations [27]. Successful outcome was reported in 83/93 gestations (89%) in women with recurrent pregnancy loss and in all 28 gestations in women with pre-eclampsia in a previous pregnancy [27].

Administration of the LMWH enoxaparin, 20 mg day^{-1}, to women with primary early RPL and impaired fibrinolytic capacity resulted in normalization of impaired fibrinolysis, conception in 16/20 (80%), and successful live birth in 13/16 (81%) [28].

We have used enoxaparin (Rhone Poulenc, France) during 61 pregnancies in 50 women with thrombophilia who presented with RPL throughout gestation and for 4 weeks into the postpartum period [31]. Enoxaparin dosage was 40 mg day^{-1}, except for patients with combined thrombophilia or in case of abnormal Doppler velocimetry suggesting decreased placental perfusion, where the dosage was increased to 40 mg twice daily. In the case of previous thrombosis, LMWH therapy was continued for 6 weeks after delivery. Of the 61 pregnancies, 46 (75%) resulted in live births compared to a success rate of only 20% of prior gestations without antithrombotic therapy in these 50 women [29]. These preliminary results are encouraging. However, the optimal dosage of LMWH was recently determined in the LIVE-ENOX study, a multicenter, prospective, randomized trial comparing two doses of enoxaparin, 40 mg daily and 40 mg twice daily, in women with thrombophilia and recurrent pregnancy loss [30] in order to maximize successful gestational outcome. The study found equal efficacy −84% vs. 78% live birth respectively suggesting that the 40mg /day dose is sufficient for women with standart risk. Women at higher risk such as combined thrombophilia may need a higher dose [30]. Whether LMWH should be used in women with thrombophilia and previous one or two fetal losses is still not widely accepted although a recent study support this notion in women with pregnancy loss after 10 weeks of gestation. Whether women with severe early onset preeclampsia should benefit by LMWH prophylaxis on subsequent pregnancy has not been formally studied. However, reports from the LIVE-ENOX trial suggest that indeed this may be the case [31].

The role of aspirin, if any, in the setting of thrombophilia and vascular gestational abnormalities remains to be confirmed. In patients with inherited thrombophilia the value of aspirin is limited [31]. In patients with antiphospholipid syndrome, aspirin is given along with LMWH. However, whether aspirin has an added benefit to heparin or LMWH alone has not been evaluated. Prospective randomized, dose-finding studies are warranted to assess the potential advantage of LMWH in women with thrombophilia and vascular gestational abnormalities.

Unresolved Issues

Role for fetal genotype?

This is controversial. While there have been reports supporting that fetal thrombophilia is important [32], there are a number of reasons suggesting that this may not be the case. First, most thrombophilic polymorphisms are mild risk factors for gestational vascular complications (GVC) and gestational VTE. Second, thrombotic changes are noted mainly on the maternal side of the uteroplacental unit. Third, LMWH that does not cross the placenta are beneficial. Thus, unless there is a severe thrombophilic defect (i.e. homozygous protein C deficiency), fetal thrombophilic state is probably not a major contributor for GVC or VTE.

Women with unexplained pregnancy loss

The panel of thrombophilia workup is constantly expanding, for example, elevated Factor VIII levels have recently been association with RFL. Where current thrombophilia evaluation is negative, the idea is that yet undiscovered thrombophilia may be implicated, since thrombotic changes can be found in women with GVC even without thrombophilia. Following preliminary experience with antithrombotic therapy in these women, a prospective randomized multicenter trial comparing enoxaparin 40 mg day^{-1} and aspirin 75 mg day^{-1} has recently been conducted in Israel, and the results should be available soon.

Future perspectives

Future research is this field will most likely focus on four aspects. First, verification of the potential associations of the various genetic thrombophilias with gestational vascular pathologies is rapidly emerging. Second, currently 30–50% of vascular gestational pathologies cannot be accounted for by thrombophilia. Whether yet unknown novel genetic or acquired thrombophilia will be found to play a role remains to be determined. Elevated Factor VIII levels, PAI-1 4G/4G polymorphism and some EPC-R polymorphisms are potential candidates [21]. Third, the pathogenetic mechanisms responsible for placental vascular pathologies in women with thrombophilia have not been fully elucidated. Furthermore, it is not known why some women with thrombophilia express vascular gestational pathologies while others do not. It is possible that this may relate to local factors affecting coagulation, fibrinolysis and vascular tone at the level of placental vessels. Finally, the role of antithrombotic therapeutic modalities deserves prospective clinical trials, several of which are ongoing, to improve outcome for a large population of women who experience poor gestational outcome.

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LYMPHOMAS

Gray zone lymphomas

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The term gray zone lymphoma is mostly used for cases of malignant lymphoma which can not be reliably classified as Hodgkin lymphoma or non-Hodgkin lymphoma [1–3]. As this differential diagnosis has direct implications for management strategies, it is a major problem area for both pathologists and hemato-oncologists [4]. This difficulty is seen in three specific areas as shown in Figure 1.

1. Gray zone between Classical Hodgkin lymphoma (CHL) and diffuse large B-cell lymphoma (DLBCL), in particular primary mediastinal large B-cell lymphoma (MLBCL).
2. Gray Zone between CHL and ALK negative anaplastic large cell lymphoma (ALCL) and/or peripheral T-cell lymphoma (PTCL).
3. Gray zone between CHL or nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) and T-cell/histiocyte-rich large B-cell lymphoma (TRLBCL)

Gray zone between CHL and DLBCL

This is the most frequent problem area in gray zone lymphomas. Typically these cases occur in the mediastinum but also, occasionally, in extra-mediastinal sites. In the mediastinum both the CHL and MLBCL are thought to arise from a common precursor, the thymic B-cell. Recent gene expression profiling studies revealed that gene expression profile of MLBCL were distinct from DLBCL occuring at extra-mediastinal sites, but appeared very similar to CHL cell lines [5,6]. This suggests that there is a close biological realtionship between these two entities and the pathological features which respresent the phenotype will overlap. The presence of both CHL and MLBCL in the same biosy or consecutive biopsies from the same patient supports this view [7]. Therefore one would expect that at either end of the phenotypical spectrum, differential diagnosis of CHL versus MLBCL will be straight forward where as in the middle, it may be impossible (Figure 2)

There are limited number of tools for the pathologist to make this differential diagnosis. In the absence of a well-characterized genetic abnormality, the diagnosis rests on morphological and immunophenotypical analysis. Virtually all cases of CHL, like MLBCL or DLBCL are of B-cell origin and are composed of large cells. However in CHL, the B-cell phenotype of the neoplastic cells (RS cells and variants) is often incomplete, lacking expression of some pan-B-cell surface markers, B-cell associated transcription factors and immunoglobulin [8,9]. The phenotypic features that are useful in histological diagnosis are listed in Table I.

The best clinical approach for the patients diagnosed as having a gray zone lymphoma is not clear. Some investigators prefer to treat these patients with protocols appropriate for DLBCL adding rituximab for those cases expressing CD20 [7].

Gray zone between CHL and ALK negative ALCL

Another problem area for diagnosis of CHL is the differential diagnosis with ALCL that does express ALK. Although there is histological overlap, immunophenotyping is extremely helpful. ALCL is now considered to be exclusively of T or null cell origin and does not express B-cell lineage markers, especially pax-5 which is present in most CHL [3,10].

Gray zone between CHL or NLPHL and TRLBCL

CHL may morphologically overlap with TRLBCL. In TRLBCL, large B cells may occasionally display the morphologic characteristics of Reed-Sternberg cells, Hodgkin's cells, or variants, but the background cells usually do not include eosinophils, a distinctive feature of CHL. Immunophenotypically, the strong and consistent expression of pan-B-cell markers

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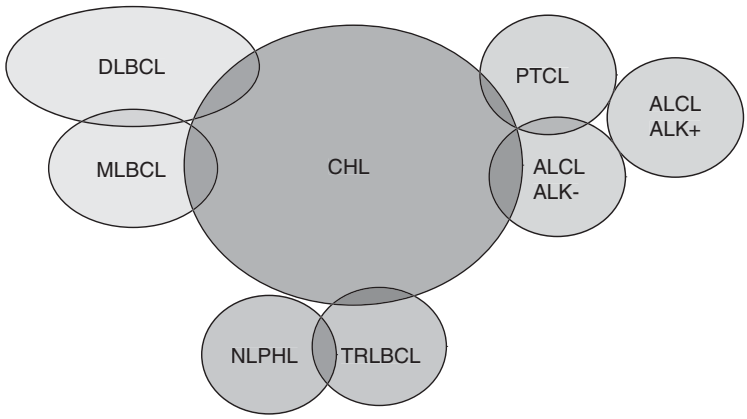

Figure 1. Gray zone lymphomas.

CHL: Classical Hodgkin lymphoma, DLBCL: Diffuse large B-cell lymphoma, MLBCL: Primary mediastinal large B-cell lymphoma, NLPHL: Nodular lymphocyte predominant Hodgkin lymphoma, TRLBCL: T-cell/histiocyte-rich large B-cell lymphoma, PTCL: Peripheral T-cell lymphoma, ALCL: Anaplastic large cell lymphoma. (Adapted from Stein et al. Eur J Hematol 2005) (3)

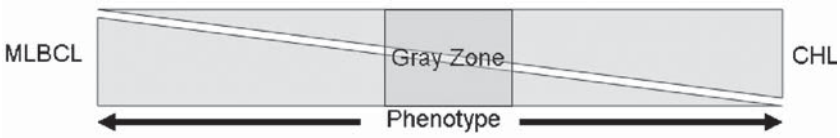

Figure 2. Phenotypic spectrum of CHL and MLBCL.

CD20, CD79a, and OCT2, the uniform expression of BCL6, the presence of immunoglobulin heavy or light chain expression, the absence or variable weak expression CD30, the lack of CD15 expression, and the absence of EBV gene products should be adequate to establish the diagnosis.

Perhaps more difficult is the differential diagnosis between NLPHL and TRLBCL. Although architectural nodularity and presence of a background of small B-cells favor NLPHL over TRLBCL, in later stages of NLPHL these features are usually lost and the accurate diagnosis may be only possible with clinical parameters.

The immunophenotypic markers for differential diagnosis of CHL or NLPHL and TRLBCL are summarised in Table II [11–13].

Table I. Features helpful in differential diagnosis of CHL versus MLBCL/DLBCL

	CHL	MLBCL	DLBCL
Morphology			
RS cells and variants	All	Occasional	Rare
Nodular sclerosis	Most	Occasional	Rare
Inflammatory background	All	Occasional	Rare
Phenotype			
CD30	+	−/+	−/+
CD15	+/−	−/+	−
CD45	−	+	+
Fascin	+	−	−
CD20	−/+	+	+
CD79a	−	+	+
Bcl-6	+/−, variable	+, uniform	+/−, uniform
Pax-5	+, variable	+, uniform	+/−, uniform
Oct-2	−/+	+	+
B ob-1	−/+	+	+
PU.1	−	+/−	+/−
EBV	+/−	−	−/+
immunoglobulin	−	−/+	+/−

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Table II. Immunophenotype of TRLBCL, CHL and NLPHL

	TRLBCL	CHL	NLPHL
Phenotype	Centroblasts, L&H-like cells, RS-like cells	Rs cells, Hodgkin cells	L&H cells
CD20	+	−/+	+
CD79a	+	−/+	+
CD3	−	−	−
CD21	−	−/+(FDC)	+(FDC)
CD10	−	−	−
BCL-6	+	−/+	+
OCT-2	+	−/+	+
CD30	−/+	+	−
CD15	−	+/−	−
EMA	−/+	−	+
EBV	−	−/+	−
clg	+	−	+

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LYMPHOMAS

Treatment of T-Cell lymphoma

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Abstract
T-cell lymphoma composes 25% of lymphoid malignancies in Japan. Peripheral T-cell lymphoma (PTCL) unspecified and adult T-cell leukemia/lymphoma (ATLL) are major subtypes of T-cell lymphoma. The Japan Clinical Oncology Group (JCOG) has conducted 7 clinical trials for aggressive non-Hodgkin's lymphoma (NHL) including T-cell lymphoma. JCOG trials revealed that patients with ATLL had an extremely poor prognosis as compared with other peripheral T-cell lymphomas. A second generation combination chemotherapy including pentostatin (JCOG9109) could not improve the prognosis of patients with aggressive ATLL with the median survival time (MST) of 7.4 months. Subsequently, JCOG developed a new alternating multi-agent chemotherapy including MCNU and carboplatin with prophyractic use of G-CSF, resulting 35% of CR rate and 31% of 2-year OS. Considering the poor prognosis of aggressive ATLL patients, allogeneic stem cell transplantation seems to be another promising approach for a cure of the disease. New active agents such as chimeric monoclonal anti-CCR antibody are under developing for PTCL and ATLL.

Incidence of T-cell lymphoma in Japan

T-cell lymphoma is a common subtype of lymphoid malignancies in Japan as compared with Western countries. The Lymphoma Study Group of Japanese Pathologists [1] reviewed 3,194 cases of lymphoid malignancies according to WHO classification [2] and demonstrated that they consisted of 69% of B-cell lymphoma, 25% of T- or NK cell lymphoma and 4% of Hodgkin lymphoma. The incidence of major subtypes of non-Hodgkin's lymphoma (NHL) were 33.3% for diffuse large B-cell lymphoma, 8.5% for marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) type, 8.5% for plasma cell myeloma, 7.5% of adult T-cell leukemia-lymphoma (ATLL), 6.7% for follicular lymphoma, 6.7% for peripheral T-cell lymphoma (PTCL) of unspecified type, 2.8% for mantle cell lymphoma, 2.6% of nasal and nasal-type NK/T-NHL, 2.4% of angioimmunoblastic T-cell lymphoma (AITL), 1.7% of T-lymphoblastic lymphoma (LBL)/T-acute lymphocytic leukemia (ALL), and 1.5% of anaplastic large cell lymphoma (ALCL). ATLL is defined as a peripheral T-cell malignancy caused by a RNA retrovirus, human T-cell leukemia virus type I (HTLV-I) which is transmitted by breast feeding and through exposure to blood and its products. Approximately 1.000.000 Japanese (mainly in Kyushu area) are seropositive for HTLV-1 and cumulative incidence of ATLL is estimated to be 2.5% among HTLV-1 carriers. ATLL occurs in adults with a median age of 55 years. Four clinical subtypes such as acute- lymphoma- chronic- and smoldering-type have been recognized. Acute type ATLL is characterized by flower cells in peripheral blood, hypercalcemia, and frequent organ involvement such as the skin, gastrointestinal tracts, lung, and central nervous system. Patients with acute- and lymphoma-types have an extremely poor prognosis; the median survival time (MST) was only 8 months.

Japanese clinical trials for advanced-stage aggressive NHL including PTCL

Most of the clinical trials for malignant lymphoma in Japan have been conducted by cooperative study groups, especially the Lymphoma Study Group of Japan Clinical Oncology Group (JCOG-LSG). JCOG is a multicenter cooperative oncology group, supported by Grants-in Aid for Cancer Research from the Ministry of Health, Labor and Welfare, Japan. JCOG has a common Data Center and consists of 13 cancer study groups including LSG. JCOG-LSG has conducted 21 multicenter trials, including eight

randomized controlled trials for lymphoid malignancies. Table I shows the summary of JCOG-LSG trials for advanced aggressive lymphoma. The JCOG-LSG began to conduct a prospective clinical trial (JCOG8101) for B- and T-cell non-Hodgkin's lymphoma (NHL) including adult T-cell leukemia/lymphoma (ATLL) from 1978 [3,4]. At that time, ATLL was generally classed as peripheral T-cell lymphoma. JCOG-LSG developed a CHOP-like chemotherapy, named LSG1, which consisted of vincristine (VCR), cyclophosphamide (CPM), prednisolone (PSL) and doxorubicin (ADM). The complete response (CR) was obtained in 63% of patients with B-NHL, 35% of T-NHL, and 17% of ATLL. In 1987, JCOG-LSG started a new second generation alternating multi-agent chemotherapy called LSG4 consisting of VEPA-B, M-FEPPA, VEPP-B against advanced aggressive NHL (JCOG8701) [5]. Estimated 5 year-overall survival (OS) after the median follow-up of 56 months was 60% in B-NHL, 35% in T-NHL and only 12% in ATLL. The results of JOCG8701 led to the following conclusions: (1) T-cell phenotype was an important pretreatment variable for aggressive NHL, (2) LSG4 regimen was effective against B-NHL. Since the clinical diagnosis of ATLL was an independent poor prognostic factor, ATLL were excluded from aggressive NHL in subsequent Japanese trials.

JCOG9002 was a randomized phase III study to investigate survival benefit of dose-intensified multi-agent combination chemotherapy [6]. Patients were randomly assigned to either LSG9 (VEPA-B/FEPP-AB/M-FEPA, every 10 weeks; 3 courses, 28 weeks in total) or modified LSG4 (VEPA-B/FEPP-B/M-FEPA, every 14 weeks; 4 courses, 54 weeks in total). Four hundred and forty-seven patients were enrolled between 1991 and 1995. Five-year OS was 57% for LSG9 and 55% for modified LSG4. It was concluded that the increase in dose-intensity of doxorubicin in multi-agent combination chemotherapy could not improve survival of patients with aggressive NHL including PTCL.

JCOG-LSG began to explore a promising dose-intensified chemotherapy against higher risk patients with advanced aggressive lymphoma. JCOG9505 was a randomized phase II study to choose a suitable dose-intensified regimen for a subsequent phase III study to compare with CHOP [6]. Seventy patients with intermediate-high or high risk aggressive NHL according to IPI were randomly assigned to either biweekly CHOP arm or dose-intensified CHOP arm. The CR rate was 60% with biweekly CHOP and 51% with dose-escalated CHOP. Grade 4 hematological toxicities were more frequent in the dose-escalated CHOP arm. It was concluded from the results of this study that biweekly CHOP is more promising for further investigations.

Based on the results of JCOG9505, JCOG-LSG initiated a phase III study, JCOG9809 in which biweekly CHOP was compared with standard CHOP in patients with advanced aggressive NHL in 1999 [7]. The primary endpoint was progression-free survival (PFS) and planned accrual was 450. Until December 2002, 323 patients were enrolled and randomly assigned to either biweekly CHOP arm or standard CHOP arm. The first planned interim analysis for 286 patients on December 18, 2002 revealed that the PFS of biweekly CHOP arm (n = 143) was slightly inferior to that of the standard CHOP arm (n = 143). The median PFS was 34 months in the standard CHOP arm and 24 months in the biweekly CHOP arm, and 2-year PFS was 54% in the standard CHOP arm and 51% in the biweekly CHOP arm. The hazard ratio of PFS between the arms was 1.10 (95% CI, 0.76–1.57). The predictive probability of biweekly CHOP superiority was estimated as only 19%. Two-year OS was 74% in the standard CHOP arm and 75% in the biweekly arm CHOP. According to the recommendations by the Data and Safety Monitoring Committee, JCOG9809 was terminated early. It was concluded that a dose-dense strategy by interval shortening of CHOP chemotherapy was unable to prolong PFS in patients with aggressive NHL. The subset analysis showed that 3-year PFS was 28% for T-NHL (n = 33) and 53% for B-NHL (n = 248). T-cell phenotype remains an unfavorable prognostic factor in patients treated with CHOP or biweekly CHOP.

The Japanese Clinical Study Group of THP lymphomas in the Elderly (JGTLE) [8] conducted a randomized phase III study of 3 CHOP-equivalent regimens, THP-adriamycin (terarubicin) -COP, dose-reduced CHOP and THP-COPE for elderly patients

Table I. Results of JCOG trials for aggressive NHL

Study	Phase	No. of Pts	(T-NHL*)	%CR	MST (mo)	%OS
JCOG8101 CHOP-like)	III	190	(21)	57	21	32 (4-yr)
JCOG8701 (2nd generation)	II	338	(42/234)	72	39	48 (5-yr)
JCOG9002 (dose-inensified)	III	447	(74 /404)	67	NA	56 (5-yr)
JCOG9505 (dose-intensified CHOP, IPI-HI/H)	R-II	70	(10/54)	56	28	42 (4-yr)
JCOG9506 (CHOP-Auto-SCT, IPI-HI/H)	II	43	(6.33)	64	NA	58 (3-yr)
JCOG9508 (CHOP, IPI-L/LI)	II	213	(16/195)	74	NA	70 (5-yr)
JCOG9809 (CHOP, Biweekly CHOP)	III	323	(33/290)	64	NA	74 (2yr)

CR, complete response; MST, median survival time; OS, overall survival, NA, nor applicable * excluding ATLL

with aggressive NHL. Between 1990 and 1992, 501 patients were enrolled and randomly assigned into each of the 3 arm. Median age was 75 years. The Complete response (CR) in all 420 evaluable patients was 45% in THP-COP group, 44% in CHOP group and 50% in THP-COPE group, respectively. A subset analysis of response according to T/B phenotype revealed that THP adriamycin-containing regimens were more effective than dose-reduced CHOP against T-NHL Overall survival of patients with THP-containing regimens was slightly superior to that of CHOP in T-NHL, although the difference was not statistically significant, maybe due to small sample size. We are now conducting a phase II study of THP-COP chemotherapy against PTCL unspecified at advanced-stage.

JCOG trials for aggressive ATLL

The main results of JCOG trials for ATLL are summarized in Table II. The first trial was a phase III, JCOG8101, which compared VEPA versus VEPA-M against advanced NHL including ATLL[3]. The CR% of VEPA-M for ATLL (37%) was higher than that of VEPA (17%) ($P=0.09$). However, median survival time (MST) of 54 patients treated with VEPA or VEPA-M was only 7.5 months and the estimated 4-year OS was 8%. In the subsequent phase II study (JCOG8701) of a multi-agent combination chemotherapy, the CR rate in 42 patients was 43%, which was improved from 28% of 54 patients in JCOG8101 [4]. However, the MST of these patients was 8 months and 5-year OS was 12%. The disappointing results against ATLL with conventional chemotherapies have led to explore new active agents. Pentostatin (deoxycoformycin, DCF), an inhibitor of adenosine deaminase was reported to be effective in a number of lymphoid malignancies by a multicenter phase I and II study, showing a response rate of 32% (10/31) in relapsed or refractory ATLL [9,10]. These encouraging results prompted us to conduct a phase II trial of DCF-containing chemotherapy (JCOG9101) as an initial chemotherapy for ATLL in 1991 [11]. In 60 eligible patients, there were 17 CRs (28%) and 14 PRs (25%). The MST was 7.4 months and estimated 2-year OS was 17%, identical to the results of previous studies. It was concluded that the prognosis of ATLL patients could not be improved by a DCF-containing combination chemotherapy.

In 1994, JCOG initiated a new phase II trial (JCOG9303; LSG15) of a multi-agent combination regimen consisting of VCR, CPA, DOX, PSL, MCNU, VDS, ETP, and carboplatin (CBDCA) for untreated ATLL [12]. The CR rate and 2y-OS of 93 eligible patients was 33% and 31%, respectively. Grade 4 hematologic toxicities of neutropenia and thrombocytopenia were observed in 65% and 53% of the patients, respectively. To confirm whether the LSG15 is a new standard chemotherapy for aggressive ATLL, JCOG conducted a randomized phase III study (JCOG9801), comparing the LSG15 with biweekly CHOP. Patient enrollment into this trial was completed in October 2003, and results of final analysis will be open at the end of this year.

Allogeneic stem cell transplantation for aggressive ATLL

Poor prognosis of patients with aggressive ATLL by conventional chemotherapy prompted us to approach to allogeneic stem cell transplantation (Allo-SCT). Fukushima et al. [13] reported a paper of retrospective study on the effect and safety of Allo-SCT for younger patients with aggressive ATLL. In this series 40 transplanted patients, median age of 44 (28–53) were reviewed. Fifteen patients were in CR, 13 in PR, 3 in SD, 9 in PD at conditioning for AlLo-SCT, Grade 3/4 acute graft versus host disease (GVHD) was observed in 7 patients. Extended chronic GVHD was seen in 11 patients. Twenty one patients died 6 of GVHD, infection (5), disease progression (3), thrombotic microangiopathy (3) and other causes (3). Three year overall survival of these 40 patients was 45.3%. These promising results suggest the possibility of graft versus ATLL effects by allo-SCT.

Okamura et al. [14] reported 16 cases of allogeneic stem cell transplantation with reduced-conditioning intensity (RIST) for patients with ATLL who were all over 50 years of age (51–67, median 57). Conditioning regimen consisted of fludarabine, busulfan and rabbit anti-thymocyte globulin. One year EFS and OS were 25% and 39%, respectively. After RIST, the HTLV-1 proviral load became undetectable in 8 patients. We are planning a phase II study of allogeneic stem cell transplantation for aggressive ATLL.

Table II. Results of JCOG trials for ATLL

Study	Phase	No of Pts	%CR	MST (mo)	%OS
JCOG 8101 (CHOP-like)	III	54	28	7.5	8 (4-yr)
JCOG 8701 (2^{nd} generation)	II	43	42	8 0	12 (4-yr)
JCOG 9109 (DCF-containing)	II	60	28	7.4	16 (2-yr)
JCOG 9303 (G-CSF-supported)	II	93	35	13	31 (2-yr)
JCOG 9801 (9303 vs. Bi-CHOP)	III	118	32	11	24 (2-yr)

CR, complete response; MST, median survival time; OS, overall survival, NA, nor applicable

Development of new anticancer agents for T-NHL

Cladribine is a chlorinated purine analogue resisitant to adenosine deaminase (2-chlorodeoxyadenosine, 2-CDA). This agent was found to be effective against hairy cell leukemia, B-chronic lymphocyitc leukemia, indolent B-NHL and cutaneous T-cell lymphoma. Japanese phase I study of cladribine, one patient with ATLL achieved PR [15]. A multicenter phase II study of cladribine as single agent showed a limited efficacy for treating relapsed or refractory ATLL (ORR 7%) [17]. CCR4 is a chemokine receptor and a kind of selective marker of Th2 phenotype of non-cytotoxic lymphocytes. CCR4 is expressed in 88% of ATLL, in 38% of PTCL unspesified, in 41% of mycosis fungoides in transformation, and in 66% ALK-negative anaplastic large cell lymphoma by immunostaining [18]. Recent studies revealed that CCR4 expression is an independent and significant prognostic factor in ATLL and PTCL [19]. Defucosylated chimeric monoclonal anti-CCR4 igG1 antibody (KM2760) has recently been developed in Japan. Phase I/II trial to evaluate the safety and efficacy of anti-CCR4 humanized antibody will be initiated in patients with peripheral T-cell malignancies.

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CHRONIC LYMPHOCYTIC LEUKEMIA

Biology and prognostic factors in CLL

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Keywords: *Immunoglobulin variable region genes, B cell receptor, ZAP 70*

The biology of CLL

The traditional view of CLL as a slowly accumulative disease of immuno-incompetent lymphocytes is being superseded as new data reveals biological heterogeneity both between patients and in individual cases at different times in the course of their disease. Some of the recent key advances are summarized below:

The B cell receptor

The immunoglobulin heavy and light chain genes which encode the antigen binding site of the B cell receptor (BCR) in CLL may be either mutated or unmutated raising the question as to whether CLL originates from a single, or more than one target cell. Microarray studies performed to address this issue have been inconclusive but do show a common "CLL signature" quite similar to that of both normal naïve unmutated B cells and mutated memory B cells. However, the pattern of surface antigen expression which is consistent with an activated B cell, the presence of short telomeres reflecting multiple cell divisions, and the finding of biased IgV_H gene usage all suggest that unmutated CLL cells have been exposed to antigen. The analysis of IgV_H gene usage in large numbers of patients with CLL has recently revealed that there are several sub-groups of patients (comprising approximately 10% of all cases of CLL) that have highly or moderately homologous CDR3 regions, encoded by V D and J genes, and critical for antigen binding. There is a <1 in 10^6 chance of normal B cells having identical CDR3's, suggesting a role for specific antigen(s) in the pathogenesis of CLL. Whether antigen is responsible for selecting B cell clones for expansion and increasing the chance of a transforming event or is responsible for the subsequent survival and expansion of the leukaemic clone remains uncertain.

The ability of CLL cells to signal through the BCR is diminished as compared to normal B cells, and is especially poor in the mutated subgroup. There are a number of hypotheses to account for this difference in signalling between the two mutational subsets. These include an inability to bind antigen through the BCR, and/or a defect in the organization of the BCR in mutated cases, and the expression of Zap 70, a key molecule in T-cell signalling, in the majority of unmutated cases. The increased cell turnover in unmutated CLL may favor the acquisition of poor prognostic secondary cytogenetic abnormalities.

Genetic abnormalities

Cytogenetic analysis, interphase FISH and comparative genomic hybridization have identified genetic abnormalities in approximately 80% of patients with CLL. The incidence of abnormalities is higher in patients with advanced disease. The nature of the initial transforming events remains unknown. Transgenic mice which over-express the TCL1 gene develop a CD5+ B cell lymphocytosis reminiscent of CLL, but there is no evidence that TCL1 over-expression is an initiating event in human CLL. In contrast to B-cell tumours arising from germinal centres, translocations involving the immunoglobulin gene loci are rare in CLL, occurring in approximately 5% of patients. The commonest genetic abnormality in CLL is deletion of chromosome 13q14. Heterozygous or homozygous loss is found in up to 70% and 20% of cases respectively depending on the methodology used for their detection. Of particular interest is the finding of 13q14 loss as the sole cytogenetic abnormality in cases of 'preclinical' CLL described below, confirming that this is an early event in

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leukaemogenesis. A minimally deleted region containing exons of two genes, RFP2 and DLEU 2, and two micro RNA's, miRNA 15 and 16, has been described but their role of 13q loss in the pathogenesis CLL is not clearly established. Trisomy of chromosome 12, deletion of chromosome 11q23 resulting in loss of the ATM gene and structural abnormalities of chromosome 17p13 resulting in loss of the p53 gene are recurring abnormalities whose incidence varies depending on the clinical stage and IgV_H gene mutation status of the disease.

Approximately 5% of patients with CLL have a first degree relative with a chronic lymphoid malignancy and research is ongoing to identify "familial" CLL gene(s) which may also be important in sporadic CLL.

Role of the micro environment

The apparent longevity of CLL cells in vivo is in marked contrast to the rapid apoptosis which occurs in vitro. This paradox is accounted for by the discovery of an increasingly complex series of interactions between receptors on the surface of CLL cells and a variety of cell types such as activated T-cells, mesenchymal stromal cells and follicular dendritic cells. Histological and immunophenotypic studies of lymph nodes and bone marrow in CLL has revealed the presence of proliferation centres or pseudofollicles. The immunophenotype of leukaemic cells within these centres differs from that of circulating CLL cells in the increased expression of the proliferation marker Ki67, the chemokines CCL17 and CCL22, and the anti-apoptotic factor, surviving. Whether unmutated CLL cells are better able to access, or to benefit from the proliferative and anti-apoptotic stimuli provided by this microenvironment is unclear.

Cellular kinetics of CLL

CLL is classically described as a slowly accumulative disease of functionally incompetent B-cells consequent upon defective apoptosis. This view has been challenged by data in which patients drank "heavy water" for an 84-day period and in whom the incorporation of ^2H into lymphocyte DNA was measured both during and after the labelling period. Surprisingly the leukaemic cell birth rate varied from 0.1% up to 1% of the entire CLL clone per day and higher birth rates correlated with disease progression.

Pre-clinical CLL

The development of sensitive flow cytometric assays for detecting minimal residual disease in CLL patients enabled this assay to be applied to normal individuals with no lymphocytosis. Small clonal B cell populations with the same immunophenotype as CLL could be detected in approximately 5% of individuals over the age of 60 and in 12% of normal relatives who had family members with CLL. IgVH gene analysis shows these clones to have mutated VH genes. The reason why only a minority of patients with pre-clinical CLL evolve into a recognizable disease remains to be discovered, but could reflect the acquisition of secondary genetic abnormalities and/or access to a permissive microenvironment.

Prognostic factors

The potential value of prognostic factors is 2-fold: firstly to predict the natural history of the disease and secondly to predict the response to therapy. Traditionally therapeutic decisions in CLL have been based on staging systems which largely reflect tumour burden. The majority of patients now present with a low tumour burden characterized by a lymphocytosis with or without non-bulky lymphadenopathy and additional markers are required to predict their clinical course. A list of prognostic factors is shown in the table.

Many studies have documented that the median survival of patients with unmutated IgV_H genes is 8–10 years compared to over 20 years for those with mutated IgV_H genes. There is still uncertainty whether a 97% or 98% homology to the germline sequence is the better discriminator between the 2 mutational subsets. It is also recognised that V_H gene usage as well as mutational status affects clinical outcome. Patients utilising the V_H3-21 gene, regardless of mutational status, have a survival comparable to cases of unmutated CLL. Although IgV_H gene sequencing is unsuitable for routine laboratories, gene expression profiling in CLL has identified a series of genes which are differentially expressed in the 2 mutational subsets. Of these ZAP70 is the most studied and shows 80–90% concordance with IgV_H gene status. There is currently an international collaboration to develop and adopt a standardized flow cytometric assay for ZAP70.

Numerous studies have shown that high expression of CD38 correlates with poor outcome in CLL. Results are discordant with IgV_H gene status in approximately 30% of cases and it is uncertain whether CD38 expression has prognostic significance in multivariate analyses which include either IgV_H gene mutations or ZAP70 expression.

Cytogenetic and interphase FISH studies have clearly shown the prognostic importance of cytogenetic abnormalities. Cases with a normal karyotype or del 13q14 as the sole karyotypic abnormality have a significantly better survival than cases with del 11q23 or del 17p13. The prognostic significance of trisomy 12 remains unclear. Loss and/or mutation of the p53 gene is found in approximately 5% of patients at presentation but in 30% of patients with fludarabine

refractory disease. Identification of p53 abnormalities by FISH, screening for mutations or a functional assay, in which upregulation of p53 and p21 is assessed following in vitro DNA damage, is of particular importance in CLL since loss or mutation of this gene predicts for poor response to alkylating agents, purine analogues and single agent Rituximab. ATM loss in cases with del 11q23 is accompanied by mutation of the ATM gene on the remaining allele in approximately 30% of cases. However ATM mutations may also be found in patients without cytogenetic or interphase FISH evidence of ATM loss. The comparative prognostic significance of ATM loss and ATM mutation is still being evaluated.

To date, a series of retrospective studies have shown that a panel of prognostic markers can predict both clinical course and for p53, response to therapy. These results are now being validated in prospective randomized trials and should demonstrate whether early intervention in asymptomatic patients with poor risk disease is of clinical benefit. The final challenge will be to identify prognostic factors that are sufficiently robust to apply to the management of individual patients.

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CHRONIC LYMPHOCYTIC LEUKEMIA

Treatment algorithms in CLL

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CLL therapy has changed dramatically over the last few years. This development has been aided by progress in 2 areas: (1) a better understanding of CLL biology (and with it discovery and characterization of new prognostic markers); and (2) emergence of novel agents. The combined impact of advances in these areas is leading to new concepts and approaches in CLL therapy.

Treatment of CLL has been traditionally revolving around chemotherapy, initially alkylators such as chlorambucil, and nowadays more heavily focused around nucleoside analogs such as fludarabine. Although overall response rates (OR) exceed 50%, complete remission (CR) rates remain modest at $<10\%$ in the case of chlorambucil and at $\sim 35\%$ for fludarabine. Fludarabine proved more effective than chlorambucil in terms of superior response rates and more favorable progression-free survival in a number of randomized studies.[1–3] The German CLL Study Group (GCLLSG) randomized 86 patients >65 years with symptomatic and previously untreated CLL to receive either fludarabine (25 mg/m^2 i.v. daily days 1 to 5 every 4 weeks for a total of 6 courses) or chlorambucil (0.4 mg kg^{-1} days 1 and 15 for 12 months).[3] CR rates (13% vs. 0%; $p=0.028$) and OR rates (85% versus 68%; $p=0.038$) were significantly higher for fludarabine than for chlorambucil. Although fludarabine-treated patients experienced more significant myelosuppression, no difference among treatment arms was demonstrated in the number and severity of infections. In addition, quality of life parameters improved significantly following treatment with fludarabine. More recently, two randomized studies have shown that the combination of fludarabine with cyclophosphamide is more effective than fludarabine alone in achieving higher CR and OR rates and prolonging progression-free survival.[5,6] However, no improvement of overall survival has ever been shown with single-agent fludarabine, and the impact of fludarabine/cyclosphosphamide combinations on overall survival remains to be determined.

Monoclonal antibodies (Moabs) have had a major impact on therapeutic options for patients with lymphoproliferative disorders including CLL. The attraction of moabs is based on selective targeting of tumor-relevant and more or less specific surface markers, and a distinct mechanism of action involving elements of human effector functions such as the complement system and ADCC (antibody-dependent cellular cytotoxicity). Alemtuzumab (anti-CD52) and rituximab (anti-CD20) remain the most active moabs for CLL. Rituximab has achieved OR rates in 51% and alemtuzumab in 87% of untreated and symptomatic CLL patients.[7,8] Furthermore, alemtuzumab remains the only approved first-line therapy for fludarabine-refractory CLL where response rates of 33% with survival benefit in responding patients has been shown.[9] Despite impressive results as single agents however, most patients will eventually relapse so that recent years have seen an emergence of combination strategies involving moabs in an effort to optimize their potential and increase response rates further. Based on in vitro data suggesting sensitization of CLL cells by rituximab to the cytotoxic and apoptotic effects of chemotherapy drugs such as fludarabine, and data showing downregulation of complement defense proteins (CD46, CD55, CD59) by purine nucleoside analogs, chemoimmunotherapy regimens have been developed that combine rituximab with nucleoside analogs such as fludarabine (FR), the combination of fludarabine plus cyclophosphamide (FCR), or with pentostatin plus cyclophosphamide (PCR).[10–12] All chemoimmunotherapy regimens show higher CR and OR rates than would be expected with chemotherapy or monoclonal antibodies alone (Table 1).

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Table 1. Chemoimmunotherapy response rates (NCI-Working Group Criteria)

	N	Age (yrs)	Rai ≥3 (%)	β2M (mg/dL)	CR (%)	OR (%)
F+R [10] ⋆	51	63 (36–86)	39	4.01	47	90
FC+R [11]	224	58 (24–86)	33	3.8	70	95
PC+R [12]	33f	62 (40–79)	52	NA	33	93

⋆ concurrent arm; NA, not available

Comparisons to historical controls with fludarabine alone and fludarabine plus cyclophosphamide, respectively, have been performed for the CALGB regimen (FR) and the MDACC combination (FCR).[11,13] In both cases, chemoimmunotherapy demonstrated an increase in CR rate (70% for FCR compared to 35% for FC; $P < 0.05$), progression-free and overall survival over chemotherapy alone In addition to clinical responses, PCR-negativity reflecting molecular responses was demonstrated in almost half of the patients treated with FCR and a positive association with molecular responses and remission duration could be shown. Several combinations of chemotherapy with alemtuzumab are currently in clinical trials. Combining fludarabine with alemtuzuamb (FluCam) in 36 patients with relapsed and refractory CLL patients, CR rates of 30% and OR rates of 83% have been reported.[14] The combination of FCR plus alemtuzumab (CFAR) proved sufficiently safe and active in relapsed patients so that soon it will be investigated in symptomatic untreated CLL patients with unfavorable β-2-M levels at diagnosis.[15]

The therapeutic potential of moabs is now extending into consolidation and maintenance therapy. Use of alemtuzumab following "debulking" combination chemotherapy has demonstrated in several studies that not only is it possible to increase the number of clinical responders, but also that molecular responses can be achieved translating into a more favorable progression-free survival.[16–18] In the only randomized study to date, patients with CLL responding to initial chemotherapy (either FR or FCR) received either alemtuzumab or were observed.[18] Of 11 patients on alemtuzumab, 2 converted to CR and 5 of 6 patients achieved molecular remissions. On the other hand, 3 patients in the observation arm progressed and no patient achieved a molecular response. At a follow up of around 20 months, the alemtuzumab group showed a significantly longer progression-free survival.

The development of chemoimmunotherapy, moabs as consolidation and maintenance treatments, and novel approaches to stem cell transplantation thus suggest that curative strategies can be designed for select patients with CLL. The challenge remains to integrate new and emerging information from cytogenetic-molecular prognostic markers (e.g. p53 deletions) to identify those patients, determine targets relevant to CLL pathobiology, and thus be able and to apply novel therapies in a disease-specific and risk-adapted manner.

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TRANSFUSION AND APHERESIS

Rationales for therapeutic apheresis

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Introduction

Bloodletting seems to have held tremendous appeal for practitioners of the past. It was a treatment favored by barbers, physicians and naval surgeons from medieval times through the Renaissance, through the Enlightenment and well into the 19th century. It's likely that the trust and confidence it inspired were based in part on its compelling rationale (the depletion of evil humors), while additional support may have come from its obvious effects on complexion and vital signs, plus a generous amount of placebo value. The advent of the scientific method exposed the theoretical basis for bloodletting for what it was – preposterous – and revealed that, with a very few exceptions, the benefits attributed to it for centuries were pure fiction. There are lessons here for contemporary physicians; namely to be clear about reasons for implementing a therapy and cautious about claims for clinical benefit.

Therapeutic apheresis may be fairly described as the modern descendant of bloodletting. Providers of this service/treatment often encounter expectations among patients and other physicians that differ very little from those associated with bloodletting in the past; i.e.; they expect that "cleaning the blood" in some vaguely understood manner will be good for what ails them. Such expectations are sometimes met in individual patients, resulting in case reports suggesting efficacy, but many of these claims have not stood the test of time.

Systematic approach to evidence

It is desirable that medical therapies have a rational basis. A framework is presented here for analyzing the appropriateness and effectiveness of therapeutic apheresis (TA) for a given application. It is argued that TA is most rational where three conditions are met: 1) the disease or symptom is caused by a blood constituent that TA can reasonably be expected to deplete; 2) TA has in fact been shown to meaningfully deplete it; and 3) documented meaningful depletion has been shown to be accompanied by clinically significant benefit.

Firm pathophysiological basis

First, there will ideally be a clear understanding of the pathophysiology of the disease in question that provides a strong rationale for TA. Since TA is usually a "subtractive" therapy, this generally requires that there be compelling evidence that the disease, or at least the targeted manifestation of it, is related to the presence of a blood constituent, preferably an abnormal blood constituent. Furthermore, classic metabolic data (e.g.; half-life, volume of distribution) should support the notion that the pathogenic constituent can be meaningfully depleted by the appropriate TA technique. Often the goal is depletion of a plasma constituent, in which case it is well to bear in mind that therapeutic plasma exchange (TPE) can only be considered reasonable for macromolecules having a relatively long half-life (preferably a week or more) and a substantial fraction of total body content (preferably at least half) in the vascular space.

Documented depletion by TA

Second, the expectation that the pathogenic blood abnormality will be corrected or meaningfully improved by TA should be confirmed by appropriate measurements. In practice, documented utility of TPE to bring about lasting depletion has been limited to antibodies (half-life of IgG = 4 weeks) and low density lipoproteins [1]. Expectations of benefit from "cleansing" the blood of smaller, shorter-lived molecules, including various mediators of inflammation, have never been substantiated by data showing

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meaningful depletion or borne out by clinical experience.

These first two conditions might be called "rationale criteria." A good example of an application having a strong rationale might be red cell exchange for complications of sickle cell disease (SCD). There is ample evidence linking the vaso-occlusive pathology observed in SCD to the presence of abnormal red cells. This provides good reason to suppose that removal of these cells and replacement with normal cells might be beneficial. Furthermore, since the lifespan of a normal red cell is measured in months, there is good reason to expect that a corrective effect of TA would persist for at least several weeks; in fact, serial electrophoretic analyses have repeatedly shown a preponderance of circulating hemoglobin A for many weeks following a red cell exchange. Thus, the rationale criteria are clearly met for this application.

Clinical efficacy

The third criterion requires analysis of clinical data and assessment of its strength. In a few illnesses in which the rationale factors are very strong, efficacy has been considered proven when TA is consistently followed by dramatic and unprecedented clinical improvement, preferably in the absence of any other therapy but at least in the absence of any other new therapy. Examples of this would include the hyperviscosity syndrome (TPE), the leukostasis syndrome (therapeutic leukapheresis) and symptomatic thrombocytosis (therapeutic plateletpheresis). Ideally, however, it is preferable that clinically (vs. statistically) significant benefit be documented in a disorder with a reasonable rationale by properly conducted, randomized controlled trials [2,3]. These can be especially important when TA is employed in conjunction with other therapies and/or used in an illness characterized by spontaneous remissions or fluctuations in activity.

Four combinations of rationale and clinical factors

The remainder of this paper will examine the role of TA in several specific diseases. The examples chosen will encompass four possible combinations of rationale and clinical data and will illustrate how both rationale factors and uncontrolled clinical data can be misleading. They will support the argument that TA is most clearly rational when all three criteria are met.

Rationale and clinical evidence both strong

Guillain-Barre syndrome (GBS) is a good example of a disease for which there is both a strong rationale for TPE and convincing evidence of clinical benefit. Studies in the 1970s showed that the disease could be reproduced in experimental animals by injection of patient plasma, thereby suggesting that a plasma factor was pathogenic [4]. Later studies have established that anti-myelin antibodies circulate during the illness [5]. These antibodies probably arise from a "cross-reactive" immune response to microbial antigens encountered in a recent infection, particularly an enteritis due to *Campylobacter jejuni* [6]. Furthermore, clearance of anti-myelin antibody has been shown to be more rapid in patients who receive TPE [7]. Finally, several large randomized controlled trials have shown that TPE shortens the time to recovery by a substantial margin [8–10]. Thus there is a complete package of evidence supporting use of TPE in GBS.

Strong rationale with no clinical benefit

Systemic lupus erythematosus (SLE) is an example of a disease that offers a strong rationale for TPE but for which the results of controlled trials show a lack of efficacy. SLE has long been considered the prototype of an autoimmune disease mediated by autoantibodies. Enthusiasm for treating SLE was thus understandably high in the early days of automated TPE [11]. There was also no shortage of data confirming the expectation that plasma levels of antinuclear antibodies and immune complexes could be lowered by TPE [12]. Thus, the rationale factors strongly support the use of TPE in SLE. There were also observational reports suggesting that TPE was beneficial for lupus patients [13]. However, these were inadequate to establish efficacy because the patients inevitably received other effective therapies and because spontaneous fluctuations in disease activity are common in SLE. It was nevertheless surprising when a randomized, controlled trial of TPE in lupus nephritis showed no benefit [14]. This conclusion was confirmed by a subsequent international controlled trial in patients with a variety of severe lupus syndromes [15]; the latter trial was stopped early when interim analysis showed no benefit from TPE and an increased risk of death from central venous catheter sepsis in patients randomized to TPE. This experience with SLE illustrates the pitfalls of accepting conclusions about efficacy without controlled data in an illness in which improvement is not unprecedented.

Weak rationale and weak clinical data

There are also diseases in which TA has been used extensively despite a rather weak rationale. Controlled trials in some of these, when finally performed, have shown little or no evidence of benefit. Multiple sclerosis (MS) is a good example of this combination of findings.

TPE has been used extensively in MS, beginning in the 1980's. Interest in trying it in a central demyelinating disorder may have arisen from the favorable outcomes neurologists were reporting with TPE in peripheral demyelinating disorders such as GBS. Whatever the reason, early advocates of TPE in MS chose to ignore the absence of evidence that an autoantibody or any other humoral factor had a pathogenic role in MS. Rather, past and current evidence implicates cellular immune mechanisms as the major cause of demyelination in MS [16]. Absent a causative plasma factor, there can obviously be no documentation of depletion of such a factor by TPE, and this has never been done in MS.

Clinical use of TPE in MS produced many observational studies suggesting benefit. These were not convincing, however, because the patients were receiving other therapies and because the disease is characterized by spontaneous remissions. When randomized controlled trials of TPE were done, the evidence for benefit was not convincing [17,18]. Although there remain pockets of enthusiasm for TPE within the MS treatment community, most neurologists have abandoned it [19]. In the case of MS, then, the lack of a compelling rationale was overlooked until controlled studies showed no clinical benefit. A similar sequence of events can be found in the early use of TPE for renal transplant rejection.

Rationale weak but clinical data supportive

The most perplexing scenario for the analytic approach advocated herein is a disease in which the rationale for TA is unclear or uncertain, but clinical evidence has nevertheless been adduced and interpreted to show efficacy. Some diseases that would formerly have fallen into this category have been reassigned to the previous one when unfavorable clinical data from controlled trials became available. On the other hand is the example of thrombotic thrombocytopenic purpura, which moved from this category to the first one (rationale and clinical data both strong) when its pathophysiology was finally worked out. There are others, however, in which controlled trials have been done and appear to support efficacy in spite of a weak rationale. It is curious that several such diseases are treated with a proprietary TA device provided by a single manufacturer. Because this is the most challenging category, and the one in which critical analysis is most needed, several examples will be offered.

ProSorba column

The ProSorba column, which contains recombinant staphylococcal protein A bound to silica particles, was originally conceived as a mean to deplete IgG antibodies and/or immune complexes from plasma separated by an apheresis instrument without the necessity for the plasma replacement fluids used in TPE. However, the IgG absorption capacity of the commercial product is quite limited – certainly less than the depletion achieved by a one-plasma-volume TPE – and it has never been shown to have a meaningful subtractive effect on any plasma constituent [20]. No other mechanism of action has ever been convincingly demonstrated for this device, though recent unpublished studies propose that leaching of protein A into patient plasma may account for some of its biologic effects.

Notwithstanding the mystery surrounding its mechanism of action, a multicenter randomized sham-controlled trial of this device was undertaken in rheumatoid arthritis (RA) and showed some temporary benefit for patients who completed 12 weekly treatments [21]. On the strength of this evidence, the device has been granted FDA approval for use in RA. In this case, then, a disease of unknown cause is being treated with a device whose mechanism of action is not clear. From an evidentiary standpoint this is suboptimal to say the least.

Photopheresis

Extracorporeal photochemotherapy (ECP), also known as photopheresis, may reasonably be mentioned in this category. In this procedure, autologous mononuclear cells separated by leukapheresis are exposed to UVA light in the presence of a psoralen and then reinfused to the patient [22]. Several theories have been proposed regarding its mechanism of action. One involves apoptosis of treated lymphocytes. Another emphasizes stimulation of monocyte differentiation into dendritic cells. A third envisions modulation of cytokine profiles from the more inflammatory Th 1 pattern to the more inhibitory Th 2 pattern [23,24]. None is proven and thus none can provide a firm rationale for any application. There seems little doubt that ECP leads to remissions in some patients with cutaneous T cell lymphoma (CTCL), an illness in which malignant lymphocytes are abundant in the blood. The ultimate effect of ECP in CTCL is down regulation of a malignant lymphocyte clone; however, down regulation of a pathogenic lymphocyte clone has not been shown in any application except CTCL and therefore the second rationale criterion has not been met for any other application. Nonetheless, ECP is widely believed to be an effective treatment for graft-versus-host disease and solid organ transplant rejection. Clinical evidence supporting these applications is almost entirely observational. A large controlled trial of prophylactic ECP in heart transplant recipients yielded conflicting signals in that mild histologic changes in routine biopsies were less frequent in ECP-treated patients but the incidence of severe, hemodynamically significant rejection was not

decreased [25]. Thus ECP is in regular use in illnesses in which its mechanism of action is unknown and its clinical utility has not been established by randomized controlled trials.

Rheopheresis

A third TA modality that currently falls into this category is called "rheopheresis". In this technique patient plasma separated by apheresis is filtered through a membrane that retains the highest molecular weight solutes. Like the protein A column, it was originally intended to deplete antibodies without the need for plasma replacement solutions, but other macromolecules are also depleted. It is currently under study as a treatment for the dry form of age-related macular degeneration (AMD) [26]. The cause of AMD is not known; however, it has been proposed that microvascular ischemia plays a role. Based on this proposal, it is further hypothesized by proponents and practitioners of rheopheresis that depletion of large plasma macromolecules could improve retinal perfusion by "decreasing plasma viscosity and depleting the serum of soluble macromolecular species such as immune complexes, IgM, fibrinogen, LDL and VLDL cholesterol, von Willebrand factor, α-2 macroglobulin, and probably multimeric vitronectin along with other acute phase reactants, chronic immunomodifiers, and cell signaling components [27]." This is a rather extensive list of possibly harmful plasma constituents. Furthermore, it is admitted that none of them has "demonstrated any causal relationship with AMD." Thus, despite the suggestive inclusion of "rheo" (from the Greek word for "flow") in the name of the procedure, the mechanism of potential benefit from rheopheresis is far from clear [27]. Plasma and blood viscosity do fall by 15–18% after a treatment, although published data do not allow estimation of how long this change persists.

A non-blinded controlled trial showed modest temporary improvement is visual activity in treated patients [28]. A small sham-controlled trial showed similar improvement in visual acuity in patients whose plasma did not pass through the macromolecular filter [27]. A larger multicenter trial is underway in which control patients have antecubital venipunctures done under a drape by attendants who then pretend to operate a concealed but noisy machine nearby. Interim results from this trial showed improvement in visual acuity in treated patients [27]. Since neither the cause of the disease nor the mechanism of the postulated beneficial effect is clear as yet, rationale factors for this procedure would have to be judged weak. If blood viscosity proves to be important, it is fair to point out that hematocrit is a more important determinant of whole blood viscosity than is plasma viscosity, and that phlebotomy (bloodletting) could be a simpler, cheaper and very likely a longer lasting means to achieve a lower blood viscosity.

Summary and conclusions

In summary, then, three criteria are suggested for assessment of applications of TA; two concerning rationale and one concerning clinical data. These criteria are satisfied for some of the conditions that practitioners of TA feel most confident about, but are not yet satisfied in other conditions in which it remains controversial. They may also be useful in evaluating new applications as they arise. Practitioners of TA should strive for a firm evidential basis for what they do, including the rationale criteria set forth herein. When these criteria are not met, there is a danger that a current practices will not differ in kind from bloodletting to remove evil humors.

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TRANSFUSION AND APHERESIS

Transfusion immunomodulation or TRIM: What does it mean clinically?

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Abstract
Evidence from a variety of sources indicate that allogeneic blood transfusions can induce clinically significant immunosuppression, as well as other effects, in recipients. This clinical syndrome is generally referred to in the Transfusion Medicine literature as transfusion-associated immunomodulation, or TRIM. TRIM has been linked to an improved clinical outcome in the setting of renal allograft transplantation. Possible deleterious TRIM-associated effects include an increased rate of cancer recurrence and of post-operative bacterial infection. The recognition that TRIM can increase morbidity and mortality in allogeneically transfused individuals has become a major concern for those involved in Transfusion Medicine. However, based on available randomized controlled trials, whether TRIM predisposes recipients to increased risk for cancer recurrence and/or bacterial infection is still unproven. In contrast, data from experimental animal studies suggest that TRIM is an immunologically mediated biological effect, associated with the transfusion of allogeneic leukocytes; an effect, which can be completely ameliorated by the pre-storage leukoreduction of blood products. Relevantly, several (n = 5) recent large observational trials have provided important evidence for the existence of deleterious TRIM and related effects (mortality and organ dysfunction) of leukocyte-containing allogeneic cellular blood products. These latter data suggest that allogeneic blood product transfusions, containing leukocytes, are associated with an increased risk both for mortality, and organ dysfunction in recipients.

Introduction

The transfusion of allogeneic blood products results in the recipients being exposed to large amounts of foreign antigens (alloantigens) in both the soluble and the cell-associated form. The presence of these alloantigens in the circulation can create conditions for a variety of possible immunological responses, which include both alloimmunization and the down-regulation of immune responses. The latter effect generally has been referred to in the literature as transfusion-associated immunomodulation, or TRIM. TRIM has been associated with alterations in immune function in allogeneic transfusion recipients, including: decreased helper to suppressor T-lymphocyte ratio; decreased NK cell function; defective antigen presentation; and reduction in cell mediated immunity [1–4].

Clinical evidence for the existence of TRIM was initially reported in 1973. In their seminal study, Opelz et al. provided evidence, counter-intuitive at the time, that recipients of allogeneic blood transfusions had improved renal allograft survival [5]. Subsequent clinical studies, as well as data from experimental animals studies corroborated these findings. In fact, in the early 1980s, allogeneic blood transfusions were often administered deliberately to renal allograft recipients in order to try to delay, or prevent, the rejection of the renal allograft [6].

It is particularly noteworthy that although the TRIM effect was widely accepted to improve renal allograft survival in renal transplant recipients in the early 1980s, the practice of using allogeneic blood transfusions as a therapeutic modality in renal transplant patients has generally not seen widespread use. This was both because of concern that allogeneic blood products might be associated with the transmission of viral infections (i.e. HIV, HCV etc.) and the availability of increasingly effective immunosuppressive agents; the latter potentially mitigating the need for the TRIM effect.

Nonetheless, Opelz et al. have reported a clear-cut beneficial effect of allogeneic blood transfusion in

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renal allograft recipients receiving contemporary immunosuppressive therapy [7]. In a collaborative study involving 14 renal transplant centers in Europe and North America, prospective renal allograft recipients of cadaveric allografts (n = 423) were randomized to receive either three unmodified allogeneic red blood cell (RBC) transfusions, or no transfusions. The one-year renal allograft survival rate was 90% in the recipients of the allogeneic RBCs vs. 82% in those renal allograft recipients who did not receive any allogeneic transfusions ($P = 0.02$). At 5 years, the corresponding renal allograft survival rates were 79% vs. 70% ($P = 0.025$) [7].

On the basis of the TRIM effect observed in renal allograft recipients, Gantt raised the intriguing question, in 1981, as to whether the TRIM effect might also be associated with an increased risk of cancer recurrence in patients undergoing surgery for resection of a malignancy [8]. Gantt's hypothesis was based on the premise that, if allogeneic blood transfusion down-regulated the host's immune surveillance mechanisms that might target malignant cells, the TRIM effect could thus enhance tumor growth in patients with a malignancy. A corollary of Gantt's hypothesis is that if allogeneic blood transfusions do indeed cause immunosuppression in a transfusion recipient, then such recipients could be at increased risk also for various infections, particularly post-operative bacterial infections.

Since 1980, more than 150 studies have examined the potential association between peri-operative allogeneic blood transfusions and either cancer recurrence and/or post-operative bacterial infections. Most of these studies are observational cohort studies comparing patients who received allogeneic transfusions with those that were not transfused [9,10]. In addition, 7 randomized control trials (RCTs) have compared the risk of cancer recurrence and/or post-operative infection in recipients of allogeneic blood transfusions compared to control subjects who did not [11].

Allogeneic blood transfusions and tumor growth

Studies in humans

As indicated above, most of the available data relating TRIM to tumor growth promotion are from non-randomized studies. These have been summarized elsewhere [4,9,11]. Of the available reports, approximately 50% of the non-randomized, mostly retrospective, studies indicate that allogeneic blood transfusions have an adverse affect on tumor-related prognosis. However, in the remaining studies no effect was observed [4,9].

The available observational studies usually compared the incidence of cancer recurrence, death due to cancer recurrence, and/or overall mortality between patients undergoing cancer resection or who did or did not receive an allogeneic transfusion [9,12–14]. These studies indicate that patients having allogeneic transfusions (compared with those not having such a transfusion) had a higher incidence of cancer recurrence, or death due to cancer recurrence; as well as a shorter overall survival after the cancer resection operation. These also indicate that patients receiving allogeneic transfusions generally differed from those not receiving such transfusions. These differences included several potentially important prognostic features, including: clinical stage of the malignancy; size, histological grade, and type of tumor; patient age; preoperative hemoglobin; duration and extent of surgery; amount of peri-operative blood loss; and the frequency of chronic systemic illness, such as congestive heart failure, lung disease, liver disease, kidney failure, or diabetes mellitus [9,10,15].

The latter caveats have led various investigators to different interpretations. Some investigators still concluded that peri-operative allogenic blood transfusions had a direct deleterious effect on allogeneic transfusion recipients [13]. Other investigators concluded that the allogeneic blood transfusions were simply a surrogate marker for a variety of adverse prognostic factors, as well as other variables that necessitated the need for peri-operative allogeneic transfusions in the first instance [10].

In some of the reported observational studies, the reporting authors used multivariant regression analysis to try to adjust for the effects of possible confounding factors. However, for most of the published observational studies, important potential confounding factors were not adequately dealt with by the investigators [11]. Thus, the TRIM effects reported as being "independent" by many teams of investigators may not be free of the effects of known confounding factors. These caveats notwithstanding, allogeneic blood transfusions often emerged in these studies as the leading predictor of cancer recurrence and cancer associated mortality in patients with a malignancy [11].

There have been three RCTs that compared the incidence of cancer recurrence in recipients of buffy-coat-reduced allogeneic RBCs with that of recipients of control blood [16–18]. All three studies enrolled patients undergoing colorectal cancer resection. The proportion of patients having allogeneic transfusions varied from 58% to 64% amongst the studies. The proportion of patients developing recurrent cancer varied from 23% to 25.5%. The findings of these 3 RCTs were combined in two meta-analyses [19,20] and the summary odds ratio (OR) of cancer recurrence in the allogeneic transfusion group compared to the control group, across the three studies, was 1.04 (95% CI, 0.81 to 1.35; $P > 0.05$) in one study [19]. In the other, the summary OR of death due to cancer recurrence was 0.98 (95% CI, 0.76 to 1.26; $P > 0.05$) [20].

It is important to note that, for ethical reasons, it is impossible to perform an RCT in which patients are randomly allocated not to receive an allogeneic blood transfusion or always to receive an allogeneic blood transfusion. However, it would be possible to randomize prospectively patients, who are required to receive allogeneic blood products, to receive different allogeneic blood products (i.e. leukoreduced vs. non-leukoreduced); at least in those countries that have not yet introduced universal leukoreduction.

It is important to note that the three RCTs mentioned above were all done in Western Europe [16–18]. The standard issue (since the early 1990s) of allogeneic RBCs in Western Europe has been as buffy-coat-reduced products. Buffy-coat-reduced cellular blood products have 70–80% of the allogeneic donor leukocytes removed. Thus, in two of these studies, the investigators compared outcomes of autologous blood to that seen in recipients of buffy-coat-reduced allogeneic RBCs. In the third study, the effect of buffy-coat-reduced allogeneic blood was compared with that seen in buffy-coat-reduced autologous blood.

Animal studies

The tumor growth promoting effect of allogeneic blood transfusions has also been studied in various experimental animal models [21]. Data from both inbred and outbred experimental animal models indicate that the tumor growth-promoting effect of allogeneic blood transfusions is an immunologically mediated biological phenomenon that is related to the presence of allogeneic donor leukocytes in the transfused blood product [21–23]. Moreover, these studies show that the tumor growth-promoting effect can be ameliorated by the pre-storage leukoreduction of the transfused allogeneic blood. Post-storage leukoreduction of the allogeneic blood was *not* as effective in ameliorating this effect [23].

To examine for the possible role of buffy-coat reduction on the allogeneic tumor growth-promoting effect of allogeneic blood, studies were done in the author's laboratory, in experimental animals, which examined this effect. In these studies, the tumor-growth promoting effect of non-buffy-coat reduced allogeneic whole blood was compared with that seen with allogeneic blood, which was buffy-coat poor. A significant reduction in the median number of pulmonary nodules was seen in rabbits that had received buffy-coat poor allogeneic blood, compared to that seen in recipients of unmodified allogeneic whole blood (34.0 vs. 74.0; $P < 0.0001$) [4]. However, the ameliorative effect of buffy-coat reduction was not complete, in that the median number of pulmonary nodules seen with buffy-coat-depleted whole blood was greater than that seen in animals that received 3 $\log_{10}$ pre-storage leukoreduced allogeneic whole blood, or those that did not receive any allogeneic whole blood (34.0 vs. 23.5 vs. 21.5) [4]. These data therefore indicate that the buffy-coat reduction of allogeneic blood has a significant ameliorating effect on tumor growth in allogeneic transfusion recipients, at least in rabbits.

Allogeneic blood transfusions and the risk of bacterial infection

The association between peri-operative allogeneic blood transfusion and the possibility of increased risk of post-operative bacterial infection following surgery has been reported in many observational studies. The available studies have been summarized elsewhere and indicate a possible association between allogeneic blood transfusion and post-operative infection [11].

Until recently, the various observational studies reporting an association between allogeneic blood transfusions and increased risk for post-operative infection were not adjusted for the effects of severity of illness and/or for the various risk factors for post-operative infection at specific sites. Some investigators partially accounted for the effects of confounding variables by excluding certain types of infection, such as urinary tract infections from the definition of post-operative infection. However, adjustments for the effects of all the possible confounding factors, in combination, has rarely been presented in the literature [11].

Recently there have been three large observational studies reported in which the authors attempted to adjust for many of the potential confounding variables. Thus, Carson et al. conducted a retrospective cohort study of 9,598 consecutive patients with hip fractures, who underwent surgical repair between 1983 and 1993, at twenty hospitals across the US. The primary outcome variable was serious bacterial infection defined as bacteremia, pneumonia, deep wound infection, or septic arthritis/osteomyelitis. The adjusted relative risk of serious post-operative infection associated with allogeneic transfusions was calculated to be 1.43 (95% CI, 1.16 to 1.78; $P = 0.001$) [24].

Similarly, Chang et al. analyzed a database of 1,349 patients, undergoing elective colorectal surgery for various diseases of the colon or rectum at 11 Canadian University hospitals. Ten prognostic variables were found to be associated with both transfusion and post-operative wound infection, with the final regression model adjusting for four of these confounders. In this study, allogeneic blood transfusions were found to be a significant independent predictor of post-operative wound infection (OR = 1.18; 95% CI, 1.05 to 1.33; $P = 0.007$) [25].

Finally, Vamvakas and Carven reported a retrospective cohort study of 416 consecutive patients admitted to one hospital for coronary artery bypass surgery [26]. The outcome variable was limited to

post-operative wound infection, or pneumonia, and adjustment was made for the effects of chronic systemic illness and specific risk factors for wound infection and/or pneumonia. In this latter study the adjusted risk of post-operative infection or pneumonia increased by 6% per unit of allogeneic RBCs and/or platelets transfused ($P = 0.0284$), or by 43% per patient receiving a mean transfusion dose of 7.2 units of RBCs and/or platelets [26].

There have been 7 RCTs reported that have compared the incidence of post-operative infection between recipients of buffy-coat-reduced RBCs, standard allogeneic RBCs, or whole blood; with recipients of autologous, WBC-reduced buffy-coat-reduced allogeneic RBCs, or whole blood [11]. These seven studies are statistically very heterogeneous. Thus, when all seven studies were considered together, to be included in a potential meta-analysis, the probability that the disagreements about the findings might have arisen by chance was smaller than 1 per 10,000 [11]. Two studies [27,28] reported a significant ($P < 0.05$) TRIM effect, two studies [29,20] reported a marginally significant ($P < 0.10$) TRIM effect and three studies did not detect any TRIM effect. More importantly, the variation in the results of these 7 RCTs range from a 7.3-fold increase in the risk of post-operative infection to no effect. The various characteristics of these seven studies are extensively examined elsewhere and the final conclusion is that these seven studies do not show unanimity of a TRIM effect and thus cannot be combined in a meta-analysis [11,31,32].

To try to explain the disagreements among the 7 RCTs, Blajchman [31] & Vamvakas and Blajchman [32] proposed a meta-analysis of the 7 RCTs using individual patient data (IPD). Such an analysis, usually is referred to as an IPD-meta-analysis, would require the re-coding of the raw data prospectively by the authors of the 7 RCTs, using a common patient data form; as well as the collection of additional data through a retrospective review of the medical records of all the patients who were enrolled in the original seven studies. The additional information would be required in order to try to explain and redeem possible disagreements amongst the various studies. Such information would probably highlight differences in severity of illness, or application of diagnostic criteria for post-operative infection, between the treatment and the control arms of the various studies; thus allowing the meta-analysts to assess the possible effects of bias and confounding. Such an IPD-meta analysis is yet to be done.

Post-operative mortality and organ dysfunction following allogeneic transfusions

In addition to showing a possible association between allogeneic blood transfusion and post-operative infection, the study of van de Watering et al. from Leiden detected an unexpected association between WBC containing allogeneic blood transfusion and post-operative mortality (This was not the primary end-point) [30]. Twenty-four of 306 cardiac surgery patients (7.8%) having allogeneic blood transfusions consisting of buffy-coat-reduced RBCs died, compared with 11 of 305 patients (3.6%) receiving buffy-coat-reduced RBCs that were also WBC-reduced (by filtration) before storage. The mortality was 10 out of 303 (3.3%) in a third arm receiving buffy-coat-reduced RBCs that were WBC reduced after storage. This overall difference in 60-day mortality was due to a highly significant ($P = 0.001$) difference amongst the three arms.

Nonetheless, because this study had not been designed *a priori* to investigate post-operative mortality as a primary, or even as a secondary outcome; this group of investigators performed a second study–an RCT that *specifically* tested the hypothesis that WBC-reduction by pre-storage leukocyte filtration reduces post-operative mortality and/or post-operative multi-organ failure. Thus, between 1999 and 2001, 496 complex cardiac surgery patients were randomized to receive either buffy-coat-reduced RBCs, or WBC-leukoreduced (by filtration) buffy-coat-reduced RBCs. The primary end-point of this study was mortality at 90 days. Secondary endpoints included in-hospital mortality, multiple organ dysfunction, infections and hospital stay. The difference in mortality at 90 days was not statistically significant (12.7% vs. 8.4%; odds ratio 1.52; 95% confidence intervals were 0.84 to 2.73). Recipients of buffy-coat reduced RBCs were found to be twice as likely ($P = 0.05$) to die within 60 days of their cardiac operation compared to recipients of WBC reduced RBCs (10.1% vs. 5.5%) [33]. Interestingly, there was no difference in hospital length of stay (13.8 vs. 13.3 days) between the two arms of this study.

It is noteworthy that a recent double blind RCT from the US has been reported, also in patients undergoing elective cardiac surgery. 562 patients undergoing cardiopulmonary bypass surgery in 3 hospitals were randomized to receive either prestorage leukoreduced RBCs or standard not leukoreduced RBCs. The leukoreduced RBC cohort ($n = 304$) showed a lower 60-day mortality (the primary end-point) than that seen in the standard RBC cohort ($n = 258$) (4.9% vs. 9.7%; $P = 0.029$). This data has only thus far been reported in an abstract, [24] but is similar to that reported in the two Dutch studies [30,33].

Recently, the results of two before/after studies which were done in Canada where universal leukoreduction (ULR) was introduced in 1999, have been reported [35,36]. Both studies show a beneficial effect of leukoreduction on recipient mortality and/or evidence of organ dysfunction. In one of these studies,

14,786 adult patients were evaluated, who received leukoreduced RBC transfusions following cardiac surgery, repair of a hip fracture, or required intensive care following a surgical intervention [35]. Those patients who received leukoreduced RBCs (n = 7804) had a lower mortality rate (6.19% vs. 7.03%; $P = 0.04$) than those who received non-leukoreduced RBCs (n = 6982). However, serious nosocomial infections were not shown to be lower in the leukoreduced cohort ($P = 0.63$) [35]. It should be noted that the relative difference in mortality between the two groups in this study is 13.6%.

In the second study, [36] in 515 premature low birth weight neonates weighing less than 1250 grams, the leukoreduced cohort (n = 246) was shown to be associated with a lower risk for bacteremia (OR = 0.59; 95% CI: 0.34–1.01), bronchopulmonary dysplasia (OR = 0.42; 95% CI: 0.25–0.70), retinopathy of prematurity (OR = 0.56; 95% CI: 0.33–0.93), necrotizing enterocolitis (OR = 0.39; 95% CI: 0.17–090), and intraventricular brain hemorrhage (OR = 0.65; 95% CI: 0.35–119). In this latter study both the crude and the adjusted rates for these outcomes indicate that leukoreduction is associated with improved clinical outcome and reduced neonatal ICU length of stay [36].

Relevantly, a meta-analysis was recently undertaken to examine whether an association exists between allogeneic blood transfusions and mortality [37]. An association between allogeneic transfusions and mortality was not detected across all clinical settings; however, subgroup analysis suggested that there was an association between WBC-containing allogeneic blood transfusions and short-term mortality in cardiac patients undergoing open-heart surgery [37]. Whether such results justify the institution of ULR of all blood products for all allogeneic blood recipients still remains open to debate.

Conclusions

A causal relationship between allogeneic blood transfusions and cancer recurrence and/or post-operative infections appear to be indicated by the observational studies reported between 1985 and 2000, *but* not by the available RCTs [9,11,31,32]. Specifically, the available RCTs (n = 3) provide no indication that peri-operative allogeneic blood transfusion causes an increase in cancer recurrence or death due to cancer recurrence, at least in patients with colorectal cancer [11].

With regard to the issue of possible TRIM associated post-operative infection, the available observational studies support the hypothesis of an increased risk of post-operative infection in recipients of allogeneic blood transfusions compared to patients who have not been transfused [11,24–26]. However, it is impossible to determine from the available data whether some of the increased risk of post-operative infection seen in association with allogeneic blood transfusions would persist if the effects of patient selection bias, observation bias, and/or the other clinically relevant confounding factors for bacterial infection were to be removed completely. In this regard it is important to note that the RCTs investigating the association between peri-operative allogeneic blood transfusion with post-operative infection were either unblinded or single-blinded. No double-blind RCTs addressing this question have been done! Moreover, because the diagnosis of nosocomial infections is often subjective, observation bias may be an important concern for several of these RCTs. Lastly, the available RCTs did not present sufficient information about severity of illness, or the distribution of risk factors for post-operative infection. In the absence of such information, the possible contributions of confounding factors and/or bias cannot be excluded from the available RCTs [11].

One possible explanation for the disagreements among the available RCTs investigating the possible impact of TRIM on infection may be that the TRIM effect is quite small (i.e. less than 10%) [11]. Such a small effect would not be detected consistently, particularly in small studies, and its detection would be highly dependent on both the size and the particular design of each individual study. Thus, to detect a 10% difference in the risk of a post-operative infection between treatment and control arms of equal size, 20,000 (10,000 per arm) patients would be required, if the overall infection rate were 20%, and if half of the enrolled patients did not receive any allogeneic transfusions. Therefore, the data from the available RCTs, which together comprise just over 3,000 patients, are likely insufficient for the determination of a small adverse TRIM effect. In fact, in this author's opinion, it is unlikely that an RCT of sufficient power, capable of detecting a TRIM effect of 10% or less, will ever be conducted! [11,31] It may be relevant, therefore, that the recently reported non-randomized observational before/after study of the institution of ULR which reported a 13.6% ($P = 0.04$) lower rate of mortality associated with the use of prestorage leukoreduced transfusions may be the largest data base available, in this regard [35]. In other words, this may be as close as we will ever get to a study of sufficient power to address this question.

Thus, the answer to the question as to whether ULR is effective in abrogating the TRIM effect is still unproven [38]. Moreover, the potential implementation of ULR will leave open the issue as to whether allogeneic blood transfusions are causally associated with TRIM. Thus, the issue whether ULR should be introduced now or await definitive double-blind RCTs of sufficient power, which examine both the existence of the deleterious TRIM effect and the efficacy of WBC reduction in abrogating these effects,

is still being debated [11,38]. Recently, Vamvakas and Blajchman presented pro and con arguments relating to the decision to implement ULR [38]. They indicated that on the one hand, it is possible to argue that the decision to implement ULR should be made on the basis of existing evidence, because better evidence is unlikely to be forthcoming soon. Alternatively, they indicated that one could argue that a policy decision should not be made until it is possible to make such a decision based on clear-cut evidence to warrant the introduction of ULR [38].

Relevantly, the United States Department of Health and Human Services (DHHS) Advisory Committee on Blood Safety and Availability (ACBSA) met on January 25 and 26, 2001 to discuss how the US Government should respond to the current debate in the US over the introduction of ULR [39,40]. ACBSA recommended, by a vote of 11 to 2, with 2 abstentions, that ULR should be implemented as soon as feasible. In separate recommendations, ACBSA also recommended that the DHHS should strive to minimize the impact of this recommendation on the supply of blood components, to ensure adequate funding for this effort, and to support continuing research in this area [39].

This decision by ACBSA was taken despite the knowledge that if ULR were to be implemented in the US and elsewhere, the question as to whether deleterious allogeneic blood transfusion TRIM effects were clinically important would still be open. In fact, it appears that the DHHS ACBSA recommendation on ULR was based on the available data indicating clinical efficacy of ULR for some indications, partial clinical efficacy of ULR for others, and no clinical efficacy for others [38,40]. Thus, ACBSA indicated that if universal WBC reduction were to be introduced in the US, it would still be important to establish definitively the existence of adverse TRIM effects and to obtain additional scientific information about the clinical impact and mechanism of the TRIM phenomenon.

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MYELOPROLIFERATIVE DISEASES

Clinical, pathological and molecular features of the chronic myeloproliferative disorders: MPD 2005 and beyond

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Abstract
The combined use of bone marrow histopathology, biomarkers and clinical features has the potential to diagnose, stage and distinguish early and overt stages of ET, PV and idiopathic myelofibrosis, that has an important impact on prognosis and treatment of MPD patients. As the extension of the PVSG and WHO for ET, PV and agnogenic myeloid metaplasia (AMM), a new set of European clinical and pathological (ECP) criteria clearly distinct true ET from early or latent PV mimicking true ET, overt and advanced polycythemia vera (PV), and from thrombocythemia associated with prefibotic, early fibrotic stages of chronic megakaryocytic granulocytic metaplasia (CMGM) or chronic idiopathic myelofibrosis (CIMF). Cases of atypical MPD and masked PV are usually overlooked by clinicians and pathologists. Bone marrow biopsy will not differentiate between post-PV myelofibrosis versus so-called classical agnogenic myeloid metaplasia. The recent discovery of the JAK2 V617F mutation can readily explain the trilinear megakaryocytic, erythroid and granulocytic proliferation in the bone marrow, but also the etiology of the platelet-mediated microvascular thrombotic complications at increased platelet counts and red cell mass in essential thrombocythemia and polycythemia vera.

Introduction

In the 1980s and 1990s, the German pathologists Burkhardt et a1. Georgii et al. [2–4] and Thiele et al. [5–10] have described typical histopathological features from bone marrow biopsy marterial for the diagnosis and classification of each of the 3 different Ph-negative MPDs ET, PV and AMM. In 1997, we introduced histopathology from bone marrow biopsy as a diagnostic clue and pathognomonic feature of each of the MPDs ET, PV and megakaryocytic granulocytic myeloid metaplasia (CMGM) [11–13]. Thiele & Kvasnicka extended the Rotterdam criteria for ET, PV and added the Cologne criteria for MGM or chronic idiopathic myelofibrosis (CIMF) [14,15]. This concept was taken over by the WHO criteria for true ET, classical PV, and prefibrotic and fibrotic stages of MGM or CIMF [16]. To overcome the shortcomings of the PVSG and WHO criteria, a new set European clinical and pathological (ECP) criteria for the diagnosis ET, PV and CIMF was proposed by Michiels and Thiele [17], which allows to differentiate between the early stages of ET, PV and MGM, to detect prefibrotic and various degrees of fibrosis in PV and MGM and to classify the early, overt and advanced stages of PV and MGM or CIMF, that have major prognostic and therapeutic implications.

Diagnosis and classification of the myeloproliferative disorders: MPD

The inclusion and exclusion criteria for the diagnosis of true ET according to WHO [55] and ECP [17] are identical except platelet count of $>600 \times 10\ 1^{-1}$ according the WHO, and $>400 \times 10^9\ 1^{-1}$ according the ECP (Table I).

A typical bone marrow picture for true ET affects mainly of the megakaryocytic cell lineage and shows increased numbers of loosely clustered enlarged, mature megakaryocytes with hyperploid staghorn-like nuclei together with normal cellularity, erythropioesis, and granulopoiesis, no increase of reticulin fibrosis, and there is no peripheral blood or bone marrow and cytogentic evidence of CML, PV, MGM, CIMG, MDS or reactive thrombocytosis.

The Rotterdam criteria of PV proposed by the Thrombocythemia Vera Study Group (TVSG) extend the PVSG criteria by including histopathology from bone marrow biopsies (Figure 1) [14].A typical

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Table I. The Rotterdam, the ECP and the WHO criteria for the diagnosis of true ET

Platelet count	$>400 \times 10^9\ l^{-1}$	Rotterdam & ECP
	$>600 \times 10^9\ l^{-1}$	WHO
Bone marrow	Increase of dispersed or loosely clustered, predominantly enlarged mature mega-karyocytes with hyperlobulated nuclei and mature cytoplasm, normal cellularity, no or borderline increase of reticulin	Rotterdam & WHO & ECP
	No proliferation or immaturity of granulo- or erythropoiesis	ECP
Exclusion	No peripheral blood, bone marrow and cytogenetic evidence of PV, CML, CIMF, MDS or reactive thrombocytosis	Rotterdam & WHO & ECP

picture in the bone marrow diagnostic for classical PV is featured by increase of clustered enlarged mature megakaryocytes comparable to ET, and a moderate to marked increased cellularity, erythropoiesis and granulopoiesis, the so-called trilinear myelopriliferation [16,17]. The megakaryocytes in PV may have a rather pleiomorphic appearance with wide ranges of sizes including small and giant forms. In our experience a typical PV picture of the bone marrow is seen in classical PV, in erythrocythemia or idiopathic erythrocytosis, in early or latent PV and in masked PV [14–22]. The combination of a typical PV picture, increased red cell mass, high hematocrit and one of the B criteria is consistent with classical PV according the WHO [16] and PVSG criteria. A typical PV picture of the bone and increased red cell mass, high hematocrit but normal platelet count and spleen size is consistent with stage 1 erythrocythemia, is not consistent with PV according to WHO and will be overlooked by the PVSG criteria. A typical PV bone marrow picture with normal red cell mass and hematocrit but with increased platelet count is consistent with ET [13]. This prompted Thiele to define this entity as early (latent) PV (Figure 1 bottom right) [14]. In our experience, early or latent PV usually presents with microvascular disturbances at platelet count in excess of $400 \times 10\ l^{-1}$, increased LAF score, low serum EPO, spontaneous EEC and no or slight splenomegaly. The combination of a typical PV picture, normal red cell mass, normal platelet but slowly progresssive splenomegaly, granulocytosis or even slight anemia is not consistent with either ET, PV but with atypical or unclassifiable MPD or masked PV [13]. Such cases may present with thrombotic complications including splanchnic vein thrombosis (Budd-Chiari syndrome, portal, splenic or mesenteric vein thrombosis) [24,25], or masked PV [26,27], which all typically show spontaneous EEC as the clue to the atypical presentation of MPD. Such cases of atypical MPD and masked PV are overlooked by clinicians and pathologists and may comprises about one quarter of patients with initially a typical PV picture of the bone marrow and usually will progress to so-called classical CIMF without overt PV. At time of classical CIMF bone marrow biopsy will not differentiate between post-PV myelofibrosis versus so-called classical agnogenic myeloid metaplasia. In this regard, we reported a case of primary myelofibrosis with splenomegaly in 1971 in a 61-year old man [28]. The spleen size had progressed to 7 and 13 cm below the costal margin (18 and 23 cm length diameter on scan) respectively associated with progressive anemia increase of platelet count from normal to $811 \times 10\ l^{-1}$ and increase of leukocytes from normal to $24 \times 10\ l^{-1}$ during a follow-up period of 18 years. Sequential bone marrow biopsies showed normal cellularity, fine reticuline fibers grade 1 according to Baumeister [29], and an increase of clustered mature megakaryocytes with hyperploid nuclei in 1971, 1978 and 1982, but hypercellularity, coarse reticulin and collagen fibrosis (dry tap) and increased clustered megakaryocytes in 1985 and 1989 [28].

The Rotterdam Criteria of Polycythemia Vera Proposed by the Thrombocythemia Vera Study Group (TVSG)

A1 Raised red cell mass
male >36 mL/kg
Female >32 mL/kg

A2 Absence of any cause of secondary erythrocytosis by clinical and laboratory investigations

A3 Histopathology of bone marrow biopsy increase of:
a. cellularity, panmyelosis
b. enlarged megakaryocytes with hyperploid nuclei; clusters of megakaryocytes
c. reticulin fibers (optional)

B1 Thrombocytosis
Platelet count >400 × 10^9/L

B2 Granulocytes >10 × 10^9/L and/or raised neutrophil alkaline phosphatase score of >100 in the absence of fever or infection

B3 Splenomegaly on palpation or isotope/ultrasound scan

B4 Erythroid colony formation in absence of EPO: spontaneous EEC

A1 + A2 + A3 is consistent with early stage PV (so-called "idiopathic erythrocytosis")
A1 + A2 + A3 + any one from category B establishes overt PV
A3 + B1 is consistent with essential thrombocythemia
A3 + B3 and/or B4 is consistent with a primary myeloproliferative disorder

Figure 1.

Bone marrow biopsy clearly differentiates between PV and congenital or secondary erythrocytosis with a sensitivity and specificity of more than 95% near to 100% as recently by Thiele et al. [18,19]. In congenital and secondary erythrocytosis, in which increased erythropoiesis is present, the number, size, morphology and distribution of megakaryocytes in bone marrow smears and biopsies remain normal [19,20,22].

The third category of chronic myeloproliferative disorders (CMPD) is usually termed agnogenic myeloid metaplasia or chronic idiopathic myelofibrosis (CIMF), but various other designations have been used such as primary myelofibrosis, myelofibrosis with myeloid metaplasia (MMM) etc [30–33]. AMM, CIMF or MMM is generally defined as a clinicopathological entity not preceded by any other MPD, CML or MDS and characterized by various degrees of anemia, splenomegaly, a leuko-erythroblastic blood picture with tear drop-shaped erythrocytes and various degrees of bone marrow fibrosis or osteosclerosis, and thus by definition disregarding the early non-fibrotic stage of the disease [16,30–33]. In the late 1970s and early 1980s Thiele et al. [5,6] and Georgii et al. [2] drew attention to an authentic dual megakaryocytic granulocytic myeloproliferation (CMGM) as a separate pathological entity among the MPDs. This condition has been labeled in 1996 as chronic megakaryocytic granulocytic myeloproliferation (CMGM) by Georgii et al.[4] and described in detail as prefibrotic CIMF by Thiele et al. [34–39] to distinguish this entity from ET. Prefibrotic CMGM or CIMF according to the Cologne [14,15], WHO [16] and ECP [17] criteria is a mixed proliferation of increased granulopoiesis and megakaryopoiesis without or with early fibrosis but dominated with immature giant megakaryocytes which are conspiciously enlarged due to increase of nuclear as well as cellular size with bulky and irregular roundish-shaped nuclei, so-called cloud-like nuclei is typical of CMGM (Table II) [34–39]. The prefibrotic and early fibrotic stages of CMGM or CIMF are frequently associated with pronounced thrombocythemia, without a leuko-erythroblastic blood picture, normal or increased LAF-score and no or minimal splenomegaly (Table II) [34–39], which according to the PVSG criteria are to be diagnosed as ET [38].

The semiqualitative grading of reticulin and collagen fibrosis has recently been improved and standardized. In each of the MPDs, myelofibrosis (MF) can be graded from 0 to 3 [40]. Myelofibrosis is not a feature of true ET according to the WHO or ECP. Very few ET patients will develop myelofibrosis during long-term follow-up [4]. Myelofibrosis is present in only a minority of PV patients at time of diagnosis, but all stages of myelofibrosis have been observed during long-term follow-up [43]. There is a conflict of opinion and observer disagreement with regard to the classification and natural history of prefibrotic and early CMGM or CIMF and its differentiation from true ET [41]. Those cases within prefibrotic CMGM or CIMF with slight maturation defect of enlarged megakaryocytes are featured by a rather slowly progressive myelofibrosis with slight splenomegaly or anemia and a life expectancy near to normal similar as in PV. Prefibrotic MGM or CIMF with slight dysmegakaryopiesis may precede classical CIMF for 5 to more than 10 years [42]. Discussions between clinicians and pathologists reveal that diagnostic differentiation between true ET and thrombocythemia as the presenting feature of prefibrotic CMGM (or CIMF) with slight maturation defect of enlarged clustered megakaryocytes and no or slight increased cellularity is subjective with a high inter-observer disagreement between pathologists.

Clinical, laboratory and pathological features of the MPDs in 2005 and beyond

Comparing the WHO/ECP with the PVSG criteria for the diagnosis of ET show that the PVSG criteria fail to distinguish ET from early PV mimicking ET and fail to distinguish ET from early stages of thrombocythemia associated with CMGM or CIMF (Table III) [42–44]. The diagnostic guidelines of the World Health Organisation (WHO) explicitly include bone marrow pathology as a positive criterion for the distinction of true ET, early and overt PV and prefibrotic or early fibrotic myeloid metaplasia or CIMF. In consideration of disease-related complications occurring at low platelet counts, the arbitrarily chosen limit for platelet count ($>600\times10^{-1}$) by the PVSG and WHO has been reduced to $400\times10[9]^{-1}$ by the European clinical and pathological (ECP) criteria for ET. The PVSG clinical criteria for ET, when compared to the WHO and ECP criteria, include true ET, early PV mimicking ET and thrombocythemia as the presenting feature of prefibrotic or early fibrotic MGM or CIMF (Table III) [42–44]. The ECP criteria clearly differentiate PV from SP, and ET from reactive thrombocytosis, initial PV and prefibrotic MGM or CIMF. The PVSG and the WHO criteria for the diagnosis of PV in Table IV used increased red cell as the main requisite, which is a very crude and overlook by definition early PV mimicking true ET (stage 0) and the erythrocythemic phase (stage 1) of PV, formerly labeled as idiopathic erythrocytosis. It became apparent that spontaneous endogenous erythroid colony formation (EEC) and PRV-1 expression are the hall mark of PV, and about 50% of ET patients are EEC positive or PRV-1 positive. The reports on EEC/PRV-1 positive ET according to the PVSG very likely represents early or initial stages of PV because of a typical PV bone marrow picture. The cohorts of early stage 0 and 1 PV

Table II. The Cologne, the WHO and the European clinical and pathological (ECP) criteria for the diagnosis and staging of MGM, CIMF or MMM-AMM

	Clinical criteria		Pathological criteria
A1	No preceding or allied other subtype of myeloproliferative disorders CML or MDS. Main presenting feature is pronounced thrombocythemia and no dry tap on bone marrow aspiration.	**B1**	Megakaryocytic and granulocytic myeloproliferation (MGM) and no or relative reduction of erythroid precursors. Abnormal clustering and increase in atypical giant to medium sized megakaryocytes containing bulbous (cloud-like) hypolobulated nuclei and definitive maturation defects.
C	Clinical stages	**MF**	**Staging of myelofibrosis (MF)**
C1	Early clinical stages Normal hemoglobin or slight anemia, grade I: hemoglobin >12 g/dl Slight or moderate splenomegaly on ultrasound scan or CT Thrombocytosis, platelets in excess of 400, 600 or even $1{,}000 \times 10^{9-1}$ Normal or increased LAF-score No leuko-erythroblastose	**MF-0** **MF-1**	prefibrotic stage CIMF: no reticulin fibrosis early CIMF: slight reticulin fibrosis
C2	Intermediate clinical stage Anemia grade II: hemoglobin >10g/dl Definitive leuko-erythroblastic blood picture and/or tear drop erythrocytes Increased LDH Splenomegaly	**MF-1** **MF-2**	early CIMF: slight reticulin fibrosis manifest CIMF: marked increase in reticulin and slight to moderate collagen fibrosis
C3	Advanced clinical stage Anemia grade III: Hemoglobin <10g/dl Definitive leuko-erythroblastic blood picture and/or tear drop erythrocytes Splenomegaly, thrombocytopenia, leukocytosis, leukopenia	**MF-3**	overt CIMF: advanced collagen fibrosis with optional osteosclerosis

The combinations of A1 + B1 establish CIMF-any other criterion confirms CIMF/AMM

and the overt stage 2 PV patients are featured by spontaneous EEC, positive PRV-1, low serum EPO levels and a typical PV bone marrow picture. The cohorts of early stage 0 and 1 PV and the overt stage 2 PV patients are featured by EEC and PRV-1 positivity, low serum EPO levels and a typical PV bone marrow picture (Table IV) [18–27]. Both the early (stage 0 and 1) and overt (stage 2) PV patients are at high-risk for potential minor and major vascular complications, because they present with elevated platelet count mimicking true ET [21–23]. About 50% of ET patients, diagnosed according to the PVSG criteria, have elevated levels of PRV-1 expression together with low serum erythropoietine (Epo) levels, and Epo independent erythroid colony formation (EEC) [45–48]. EEC/PRV-1 positive ET according to PVSG criteria is associated with a higher risk of developing microvascular and major thrombotic com-

Table III. Clinical, pathological and molecular features of true ET, initial PV mimicking ET and false ET when the PVSG and WHO/ECP criteria are compared

Diagnosis	Hereditary ET	True ET	Initial PV mimicking ET	ET-MGM Prefibrotic Early Fibrotic CIMF
Incidence (%)	<0.001	20–30	20–30	50–60
Serum EPO	Normal	Normal	Decreased ◆	Normal
Thrombocytes	◆/◆◆	◆/◆◆	◆/◆◆	◆/◆◆
Erythrocytes	N	N	*N/t*	
Hematocrit	N	N	N/*	N/#
Bone marrow:	ET picture	ET picture	PV picture	MGM picture
Cellularity myelopoise	N	N	◆/◆◆	◆/◆◆
Erythropoiesis	N	N		
Megakaryocytes				
Megakaryocytes	Normal	enlarged / giant and mature	megakaryocytes	abnormal
Clonality	polyclonal	poly/monoclonal	monoclonal	monoclonal
JAK2V617F	–	+	+	+ versus –
EEC	–	+	++	–
PVR-1		+	++	+

Table IV. Diagnosis of Poiycythemia Vera (PV): therapeutic implications

Poiycythemia Vera	Evolution		Manifestation	
Staging of PV New concept	ECPO Aspirin/ phlebotomy A/P	ECP 1 Aspirin/ phlebototm A/P	ECP 2 PVSG WHO A/P	ECP3 PVSG WHO IFN/HU
	initial PV mimicking ET	Erythrocythemic PV	Thrombo/erythro/leuko cythemic PV	
Estimated incidence (%)	20–25	20–25		40–60
Hemoglobin g/dl	N/*			
Serum EPO	◆			
Hematocrit	male 0.43– <0.51 Female0.42– <0.48	male >0.51 female >0.48		
Red cell mass	N			
Thrombocytes (x 10^9/l)	400–600 >600	<400	>400	>1,000
Leukocytes (x 10^9/l)	N	N	N	>15
Spleen on echogram (cm)	N-15	N	N-15	>15
Bone marrow:	PV picture	PV picture	PV picture	PV MF picture
JAK2V617F	+	+		++/LOH
EEC	+	+	+	+
PRV-1	+	+	+	+
LAF score				
Myelofibrosis (MF)	0	0	0/1	1/2

plication as compared to EEC/PRV-1 negative ET [47,48]. PRV-1-positive ET comprises a pathophysiologically distinct subgroup of ET patients that is at risk for the development of thrombotic complications and for emergence of PV that may reflect early or initial PV [48]. Early or initial PV according to WHO [16] and ECP [17] criteria is typically featured by a PV picture in the bone marrow, positive results for EEC and PRV-1, and/or low serum Epo levels, and a much higher thrombotic risk, which is related to increase of hypersensitive platelet counts (thrombocythemia) and slightly increased values for hematocrit up to 0.50, and therefore candidates for low dose aspirin and phlebotomy (Table II).

The clonal and molecular etiology of ET, PV and MGM

The MPDs represent clonal proliferation of the hematopoietic stem cells [49–53]. The clonal nature is found in nearly all PV and CIMF patients, while a variable proportion of ET patients are polyclonal. A key observation since 1974 [19] is the spontaneous growth of EEC as a hall mark of PV, but also found in about half of ET patients and in a proportion of CIMF patients [24–27,45,55]. Apart from spontaneous EEC, PV bone marrow cells are hypersensitive to insulin growth factor-1 (IGF-1) [56–58]. IGF-1 sensitivity ratios reached as high as 20.000 times the no and this level of cytokine hypersensitivity in the erythroid lineage of PV is specific to IGF-1. In addition, PV bone marrow cells are hypersensitivity but to a less extent, to other hematopoietic growth factors including IL-3, GM-CSF and SCF. Spontaneous endogenous megakaryocyte colonies (EMC) (CFU-Meg) in the absence of exogenous growth factors has been described in ET patients [59–62]. The median TPO sensitivity ratios were more than 50 times the normal and this was highly specific with respect to cytokine, disease and cell lineage, suggesting a lineage restricted hypersensitivity of hematopoietic progenitors to normal endogenous TPO in thrombocythemia (ET and early PV mimicking ET) [62]. In patients with AMM/CIMF clear evidence of hypersensitivity was found for SCF, a cytokine active in several different cell lineages [63]. These results prompted Axelrad to propose a model that the clinicopathological phenotypes of the clonal MPDS (ET, PV and CIMF) are related to, and perhaps determined by specific hypersensitivities of their progenitor cells to normal endogenous cytokine: EEC-IFG-1 hypersensitivity for PV, TPO-hypersensitivity for thrombocythemia (ET and early PV mimicking ET) and GM-CSF and SCF hypersensitivity for granulocytosis in PV and for CIMF/AMM respectively. In the studies of Pahl et al. PVR-1 gene expression has been detected in nearly all PV and in about half of the ET patients, who also demonstrated spontaneous EEC, and there was a near to 100% concordance between EEC and PRV-1 positivity in PV and ET patients as well as EEC negativity and normal PRV-1 expression in ET patients [64–67]. The EEC/PRV-1 positive disease may present clinically as an ET (early PV mimicking PV), that will develop into PV, as PV or as a variant of CIMF having quickly passed the hypercellular polycythemic stage (Tables III and IV).

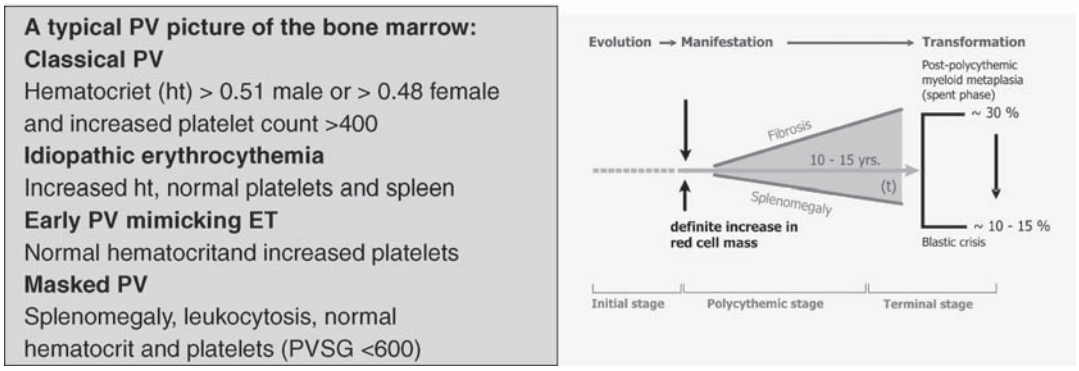

Figure 2.

The discovery of JAK2 V617F gain of function mutation by Vainchenker in June 2004 has become a real evolutionary event for a better understanding towards an unifying concept on the molecular etiology for ET, PV, and MGM, or CIMF as well as for the clinical manifestations of platelet-mediated thrombosis, for the increased red cell mass complicated by major and for secondary myelofibrosis [68]. JAK2 plays an essential role in hematopoiesis by mediating signals from several hematopoietic cytokines including EPO, TPO IL-3 G-CSF, GMSCF etc [69–71]. The JAK2 mutation makes the mutated hematopoietic progenitor cells hypersensitive for TPO, EPO. IL3, G-SCF and GM-CSF and thereby leading to growth advantage of the mutated above the normal trilinear hematopopoietic cells in the bone marrow. The discovery of the JAK2 V617F mutation by Vainchenker [68] was rapidly confirmed by several investigators [72–76]. According to the PVSG criteria, half of the ET and MF patients and the majority of PV patients have the mutated the JAK2 allele. By pooling the currently available data that were generated by DNA sequencing, the frequency of JAK2 V617F is 73% in PV, in ET, and 43% in CIMF. A much higher frequency of JAK2 in MPD (97% in PV, 57% in ET and 50% in CIMF) was described in one study that used allele-specific polymerase chain reaction (PCR) analysis in addition to sequencing. The mutation was absent in more than 600 healthy controls, in patients with Ph^{1+} CML, and in patients with reactive thrombocytosis. The mutation has been found rarely in CML, MDS, hyper-eosinophilic syndrome, chronic neutrophilic leukemia, chronic myelomonocytic leukemia, but somewhat more frequent in CML-like MDS and unclassified MPD. The acquired JAK2 V617F mutation is located on chromosome 9p. A minority of ET and about half of the PV and MF patients have both JAK2 alleles mutated, which is the consequence of mitotic recombination between homologous chromosomes 9p in a cell heterozygous for V616F and results in loss of heterozygosity of chromosome 9p (9pLOH). The 9pLOH is a second genetic event of duplication of chromosome 9p bearing the mutated JAK2 and therefore homozygous.

The JAK2 V617F mutation affects the trilinear hematopoietic bone marrow cells and is detectable in platelets, erythroblasts and granulocytes. The gain of function mutation is in line with the concept of Dameshek that all "stops" to blood production in the bone marrow seem to have been pulled out by one factor JAK2 V617F causing, due to hypersensitivity of hematopoietic progenitor cells to growth factors, trilinear myeloproliferation. The hypothesis may be that heterozygous JAK2 mutation with low activity may be enough for megakaryocyte proliferation with increase of hypersensitive platelets (ET) with no or slight increase of erythropoiesis (initial early PV), and that homozygous JAK2 mutation with moderate to high activity surely will produce pronounced trilinear megakaryocyte, erythroid or even granulocytic proliferation with the clinical pictures of PV, atypical granulocytic leukaemia, unclassifiable MPD with secondary myelofibrosis [76–78]. The sequential occurrence of heterozygous and homozygous V617F mutation can readily explain the spontaneous megakaryocyte and erythroid colony formation (EEC), and the hypersensitivity of granulocyte precursors to growth factors (increased PRV-1 expression) with the sequential production of increased hypersensitive platelets as a first step, increased hematocrit as a second step aggravating the microvascular disturbances of thrombocythemia into the macrovacsular complications of polycythemia vera. Similarly, the sequential occurrence of heterozygous and homozygous V617F mutation can also explain the dual granulocytic megakaryocytic proliferation associated with increased leukocyte activation and production (leukocythemia) and secondary myelofibrosis without features of PV because of progressive splenomegaly. Two main questions to be answered are the following. First, are cases of true ET either clonal or polyclonal positive or negative for JAK2 V617F. Second, are cases of prefibrotic or early fibrotic CMGM or CIMF JAK2 V617F positive, are additional genetic defect needed for progressive MPD disease or do indepen-

dent genetic defects give rise to JAK2 negative and Philadelphia chromosome negative PV, and AMM with predicted poor prognosis (Figure 2). The genetic aetiology and natural history, as well as the pathophysiology of clinical manifestations and the haematological peripheral blood and bone marrow features of JAK2 positive and JAK2 negative MPDs have to be determined in a large prospective study of newly diagnosed and previously untreated Philadelphia chromosome negative MPD patients to be followed-up for 5 to more than 10 years [79].

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NUTRITIONAL ANEMIAS

Introduction—The global problem of nutritional anemias

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This presentation provides a basic introduction to the review by Dr R Gräsbeck which follows. Reports from World Health Organization (WHO) and various other publications give an estimate that two 2 billion people (20–25% of the world population) are anaemic. Up to 80% of cases occur in developing countries. The main cause is iron deficiency anaemia, especially in children and pregnant women, whilst iron deficiency without anaemia is even more widespread and world-wide. Less prevalent, but nonetheless significant, are nutritional anaemias due to folate or cobalamin deficiency. Other common causes of anaemia in different regions of the developing countries are due to hookworm infection, schistosomiasis, malaria, chronic infections (notably AIDS and tuberculosis), thalassemia, sickle cell disease and other haemoglobinopathies.

Several WHO units are concerned with the problems of nutritional anaemias; relevant publications include the following:

> Iron Deficiency Anaemia: Assessment, Prevention and Control (WHO/NHD/01.3) The clinical use of blood (WHO/BTS/99.2)
> Nuitrition for health and development (WHO/NHD/00.6)
> And jointly with The United Nations Children's Fund (UNICEF) and International Nutritional Anaemia Consultative Group (INACG): *Guidelines for Use of Iron Supplements to Prevent and Treat Iron Deficiency Anemia*
> These and other documents can be downloaded from the WHO website (www.who.int/nut/ida/publications) and from the UNICEF and INACG websites.

In assessing the significance of anaemia account must be taken of both mortality and morbidity, as reflected by disability-adjusted life-years. (DALY). This is a measure of the total impact on population health of the illness, combining the time lived with a disability and the time lost due to premature mortality. Accordingly to the WHO annual report (2002), globally iron deficiency is the cause of death in 1.3% of males and 1.8% of females. In terms of DALY, iron deficiency represents $35 \times 10[9]$ years of quality life lost annually, i.e. 15.9% of the global burden. Its geographic distribution is as follows:

SE Asia	40%
Africa	29%
Western Pacific	13%
E. Mediterranean	11%
Americas	4%
Europe	3%

Iron deficiency anaemia in childhood gives rise to physical and mental/cognitive retardation. If untreated, continuing morbidity will result in a future inefficient and unproductive adult population. To sustain national development requires a physically healthy and mentally alert population from generation to generation. Thus, an important priority for health programmes should be eradication of anaemia, and especially iron deficiency anaemia. This requires correct diagnosis, adequate diet and appropriate therapy-a task that requires collaboration and coordination by haematologists, paediatricians and public health authority, with an understanding by government authorities of the importance of this problem.

The reliability of screening for anaemia by clinical features alone is debatable. Errors are unlikely to be made in severe anaemia when conjunctival, mucosal, nail bed and/or palmar pallor is obvious, but clinical diagnosis is less reliable with moderate anaemias, when less than half of the cases may be correctly diagnosed; conversely, in one study a false positive diagnosis of anaemia was made in 16% of cases where haemoglobin concentration was normal.

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It is thus necessary to measure haemoglobin in point-of-care screening, whether at clinics (especially childhealth and antenatal) or in epidemiological field studies. As anaemia must frequently be diagnosed by health workers without access to laboratory facilities, a method is required that is simple to use with minimal training, is sturdy but readily portable-preferably pocket-sized, does not require electricity, is stable during use, is as cheap as possible and able to provide results rapidly and sufficiently reliable for the intended purpose.

To meet these criteria, SM Lewis and GJ Stott, in collaboration with WHO, developed the Haemoglobin colour scale [1,2]. Its validation in a multicentre study and data on its utility in various clinical trials have been reported –a resume of these studies and full bibliography appear at *www. who sites ['search for'] haemoglobin colour scale*. From a drop of blood on a test strip, this gives an assessment of anaemia in graded shades representing Hb in g dl^{-1} at >12(normal), 10–12 (mild anaemia), 8–10 (moderate anaemia), 6–8 (marked anaemia), 4–6 (severe anaemia) and <4 (critical) It is, thus, a device for estimating the severity of anaemia in clinical terms, and it is not intended to compete with laboratory-based haemoglobinometry.

HemoCue is a haemoglobinometer that also uses a drop of blood taken up in a special micro-cuvette, and does not require dilution. This well established instrument which has a place in the laboratory as well as in point-of-care testing, provides haemoglobin measurement with an accuracy within 0.1 g dl^{-1} and a precision of <2%. Other simple methods on the market include (a) the Lovibond comparator in which accurately diluted blood is spread in a capillary cell and compared against a set of colour discs, and (b) the **DHT** meter in which a blood sample is diluted and read in a cuvette on a spectrometer as HbO_2.

The advantages and disadvantages of these methods are summarized in Table I.

Subsequent stages in investigation depend on what facilities are available. Romanowsky-stained blood films may be helpful in identifying the morphological appearances of the red cells. These are, of course, illustrated in currently available atlases, whilst WHO has published a set of laminated cards (*Bench Aids for the morphological diagnosis of anaemia)*, which show the characteristic features of various anaemias.

A full blood count may be helpful in determining whether anaemia is due to iron deficiency, and, conversely, whether iron deficiency is present without anaemia. MCV and MCH are sensitive early predictors of iron deficient erythropoiesis [3], but full assessment of iron status requires tests of varying complexity, as listed in Table II. These must, however, be interpreted with caution. Thus, when interpreting red cell parameters (a) iron deficiency must be distinguished from thalassaemia, (b) to avoid misleading results due to diurnal variations, blood for serum iron and ferritin should always be collected at the same time of day with the subject at rest and prior to eating, (c) High serum ferritin may be due to infections; ESR and/or C-reactive protein measurement provides helpful supplementary information for assessing the significance of the measured ferritin value [4]. Bone marrow aspirates stained for iron have been shown to provide the most reliable identification of iron status [5] – but this is not practical as a routine test, especially in young children, nor in rural clinics.

There is evidence that iron deficiency without anaemia may result in retarded cognitive development in children and reduced work capacity in adults. The extent of these problems when there is no anaemia is debatable [6]. A concise account of current views has been published by J Umbreit [7].

What of the future? Reliable non-invasive methods for measurement of haemoglobin at the skin surface have already been developed [8]. This has important implications for anaemia screening at a global level. Finally, nanotechnology is being used for developing micro-biopsy probes for analysis of cell chemistry and functional integrity of individual cells [9]. Application of this should eventually be able to provide information on the effects of iron deficiency or iron depletion on the biology of the cell.

Table I.

Method	Hemo Cue	DHT Hbmeter	HCS	Lovibond
Dilution required	No	Yes	No	No
Basic cost (ε)	350–450	540–590	4.5	360
Cost of disposables+ (ε)	0.6	Trivial	0.01	None
Test time	2 min	2 min	1 min	2 min*
Accuracy	1–2%	2%**	Within $10g^{-1}$	Within $10–15g^{-1}$*

+ i.e Reagent, cuvette or test strip

* But requires washing of capillary cell immediately after each measurement

** Depends on reliability of dilution technique

Table II. Measurement of iron status.

	Early Fe deficient	Fe depletion	Fe-deficient erythropoiesis	Anaemia
Hb	N	N	N	↓
MCV				
MCH				
MCHC	N	N	↓	↓
Hypochromic cells	< 5%	< 5%	< 5%	↑
Serum ferritin (>15 μg/l)	↓	↓	↓	↓
Serum iron (>10 μmol/l)	N	N	↓ ++	↓ +++
TIBC (>55 μmol/l)	N	N	↑ +	↑ ++
Serum Tf receptors	↑ ±	↑ +	↑ +++	↑ +++
RBC ZPP <30 μmol/mol)	N	N	↑ ++	↑ +++
Bone marrow Fe (++)	+ →±	0	0	0

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NUTRITIONAL ANEMIAS

Megaloblastic Anaemia (MA)

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Megaloblastic Anaemia (MA) is caused by retarded purine and pyrimidine (notably thymidylate) synthesis. Therefore DNA does not replicate, but RNA and protein (including haemoglobin) synthesis continues leading to typical cell changes, especially in rapidly dividing tissues such as the bone marrow, testicles, gastrointestinal and bronchial epithelia. Excepting some rare metabolic errors, MA is due to lack of or inability to absorb or to deliver to tissues and subcellular structures folate (FA) or cobalamin (Cbl or vitamin B_{12}) or to produce enzymes which contain their coenzyme forms. Typical changes in the peripheral blood are pancytopenia, high MCV, MCH and red cell diameter (MCD) indexes and granulocytes with polylobulated nuclei. Low haemoglobin concentration is a late sign of deficiency. Specific treatment causes reticulocytosis and haematological remission, probably the most reliable diagnostic evidence for deficiency. In Cbl deficiency there is typical neurological damage; in children especially the brain is vulnerable.

The mechanism causing MA (which does not occur in non-primates) is a disturbance in the regeneration of active tetrahydrofolate (THFA) needed for transfer of one-carbon units. Methyl-Cbl is a cofactor in the transfer of methyl from 5-methyl-THFA to homocysteine (Hcy), which becomes methionine. The latter is then converted to S-adenosylmethionine and used for methylation reactions. Serum-Hcy increases both in folate and Cbl deficiency. Deoxyadenosyl-Cbl (Ado-Cbl) is a mitochondrial coenzyme needed for the conversion of methylmalonyl-CoA to succinyl-CoA, then processed in the Krebs cycle. In Cbl deficiency, serum-methylmalonate (MMA) increases. Accordingly, Hcy and MMA are used to diagnose FA and Cbl deficiency. The mechanism causing the neurological damage in Cbl deficiency is not quite clear, MMA may damage the myelin sheaths.

The traditional ways of diagnosing these deficiencies is to assay serum Cbl and FA and red cell FA (all mixtures of numerous compounds), then to investigate the cause of the deficiency by measuring radio-vitamin-B_{12} absorption (especially the Schilling test), assaying intrinsic factor (IF) antibodies, performing gastric function tests, etc. During recent years, transcobalamin (TC or TCII) bound Cbl has become popular as the rest of the serum vitamin is bound to haptocorrin (HC, R-protein or TCI) and does not reflect the rapidly mobilizable body stores.

At present there is no generally accepted gold standard test for diagnosing Cbl or FA deficiency, nor agreement on decision limits. The matter is complicated by the fact that based on the tests and therapeutic trials, large sectors of the population, especially the aged, have been considered to suffer from FA or Cbl deficiency, which may manifest itself as atherosclerosis, mental retardation, Parkinson's disease, osteoporosis and infertility, maybe even problems with vision. In addition to low intake and gastric and pancreatic maldigestion, possible components in the causation of these conditions are slowly functioning genetic variants of enzymes and transport proteins involved in the metabolism of the vitamins. As lack of FA during pregnancy is known to cause neural tube defects, fortification of foodstuffs has been instituted, –a debatable practice, as FA tends to worsen the neurological damage in Cbl deficiency. Considering the very large sectors of the general population suspected to be deficient, the cost of the tests and their fallacies, one might consider prophylactic treatment of selected groups with FA and Cbl,

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which are relatively cheap. Sufficient amounts of Cbl have then to be given to prevent neurological damage.

These problems are of less importance in children, but may become important if one begins to look for predisposition to adult diseases. FA deficiency is a rare cause of MA in Scandinavia. In children deficiency may become apparent following treatment of iron deficiency anaemia in coeliac disease. Relative FA deficiency has been observed in babies that are not breast-fed, who have undergone extensive surgery or are premature. For monitoring these cases, the MCV index is useful. MA is sometimes observed in analogous adults or who are alcoholic or take antiepileptic drugs. Intake of poor food, boiling of vegetables and intestinal damage due to tropical diarrhoeal diseases probably partly explain the higher frequency of FA deficiency elsewhere in the world.

Cbl deficiency is also rare in children. Overt MA is usually preceded by failure to thrive and gastrointestinal and respiratory infections. Fish tapeworm anaemia was once frequent in Finland and not uncommon in children. An extensive field study permitted correlating the serum Cbl level with the blood picture, and revealed that the red cell count, the MCV, MCH and MCD indexes responded earlier to low Cbl levels than haemoglobin. The mean red cell diameter was measured with a halometer, a simple and forgotten instrument that can be used under primitive conditions. Other causes include selective Cbl malabsorption, often combined with proteinuria and very early shown to be due to an error in the intestinal receptor for the Cbl-intrinsic factor (IF) complex. Today we know that the receptor has two components, cubilin which are lacking in the patients first described by myself and amnionless, which is lacking in Imerslund's Norwegian patients, who, incidentally, have anatomical anomalies. Lack of the receptor in the kidney tubules explains the proteinuria.

Genuine pernicious anaemia with atrophic gastritis and anti-IF antibodies occurs in children, but is rare (eradication of Helicobacter may reverse the atrophy). More common is congenital lack of IF. These cases have been mistaken for selective malabsorption, especially as Schilling tests and other relevant assays have become unavailable. The proper diagnosis can be provided by genetic tests. Nutritional lack of Cbl is not uncommon in breast-fed children of Vegan mothers. Neurological and especially brain damage is a real threat here.

That congenital lack of TC can produce MA was once predicted by the author, but no cases have been reported from his part of the world. The same applies to mutations in the enzyme systems producing and requiring the Cbl coenzymes. Some of these conditions have MA and respond to large doses of Cbl. Low, but largely innocuous serum-Cbl is observed in congenital HC deficiency. However, HC may have the important task of removing antagonistic Cbl-related compounds. Their role in producing Cbl deficiency is largely unknown but worth looking into. We have recently biosynthesised ^{32}P-Cbl which may provide a tool for such studies.

Under primitive conditions, MA may be best diagnosed counting the red cells, leukocytes and platelets in peripheral blood and determining the MCV, MCH or MCD indexes, inspecting the morphology of the cells in a blood smear and observing the effects of a therapeutic test, giving first small amounts of FA, then Cbl.

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HEMOPHILIA CARE IN 2000S

Optimizing clotting factor replacement therapy in hemophilia: A global need

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Morbidity in hemophilia is predominantly related to musculoskeletal dysfunction consequent to damaged joints and soft tissue from repeated bleeding. The most critical aspect of the management of hemophilia therefore is the replacement of clotting factor concentrates (CFC) in ways that adequately prevent such bleeding and its complications [1]. In spite of the fact that CFCs have been available for over three decades, optimal ways to use them to achieve these ends have not been defined. While some models of replacement protocols that almost completely prevent bleeding have been described, the dosage used (5–9,000 IU/kg/yr) [2] and the associated costs are so high that they remain impractical in most parts of the world, even in many developed countries. More modest replacement protocols (2–4,000 IU/kg/yr) [3] with apparently similar long term musculoskeletal outcomes have also been described but systematic comparison between these two approaches particularly with regard to outcome over 3–4 decades remains to be done.

Current protocols for replacement therapy with CFC in hemophilia have several unresolved issues:

1. Is the aim preservation of normal joint architecture or maintenance normal overall musculoskeletal function? This is extremely important as the dosage that may be needed to achieve the former (if it is at all possible in the long term) may be much higher and often impractical than the latter approach.
2. Should treatment be started before or after the first (or the first two or three bleeds)? Should only joint bleeds be considered significant or subcutaneous bruises in little children also counted?
3. Do all patients with severe hemophilia need to be on regular prophylaxis? How should we identify 10–20% of patients with factor levels $<1\%$ who have clinically mild disease?
4. What should be the replacement protocol for prophylaxis? Is it necessary to maintain $>1\%$ levels at all times? Once initiated, till what age should prophylaxis be advised?
5. Should treatment be initiated with less frequent (even once a week) dosage and increased only for those who have breakthrough bleeds?
6. What should be the dose to treat breakthrough bleeds? Is there adequate data to support the dose of 25–40 IU/kg being commonly used at present? How many doses are needed? How many breakthrough bleeds are unlikely to change long term outcome and hence acceptable before intensification of prophylaxis regimen is needed?
7. What is the optimal dosage for hemostasis for surgery in hemophilia? There is a very large variation in the dosage used for similar procedures through out the world with apparently no significant difference in complications.
8. What are the optimal ways to induce tolerance in patients with different types of inhibitor profiles? Will the current studies be able to address the relevant issues?

Why is optimization necessary for replacement therapy with CFC in hemophilia? There could be several reasons. As for any pharmacologic therapy, the aim here too should be to use adequate and not excessive or inadequate dosage [4]. Venous access and the logistics of thrice a week therapy (as per current paradigms of intensive prophylaxis) in children below 2 years of age is not easy and results in significant inconvenience to the family and morbidity in the child [5]. From a pharmaco-economic point of view, the average cost of treating a person with hemophilia varies between \$50–150,000 per year [6]. This is not

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an insignificant amount anywhere in the world. While several economically sufficient countries are providing or attempting to provide care at this level (with a 2 to 3-fold difference in total CFC dosage/capita even among them), obtaining insurance for such costs has limitations. It is even more important therefore that such an approach be justified with suitable data. If not, not only is it inappropriate use of important resources but competing health care needs and demands may eventually impact on the sustainability of any program whose very basis is questionable. Some examples of this are already beginning to surface in different parts of the developed world.

The importance of these issues is even greater in economically less sufficient countries. Here, in the foreseeable future, the dosages described above are not likely to be used. It is therefore important that these countries generate data on long-term musculoskeletal outcome with different dosage in the range relevant to their practice [7]. This is completely lacking at present. It will be important to know whether there is a critical 'minimum-dose phenomenon' below which the extent of joint damage may be similar. It is possible that long-term outcome may only show significantly better results if dosages are above 500 or 1000 IU kg^{-1} per year and below that it may not matter whether it is 100 or 500 IU kg^{-1} per year even though the cost of providing CFC may be 5 to 10-fold different. Such information will be critical for health planners in deciding the minimum quantity of CFC to be provided if significant musculoskeletal morbidity is to be avoided.

It is therefore obvious that optimization of CFC replacement protocols is necessary all over the world. It is in the interest of patients, physicians and health care managers to ensure that large multi-center prospective studies are initiated to answer the relevant questions that will provide data for evidence based management of hemophilia.

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INFECTIONS IN HEMATOLOGICAL MALIGAIANCIES

Febrile neutropenia—Seven frequently asked questions and answers

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Introduction

Febrile neutropenia (FN) is one of the commonest complications of antineoplastic chemotherapy. New antimicrobials and new strategies created by the EORTC Antimicrobial and Invasive Fungal Infections cooperative groups, and MASCC (Multinational Association of Supportive Care in Cancer) [1–14] have been successful in decreasing the mortality from 15–20% in 1960–1980 to 2–5% in 2000–2004 [12–14]. Within last 5 years however many new strategies were introduced. Here are the most frequently asked questions/FAQs concerning FN:

1. Is there a change in etiology of infections in cancer patients?
2. Is there an increase of antimicrobial resistance among pathogen in FN?
3. Is prophylaxis with antimicrobials necessary?
4. Is combination therapy with aminoglycosides better than monotherapy?
5. Is addition of an glycopeptide in empiric therapy necessary?
6. Is oral therapy possible?
7. Do we need new antibiotics or new strategies?

Those are frequent questions asked by oncologists and hematologists to ID and microbiology staff, and always new questions will arise.

Is there a change in etiology?

In 1960–1980, first EORTC studies showed predominant gramnegative etiology [1–5]. In 1990–2000 grampositive cocci, mainly *streptococci* and *viridans streptococci* represented up to 70% of all bloodstream isolates, probably because of quinolones were introduced for prophylaxis supressing gramnegative bowel flora. Nowadays, again, gramnegative bacilli are emerging, mainly *Pseudomonas aeruginosa* and *Enterobacteriaceae*, represents about 50% of all documented infections [11–14]. Yeasts since 1980 and molds after 1990 are emerging as well, and represents more than 10% of all isolates, mainly in profoundly neutropenic patients after BMT and those with acute leukemia [15].

Is there an increase of resistance?

Is antimicrobial resistance in isolates from cancer patients a real problem or is just a propaganda of fear from the microbiologists? In 1990–2000, there was a significant increase of betalactam (methicillin, ampicillin and penicillin) resistance in *Streptococci* (MET, OXA), *viridans streptococci* (PEN) and *Enterococci* (AMP) probably due to empiric therapy with cephalosporins and penicillins (plus aminoglycosides) proven in first EORTC trials [1–10]. *E. scherichia coli* resistant to ciprofloxacin emerged after 1992 due to massive use of quinolones in prophylaxis [16]. Similarly, however in much less extend non albicans *Candida* spp. With decreased susceptibility to azoles emerged, possibly because large use of ketoconazol, fluconazol and itraconazol prophylaxis in leukemia and BMT [15]. All those resistance pathways were controlled due to increased use of glycopeptides (vancomycin, teicoplanin) and oxazolidinons (linezolid). Voriconazol decreased unacceptably high mortality in *C. glabrata*, *C. krusei* and *Aspergillus*/*Fusarium* spp. infections. However, because of much less innovation in new strategies (prevention other than chemoprophylaxis) and new antigramnegative compounds (meropenem was the last one, 18 years ago!). Multiresistant gramnegative bacilli such as meropenem resistant *Ps. aeruginosa* and *Acinetobacter baumanii*, quinolone resist. *E. coli* and ESBL positive (including cefepime resistant *Klebsiella*/*Enterobacter*,

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and voriconazol resistant *Zygomycetales* (*Mucorales*) emerged within the last 5 years. Last year isolates resistant even to colimycin (*Ps. aeruginosa*) emerged, and those organisms were resistant to all available antibiotics, so 2004/2005 are the first years of post-antibiotic era [17].

Is antibiotic prophylaxis necessary?

EORTC study (trial IX) documented borderline efficacy of quinolones plus V-Penicillin in prevention during afebrile neutropenia in 503 patients and since this time 1992 most centers uses oral quinolones in long lasting and profound neutropenia, in combination with fluconazol when Slavin et al. in 1995 reported decrease of mortality in BMT patients in ciprofloxacin plus fluconazol prophylaxis [18]. When Cruciani et al. [19] summarized in metaanalysis in 1996, 19 randomized studies with 2112 patients, and showed no benefit on mortality (despite decrease of grampositive bacteremia) a more conservative approach is advisable.

Akova et al. in 2005 from Turkey, on behalf on EORTC group [20] documented that superinfections and breakthrough bacteremias/fungaemias (occuring despite of quinolone or azole prophylaxis), are associated with higher mortality (5.5 vs. 2.6%, $P < 0.001$). In addition, resistance in *E. coli* to ciprofloxacin in same hematologic units increased from 1 to 15–20%. Therefore growth factors, isolation/cohortation, LAF and other non-antibiotic strategies are advisable. Antifungal chemoprophylaxis in centers without emergence of azole resistant *Candida* spp., *Aspergillus* and *Mucorales* with fluconazol or voriconazol may be still usefull in combination with other regimen-based strategies such as sterilisation, disinfection, isolation and microbiologic surveillance. However, new "anti-grampositive" quinolones may bring a new approach also into the field of "classical" chemoprophylaxis. Renter et al. after 10 years of Cruciani et al. meta-analysis showed significant benefit of levofloxacin in both prevention of infections and also reduction of mortality [21].

Is combination with aminoglycoside better than monotherapy?

Several meta-analyses published by at least 3 groups showed no benefit of adding an aminoglycoside to cephalosporin on piperacillin [22,23] within the last 3 years analysing 7807 neutropenic and 7586 other bacteremic patients showing no benefit on mortality, but higher toxicity related with combination therapy. The results of EORTC ATCG study from 1987 [5] favoring long course of amikacin in bacteremia and FN cannot be extrapolated to clinical practice anymore. However, this EORTC study [5] was done at a time with much less potents betalactams (no IV generation cephalosporins and carbapenems were available in 1987) and much less AGL resistance, than 18 years later.

In addition, another meta-analysis [23] showed no benefit on prevention or decrease of ATB resistance in those receiving combination therapy. Therefore, monotherapy with betalactam (IV gen. cephalosporins, carbapenem, piperacillin-tazobactam) is less toxic, equally effective and not related with high resistance and adding an aminoglycoside is not necessary in empiric therapy of febrile neutropenia and should be used only in cases of documented Ps. *aeruginosa* or other MRGN bacteremia upon susceptibility results.

Is addition of an glycopeptide in empiric therapy necessary?

Similarly to the previous question, the answer is no. Early EORTC study from 1991 [8] did not support the initial use of glycopeptide. Vardakas et al. [24] 15 years later summarized results of 2413 patients and showed that despite initial vancomycin, added to betalactam was related to better outcome (OR 1.63) in those with bacteremia and severe leukemia. Overall treatment success was similar [24]. Last published EORTC study in 2003 [14] favored to demonstrate that, addition of vancomycin is of benefit in febrile neutropenia [14]. Therefore, use of glycopeptides or oxazolidinons should be limited only to documented grampositive bacteremia due to MRSA or Amp/Gen–resistant *Enterococci*, PEN-R *viridans streptococci* and other multiresistant grampositive cocci.

Is oral therapy possible?

There were several attempts to simplify initial combination intravenous therapy with a betalactam plus aminoglycoside during last 30 years. In 1991, EORTC the first study trying to demonstrate equality of ciprofloxacin monotherapy (intravenous) failed, so 2 years later the scientific community was hapy to demonstrate, that once-a-day regimen (ceftriaxon plus amikacin) is equally effective than thrice (3x) daily. Second step was monotherapy study with carbapenem [12] and piperacillin-tazobactam [14] showing that monotherapy is equal to combination therapy. During those studies, stratification studies has been conducted both in US [25] and MASCC in Europe [26] trying to identify subpopulation of patients, who may not need intravenous antibiotics. Such a group of patients was previously defined as "low risk group" (without stomatitis, catheters, hypotension, old age, etc.) and "historical" first study [13] showed the possibility to treat such low risk group of febrile neutropenia patients with oral ciprofloxacin plus amoxycillin/clavulanate. Next step was undertaken within the last 3 years by the EORTC

study with a design of "oral monotherapy" study with moxifloxacin, however, the preliminary results will be available in 2006.

Explosion of new antibiotics and antifungals: Do we need new molecules or new strategies?

Last EORTC studies showed, that trials with "new molecules" are not always the solution of particular problems with febrile neutropenia, because they being only temporary solution (until resistance emerges).

If we look to the design of EORTC ATCG or IFICG (antimicrobial and invasive fungal infections therapy cooperative groups) from 14 studies, 6 are on new strategies (e.g., monotherapy versus combination, or oral versus intravenous, etc.). After 1990 an explosion of new antibacterial agents has been noted accompanied by advent of 6 new antifungals after 2002. Unfortunately, all antibiotics, developed after 1990 were developed only for management of grampositive multiresistant cocci – 4 new quinolones against PEN-R *pneumococci*, oxazolidinons against MRSE, daptomycin and tigecyclin against MRSA and VRE, quinupristin/dalfopristin against VRE and PRP. Six new antifungals (3 new azoles – Voriconazol against *Aspergillus*, *Fusarium* and Non albicans *Candida*, Ravuconazol and Posaconazol against *Mucorales* and *Aspergillus*, Caspofungin, Micafungin and Anidulafungin against *Aspergillus*) came to the drug market within the last 3 years. Both group of molecules were successful in decreasing the mortality of grampositive bacteremia, and after 17 years from the first EORTC "fungal" study in 1989 [6] also mortality on mold infections [27–30]. New antifungals, mainly lipid formulation of Amphotericin B and new azoles, are expensive, and therefore again search for preventive strategies and/or early diagnosis and dosing optimalization [28] are advisable. The problem however is that from 12 new group of "molecules", introduced after 1990, none is against gramnegative bacteria, and therefore we have currently nothing against meropenem and colimycin resistant *Pseudomonas aeruginosa* and *Acinetobacter baumanii*.

Conclusion

We have limited our mini review to 7 most frequently asked questions, what we receive from oncologists and hematologists. We did not have space to answer problems of antiviral therapy what requires separate review and only marginally discussed fungal infections. Despite limitations, some answers are better than none, because the slogan "no news – good news et al." used in diplomacy and politology is not valid in management of FN.

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INFECTIONS IN HEMATOLOGICAL MALIGNANCIES

Management of invasive fungal infections in neutropenic patients

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The risk of infection in hematological disorders is high and changes according to the underlying disease or the type of treatment [1]. Fungal infections is a challenging field for physicians dealing with hematological patients because the underlying disease itself causes a substantial increase in the risk of infections, and the presentation and the course of infections, can be puzzling. This brings out the importance of experience and evidence in this area. Classically, fungal infections can be defined as infections occuring in patients with prolonged neutropenia but special procedures such as stem cell transplantation (SCT) can change this. The changing pattern of transplant approaches such as nonmyeloablative (NMA) transplantation, new drugs, and new indications caused some major changes in the timing of fungal infections [2–4]. The risk of having a fungal infection is around 5–8% in acute leukemias and 7–20% in allogeneic stem cell transplant recipients. The risk in autologous transplants is similar to acute leukemia patients. Although the numbers represent a small proportion of infections in SCT patients, there are a lot of problems facing the physician; the mortality of fungal infections in SCT is very high (over 80%) in SCT patients, the fungal infection is sometimes very difficult to diagnose, very difficult and expensive to treat, and prolongs the hospital stay [5–7].

Changing pattern of invasive fungal infections

There are two major changes in the pattern of invasive fungal infections in cancer patients:

1. The incidence of invasive *Candida* (IC) infections is reduced in the last ten years after the utilization of azole prophylaxis. While the rate of IC in SCT was around 20% in allogeneic SCT patients not receiving prophylaxis, this rate remained below 5% after the introduction of azole prophylaxis. This also reflected itself in the mortality rates, before prophylaxis, the mortality of IC in SCT was 40–50%, which decreased to 20% after azole prophylaxis. Azole prophylaxis also changed the distribution of Candida species infecting the SCT patient. While *C. albicans* and *tropicalis* are decreasing, there is an increase in non-albicans *Candida* infections. *Aspergillus* is now the dominating fungal pathogen in SCT patient with a very high mortality rate. The use of new antifungals also brings out the possibility of breakthrough infections by pathogens resistant to these new agents. Recently breakthrough Zygomycosis after Voriconazole treatment were reported [8,9].
2. New transplant approaches caused a time-shift in the occurrence of fungal infections. The initial studies performed in NMA transplants showed that there was a decrease in the rate of early Aspergillus infections in NMA patients [3]. When these patients were followed for longer periods, it was observed that approaches such as NMA transplants and donor lymphocyte infusions delay the occurrence Graft versus Host disease (GVHD) and also delays the occurrence of Aspergillus infections. Although the cumulative rate of Aspergillus infections does not decrease, there is a slight decrease in Aspergillus related mortality [3,4]. Not only the type of transplant, but also the development of GVHD, corticosteroids, T-cell depleted grafts, advanced age and CMV infection are risk factors for Aspergillus infections. The increase of invasive fungal infections in SCT patients who had received the anti-tumor necrosis factor-alpha antibody-Infliximab demonstrates the importance of non-myeloid immunity in SCT patients. As the rate of Candida infections are low in SCT

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patients it is not easy to comment on the Candida infections in autologous patients [3,4].

Problems in the diagnosis of invasive fungal infections in SCT patients

Most of the SCT patients with invasive fungal infection present with fever and/or pneumonia and the differential diagnosis are not easy in this situation. Although EORTC/MSG proposed an approach based on the diagnosis of the fungal infection as possible, probable and definite, this is mainly useful in clinical trials and in evaluating the data [10]. Still, the diagnosis of fungal infection relies on non-invasive methods such as galactomannan and lately beta-glucan levels, imaging techniques such as high-resolution tomography and molecular studies [11,12].

The presence of relatively specific findings such as air-crescent sign and halo sign helps the diagnosis of fungal infection but in cases of angioinvasive aspergillosis, imaging techniques can be misguiding. The initial studies on galactomannan was found to be promising with high sensitivity and specificity rates, but the following studies showed that, factors such as the determination of the cut-off levels and consecutive sampling is important in the use of Galactomannan in invasive fungal infections [11]. It is early to comment on the role of Beta-glucan test in the diagnosis of invasive fungal infections, and this test is helpful in pan fungal diagnosis [12]. The help of molecular methods are limited at this point.

Treatment options, new and old drugs

Preemptive therapy

When treating a cancer patient with antifungal drugs, one of the most important problems is the number of patients receiving an antifungal treatment only because of prolonged fever and neutropenia; this empiricism originates from the studies done in the 1980s. The EORTC study done in 1989 showed that adding an antifungal to the empiric treatment of neutropenic patients with fever persisting more than 4 days had a positive effect on the number of fungal infections and a marginal benefit on the number of deaths attributed to fungal infections. Although this is an accepted practice, most of the time it is not easy to feel comfortable to use such expensive and toxic agents in a patient with good clinical status but prolonged fever. To start preemptive antifungal therapy when there is no clinical sign (except fever), we need a marker showing fungal infection in the body. Monitoring galactomannan levels can be a candidate to start preemptive treatment.

Treatment of fungal infections

Empirical treatment:

Conventional Ampho-B is being used as the initial empirical treatment of fungal infections in febrile neutropenic patients but commonly the adverse events related to conventional Ampho-B precludes the use of this drug and cause a shift to another antifungal drug. When conventional Ampho-B is compared with Liposomal Amphotericin-B, they have equal efficacy overall and liposomal form is superior in terms of breakthrough fungal infections and toxicity profile [13].

When liposomal Ampho-B was compared to lipid complex form at doses of 5 mg/kg; liposomal form was found to be superior in terms of overall success (42% *vs*. 33.3%) and drug-related fever, and discontinuation of the drug was higher in the lipid complex form [14]. In the last few years, new generation of antifungals caused a major change in the practice of empirical treatment of fungal infections. Voriconazole; a new azole is compared with liposomal Ampho-B in empirical treatment. They were found to be equally effective with fewer deaths in the Liposomal Ampho-B group, less breakthrough invasive fungal infections in Voriconazole group [15]. The main side effects of Voriconazole are visual disturbances and hallucinations; both reversible. Caspofungin, an echinocandin was also compared with liposomal ampho-B in the empirical setting. They were equally effective. Caspofungin group experienced more eradication of baseline fungal infections and less nephrotoxicity. In both trials overall success was between 30–50% [16].

Candida:

Treatment of *Candida* spp. can be separated in 3 related topics:

1. Prophylaxis
2. Treatment of azole sensitive Candida
3. Treatment of azole resistant and refractory Candida

Prophylaxis. Prophylaxis has shown to be effective against Candida infections in stem cell transplant patients by Fluconazole [17]. Fluconazole prophylaxis reduced Candida albicans infections but caused an increase in non-albicans *Candida* spp. and also azole-resistant Candida. Oral Itraconazole was also used for prophylaxis against both Candida and Aspergillus [18]. Although the results are encouraging, the erratic bioavailability and intolerance of patients to the oral forms is a major problem [18].

Treatment of azole sensitive Candida. Azole sensitive Candida left its place to Aspergillus after the intro-

duction of fluconazole prophylaxis [19]. Fluconazole, being safe, effective, orally available and cheap, is the main drug of choice. The main clinical presentation is acute disseminated candidiasis and esophageal candidiasis. The success of fluconazole in this group of patients is over 70%. When compared to fluconazole, Caspofungin, voriconazole, amphotericin-B, micafungin and anidulafungin both showed similar response rates, while Caspofungin is better against *C. glabrata* (95% *vs.* 67%) [20–24].

Treatment of azole resistant Candida. After fluconazole prophylaxis, azole resistant *Candida* spp. such as *C. kruzei* increased. New antifungals such as posaconazole, caspofungin, micafungin and anidulafungin are effective against azole resistant and refractory Candida [20–23].

Aspergillus

Aspergillus is the main pathogen responsible for fungal infections in cancer patients and it is the first cause of infectious death after stem cell transplant [24]. The incidence of invasive aspergillosis (IA) increased in the last 10 years and the incidence is 3–8% in acute leukemia, 1% in autologous transplants and 5–25% in allogeneic transplants. The number of unrelated transplants and older patients treated are increasing and an increase in the number of Aspergillus infections seems to be unavoidable.

The mortality rates are unacceptably high, especially in stem cell patients and probability of survival remained below 25% in patients with probable and proven Aspergillosis. The diagnostic approaches are limited and there is no golden standard except showing the mould in cultures, which is nearly impossible in most of the cases [2,5]. Ampho-B is effective against Aspergillus and most of the studies performed with liposomal ampho-B showed a response rate between 55–70% in filamentous infections. Although fluconazole has no effect on Aspergillus, Itraconazole and Voriconazole are highly effective. The use of Itraconazole is limited due to bioavailability and intolerance but voriconazole is a good alternative. The comparison of Voriconazole and liposomal ampho-B in invasive aspergillosis demonstrated a good efficacy of Voriconazole (53% vs. 32%) with few side effects [25]. Caspofungin received approval from the FDA after the study performed in refractory invasive aspergillosis [26]. In this study patients with documented Aspergillosis, not responding to any other antifungal received Caspofungin and 50% responded (26/52).

Combination therapy

One of the best advantages of the new antifungals is better toleration and fewer side effects. This has prompted the use of a combination of antifungal drugs, especially in refractory patients. In a retrospective analysis, the addition of caspofungin to amphotericin resulted in improvement in 42% of 48 patients who had progressing proven or possible aspergillosis despite liposomal amphotericin monotherapy. The results of the preclinical data does not necessarily convert to clinical efficacy. Although there is no data that strongly supports the use of combinations in antifungal treatment, there are many limited reports that supports the use of combinations as salvage treatment. There is still a need for a large, multicentric study that will assess the role of combinations in fungal infections.

Conclusion

There has been a remarkable progress in developing new drugs in the treatment of fungal infections. The demonstration of efficacy of posaconazole, anidulafungin and miconazole in the treatment of common and rare fungal pathogens shows that there is still more to say in this area. The main problems are the lack of definite markers to diagnose and to monitor fungal infections, the high cost of antifungal treatment, the toxicity of the antifungal agents, the future of combination therapies and the high rate of mortality especially in refractory patients. New diagnostic tests, more data coming from new trails and old studies will help us to achieve better results, and save more patients.

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Late effects of hematopoietic stem cell transplantation

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Introduction

Hematopoietic stem cell (HSC) transplantation has evolved into a modern therapeutic modality for variety of life-threatenning hematologic, neoplastic and immunologic diseases. Around the world thousands of patients are currently alive more than 5 years following stem cell transplantation and increasing data have been collected for the long term complications [1,2].

Long term complications of hematopoietic stem cell therapy are categorized as regimen related toxicity, immunodeficiency, infection, chronic graft-versus host disease (GVHD), bone disease, relapse of malignancy and secondary malignancies (Table I).

Regimen-related toxicities

Late complications after allogeneic and autologous HSC transplantation may arise from chemotherapy and/or radiotherapy associated organ toxicity [3]. These potential late effects of high-dose conditioning regimens include cataracts, neurological problems, gonadal conditions, endocrine conditions, growth and development problems (Table I). These regimen-related complications are briefly reviewed below.

Cataracts

Cataract formation is a well-known consequence of corticosteroid use and total body irradiation (TBI). In the analysis of 492 adults followed for a median of 6 years after bone marrow transplantation, cataracts developed in 159 patients (32%). [4]. The probability of cataract formation at 11 years after transplantation was 85% for patients receiving 10 GY single dose TBI, 50% for the patients receiving more than 12 Gy and was 19% for the patients who were not conditioned with TBI [4]. In the cohort developing cataracts, the severity was greater in patients given single-dose TBI than those given 12GY (33%) or no TBI (23%) [4].

Steroids administered given after Day 100 also appeared to increase the risk of cataracts seen after TBI. Patients who were treated with steroids post-transplantation had a significantly higher probability of cataracts (45%) than those who did not receive steroids (38%). In these studies cataract formation appeared to reach a plateau phase at 7 years after transplantation with a median time of 2 to 5 years [4].

Neurological complications

Late neurological complications may result from previous cranial radiation, recurrence of the primary disease, intrathecal therapy and other drug toxicities. Leukoencephalopathy may develop in patients who receive intrathecal methotrexate and cranial irradiation and risk may increase in young patients who receive more than six doses of I.T.methotrexate after the transplant [5]. Late neurologic abnormalities may also be seen in patients receiving nitrogen mustard as part of the preparative conditioning regimen [6,7].

Endocrine and growth abnormalities

Myeloablative conditioning regimens may also affect the endocrine system, growth and development. In adult bone marrow transplant patients two-thirds of the recipients may have elevated TSH with normal blood T4 and T3 levels [6]. In the same study it was also shown that 20 to 25% of the patients will develop definite hypothroidism [6]. Thyroid deficiency was reported in 31 to 43% of patients after single fraction of TBI [7]. In children who were given conditioning regimens with fractionated irradiation developed less frequent hypothyroidism [7]. Thyroid hormone replacement should be administered if the diagnosis of hypothyroidism has been confirmed.

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Table I. Late complications of stem cell therapy

Regimen-related toxicity
Cataracts
Neurological conditions
Gonadal conditions
Endocrine conditions
Growth and development
Immunodeficiency
Infection
Chronic graft-versus-host disease
Bone disease
Relapse of malignancy

Graves' disease has not been commonly seen in the bone marrow transplant recipients. In view of these clinical studies mentioned, thyroid function studies must be done annually in all bone marrow transplant recipients.

Growth hormone deficiency has also been reported following TBI [8]. It may be higher than 90% in the pediatric patients conditioned with TBI and also had a history of cranial irradiation for CNS leukemia [8]. In some children who were treated with corticosteroids for chronic graft-versus-host disease (cGVHD) growth rate may be lower compared to the period during which steroids are discontinued. Finally, children who are prepared with busulfan may also develop growth hormone deficiency and may require growth hormone replacement therapy [8].

Some children who received pre-transplant cranial irradiation may exhibit learning disabilities within the period of 24 to 42 months post-transplant. In addition, some patients may develop variety of coping mechanisms to deal with intensive medical care and life-threatening illnesses. Concurrent drug therapies such as prednisone may cause emotional problems, rarely psychosis and cyclosporine may cause tremors, seizures, muscle cramps and lethargy.

Gonadal dysfunction

Gonadal function abnormalities are quite frequently observed as a result of myeloablative chemotherapy and radiotherapy. Gonadal dysfunction has been closely associated with alkylating agents, may be related to patient's age and intensity of the chemotherapy regimen. Female patients may have anovulation, low estrogen levels and elevation of serum gonadotropins [9]. Patients who were conditioned with TBI containing preparative regimens rarely have return of their fertility [10].

Children who are eight years and older should be examined annually and assessed by Tanner Development Scores for grading secondary sexual development [11].

Hormone replacement with cyclic estrogen/progesterone therapy to prevent osteoporosis and its complications of early menopause. Most women prefer Premarin 0.625 mg to 1.25 mg per day (P.O) and Provera 2.5 to 5.0 mg days 1–14 (P.O.). Both of these medications are well-tolerated. Provera should not be given to women who had hysterectomy.

Gynecological and obstetric conditions

Post-pubertal female patients who received TBI containing regimes have been shown to have climacteric abnormalities [12]. Appropriate and early hormone replacement with estrogen and progestrone may reduce the risk of osteoporosis and eliminate the unnecessary discomfort. Although pre-term delivery and low-birth weight children were reported to be increased than expected in the bone marrow recipients, the incidence of congenital abnormalities did not appear to be different than the rates observed for the population [12].

Immunodeficiency

Both allogeneic and autologous hematopoietic stem cell recipients experience impaired immunological changes for 6 to 12 months post-transplant [13]. HLA-disparity of the allogeneic donor and presence of chronic GVHD will also play a very significant role in the evolution of both cellular and humoral immunodeficiency [14]. Low levels of $CD4^+$ T-helper lymphocytes may stay low within the first six months after transplantation. Normal numbers of peripheral blood B-cells can be quantified one to two months after bone marrow transplantation. In general, the patients are expected to reach normal levels of serum IgG within 2–3 months, serum IgM in 9–12 months and IgA levels in 2 to 3 years. If the patients develop chronic GVHD the normalization of the serum immunoglobulin levels will be significantly prolonged [15]. During the first three months after transplant the recipients with hypogammaglobulinemia (serum IgG levels less than 400 $mgdl^{-1}$) should receive I.V. immunoglobulin therapy which ultimately will reduce the risk of infection.

Infection

All marrow transplant recipients undergo a time of immune deficiency which is most severe in the first 6–12 months post-transplant. It is during this time that most bacterial fungal and viral infections occur. After 12 months post-transplant most patients will achieve adequate immune reconstitution. However, the tempo of immune recovery is delayed in patients who develop chronic GVHD.

Fever of unknown origin

Fever in a marrow transplant recipient should be considered a sign of infection until proven otherwise. Prompt and thorough evaluation of fever in an

immunocompromised host should include blood cultures (and other sites of culture as indicated), chest radiograph and serial physical examination. If sudden, overwhelming sepsis syndrome (with pneumococci or other encapsulated organisms) has been observed, empiric antibiotics may be indicated after obtaining cultures. Empirical antibiotic coverage for a broad-spectrum of bacteria to include gram-negative and encapsulated organisms. Organisms should be tested for antibiotic sensitivities. Initial therapy of the febrile neutropenic patient should be directed by clinical findings and concurrent antimicrobial therapy. A combination of third generation cephalosporin with an aminoglycoside is commonly utilized for the initial treatment but physicians should make their choices depending upon the microbial sensitivities of their individual institutions.

If fungal infection is presumed, consideration should be given to CT of the sinuses and lungs.Especially in patients with cGVHD, empirical addition of Pneumocytiis Carinii specific treatment (i.e., trimethoprim-sulfamethaxazole or pentamidine isethionate) is advised if the clinical presentationis consistent with this diagnosis and prophylactic treatment is not being administered.

Empirical antibiotic therapy regimen should be modified or discontinued depending on the culture and diagnostic procedure results.

Pneumonia

Approximately half of penumonias after BMT are due to infection. The true incidence of bacterial bronchopneumonia is unknown but estimates range from 2% (documented by open lung biopsy) to over 25% (from clinical response). Aspergillus infection most often involves lung and is seen in approximately 10%.

Cytomegalovirus pneumonia

Late cytomegalovirus (CMV) pneumonias occur in around 10% of transplant patients who had positive CMV-serologies before transplant or whose donor was CMV-seropositive. This incidence is highest in patients with chronic GVHD. Pneumonia usually occurs concurrent with evidence of CMV reactivation in blood, documented either through antigenemia or PCR. Weekly blood monitoring for re-activation is very helpful in diagnosing end-organ CMV disease. Importantly, if pneumonia develops, the risk of mortality increases to 50%. CMV pneumonia is diagnosed by lung biopsy. Cytological, immunofluorescent monoclonal antibody or viral culture evidence of CMV infection with clinical evidence of pneumonia is considered CMV pneumonia. The preferred treatment is intravenous ganciclovir. An immunoglobulin regimen is combined with the antiviral ganciclovir.

Pneumocytiis carinii pneumonia (PCP)

It occurs in 7% of recipients not receiving adequate prophylaxis. All patients are to receive Pneumocytiis Carinii pneumonia (PCP) prophylaxis through day 120 post transplant, or longer if they continue on prednisone treatment. PCP prophylaxis should continue beyond 6 months if patients is still on immunosuppressives, during treatment for chronic GVHD and for 6 months after discontinuation of treatment. Systemic intravenous therapy should be initiated with either trimethoprim-sulfamethoxazole or pentamidine isethionate. Trimethoprim-sulfamethaxazole should be given 15–20 mg kg^{-1} per day of the trimethoprim component, in divided doses every 6–8 hours, for 14–21 days.

Varicella zoster

Varicella zoster virus (VZV) infection develops in 40–50% of marrow transplant recipients within the first year after transplant (peak risk 2–8 months). Importantly, around 10% of patients present with back or abdominal pain before the skin lesions, and 30–50% of patients develop disseminated infection. Patients with suspected prodromal Zoster or documented Zoster during the first year after transplant should start I.V. acyclovir (ACV) therapy immediately and have whole blood sent for VZV PCR (cutaneous lesions may not even occur). ACV is given at 10 mg kg^{-1} dose as a 1 hour infusion every 8 hours $\times$ 7 days or until the lesions crust over. Dose adjustment of ACV is needed, if renal function is impaired.

Reactivation of VZV is being seen with increasing frequency after BMT, especially among those with unrelated donors and prolonged immunosuppression. All BMT recipients should avoid exposure to persons with chickenpox or shingles. Recurrent VZV may be seen in 10–20% of ACV-treated patients, and these patients should be retreated.

Transplant patients who have been exposed to active chickenpox infections or other patients with zoster need to be observed for the development of lesions if they were VZV-seropositive pretransplant or if they have already had an episode of zoster after transplant. For VZV-seronegative exposed patients, VZV immune globulin (VZIG) prophylaxis should be administered within 96 hours of the exposure.

When patients return to home community, recurrent exposures to varicells are expected. In selected individual patient situations, multiple or high risk exposures may occur for patients who are not likely to be immediate candidates for the vaccine. To prevent infection from occurring in these patients, two approaches can be utilized. One is the use of oral prophylactic acyclovir or valaciclovir until eligible for the vaccine, unless patient is receiving other medications with activity against the herpesviruses

(e.g., ganciclovir, foscarnet, or cidofovir). The other approach is to administer monthly injections of VZIG.

Infection prevention

Patients should avoid frequent contact with anyone with viral infections or other communicable disease. Handwashing is crucial in the prevention of communicable diseases. Patients who are receiving treatment for chronic GVHD should continue antibiotic prophylaxis for 6 months after discontinuing all immunosuppression.

Antibiotic prophylaxis

All patients should receive PCP prophylaxis throughout their immunosuppressive regimen. Those patients who are on corticosteroids should remain on PCP prophylaxis until steroids discontinued. Patients on treatment regimen for chronic GVHD should follow recommendations for antibiotic coverage. Patients will receive prophylaxis with daily penicillin and once-a day double-strength Trimethoprim-sulfamethoxazole.

Patients not able to receive trimethoprim sulfamethoxazole should receive PCP prophylaxis with dapsone (50–100 mg, 3–7 times a week, P.O.) or intravenous Pentamidine (4 mg kg^{-1} up to 300 mg per dose) every 2 weeks *not to exceed total dose of 3 grams*.

Post-transplant vaccinations of seronegative transplant recipients should be done after 2 years post-transplant or 12 months after discontinuation of all immunosuppression, whichever occurs later. VZV vaccine should be given concurrently with MMR vaccine or at least 4 weeks apart from the MMR vaccination.

Immunizations

During the first year, patients are generally unable to develop antibody responses to such immunizations as pneumococcal polysaccharide antigen (Pneumovax) or other inactivated vaccines. Beyond 1 year post-transplant, patients free of chronic GVHD develop specific IgG antibody titers to recall antigens such as tetanus toxoid, and measles virus, but titers will drop over time without re-immunization. Most recipients with chronic GVHD fail to develop titers to these antigens at all. Although patients with chronic GVHD may have an inadequate immune response, we recommend a complete vaccination series because this cohort is at highest risk for development of some of the preventable diseases.

We recommend vaccination after the first year post-transplant for optimal antibody response. Booster immunization should include; influenza (yearly), pneumococcal, Haemophilus influenza, hepatitis B, diphteria, pertussis (only for patients <7 years old), tetanus and inactivated polio. Whether patients with chronic GVHD will develop an antibody response is not known in all cases. Therefore, antibody titers can be helpful if drawn before and 4 weeks following vaccinations to evaluate antibody response. If antibody response is low or unknown, repeat vaccinations up to 2 times in 2 month intervals, except for influenza and pneumococcus.

Should the oral polio vaccine (OPV) be given to family infants or others in close contact with the patient within the first year after transplantation, the patient should be isolated from the person vaccinated for 8 to 12 weeks which is the period of potential live virus shedding.

Chronic graft-versus-host disease

Incidence

Chronic GVHD is a clinicopathologic syndrome which is the major determinant of long-term outcome (mortality) and quality of life (morbidity) after allogeneic bone marrow transplantation. Chronic GVHD may develop within 3 to 18 months after allografting and occurs in approximately 33% of HLA-identical sibling recipients and 50 to 70% of recipients of unrelated or mismatched-related marrow grafts (Sullivan et al.1991). Increasing patient age and degree of prior acute GVHD are known risk factors for developing chronic GVHD, 20–30% have de-novo late onset without preceding acute GVHD. In addition, allogeneic peripheral blood stem cell recipients appear to have a higher incidence of chronic GVHD than bone morrow recipients (Storek et al.1997).

Chronic GVHD may manifest in two ways: "Limited disease" is defined as the presence of signs and symptoms of GVHD limited to skin and/or liver involvement. "Extensive disease" is defined as the presence of signs and symptoms consistent with GVHD involving multiple organ system with at least one biopsy showing characteristic pathological GVHD findings. Individuals with the extensive chronic GVHD have an unfavorable natural history (18% disability -free survival without immuno-suppressive treatment (Sullivan et al. 1981; Sullivan et al. 1981; Sullivan 1994).

There are three typical patterns of onset of chronic GVHD. Progressive chronic GVHD is defined as direct continuation of signs and symptoms of acute GVHD and it is associated with the highest mortality rate. Quiescent onset of chronic GVHD is observed after the complete resolution of prior acute GVHD. De novo onset of chronic GVHD is defined as onset of the disease without any prior history of acute GVHD. De-novo onset of chronic GVHD has the best prognosis (Sullivan et al. 1981; Wingard et al. 1989; Atkinson 1990).

Chronic GVHD is a pleiotropic disease with clinical and pathological signs and symptoms similar to several naturally occurring autoimmune disorders. Organ involvement in extensive and chronic GVHD includes the skin, mouth, eyes, sinuses, gastrointestinal tract, lungs, muscles, tendons, serous surfaces and vagina (Sullivan et al. 1981; Sullivan 1994).

Clinical manifestations

Dermal. Dermal involvement is the most frequent clinical feature of chronic GVHD. Erythema, dyspigmentation, poikiloderma, and violaceous papules resembling lichen planus may be observed. Lichenoid lesions can be generalized and coalesce to form plaques. If no therapeutic intervention is given, the skin becomes progressively indurated and sclerotic, leading to joint contractures and profound disability. The sclerosis can be associated with skin ulcers, alopecia and anhidrosis. Progressive hair loss with scarring alopecia can be observed in patients with chronic GVHD.

Oral. Oral lesions include erythema, atrophy and lichen planus-like findings. Severe mucous membrane involvement with chronic GVHD may lead to a Sjogren's syndrome -like disease findings with xerostomia and xerophtalmia. Presence of "oral sicca" syndrome in these patients may lead to poor oral hygiene and dental caries.

Ocular. Abnormalities include kerato-conjuctivitis sicca, conjuctivitis and uveitis. Schirmer's testing of lacrimal gland function may show wetting $<$5mm at 5 minutes or $<$10 mm with signs of keratitis diagnosed with slit-light examination. Other symptoms will include blurring, dryness, "gritty eyes" and/or photophobia. Artificial tear replacements may be required to prevent corneal abrasion.

Pulmonary. Chronic GVHD may be associated with recurrent sinopulmonary infection and progressive obstructive lung defects. Clinical and pathologic features are characterized by the presence of bronchiolitis obliterans (Clark et al. 1987; Clark,Crawford, Madtes, and Sullivan 1989). Progressive bronchiolitis obliterans affects 5–10% of all patients with active chronic GVHD.

Hepatic. Liver function abnormalities are common and are predominantly cholestatic in nature but hepato-cellular dysfunction may make it difficult to distinguish chronic GVHD from viral or drug-induced hepatitis. Ursodeoxycholic acid may be of benefit as bile displacement therapy. (Fried et al. 1992).

Musculo-Skeletal. Arthralgias, synovial effusions, arthritis, tendonitis and fascütis have been associated with chronic GVHD (Janin et al. 1994). Proximal muscle weakness with increased CPK, aldolase and EMG findings are consistent with myositis. Muscle cramping can also occur. Muscle biopsy may be required to confirm the diagnosis if the muscle is the only organ involved.

Gastro-Intestinal. Chronic GVHD rarely involves the intestine. Weight loss often is related to loss of appetite and increased metabolic needs. Esophageal complications may include desquamative esophagitis causing web formation and gastro-esophageal reflux. Classic findings of malabsorption may be noted due to bacterial overgrowth in the gut, pancreatic or hepatic disease.

Other sites. Vaginal stenosis, dryness and inflammation can all be seen during the course of chronic GVHD. Peripheral neuropathy and myaesthenia gravis are less common manifestations.

Infections. Due to prolonged time to immunologic recovery, infections may be common in patients with chronic GVHD. Chronic GVHD, its treatment with corticosteroids and associated hypogammaglobulinemia contribute to this risk. Infections with encapsulated Gram-positive bacteria are most common and require daily penicillin or trimethoprim- sulfamethaxazole prophylaxis (Sullivan et al. 1986).

Outcome. In an analysis of 164 consecutive patients with extensive chronic GVHD, older patient age, progressive onset of GVHD, failure to respond to 9 month therapy and continued thrombocytopenia (platelets $<$less than 100.000 μl^{-1}), hyperbilirubinemia and lichenoid histology have been reported to be associated with an inreased non-relapse mortality and poor prognosis. Among unrelated donor marrow transplants a prolonged course of interferon-alpha given before transplant in patients with chronic myeloid leukemia (CML) resulted in poorer survival due to chronic GVHD which was refractory to immunosuppressive treatment (Morton et al. 1997a; Morton et al. 1997b).

Treatment

As noted above, without treatment only 18% of patients with extensive chronic GVHD survived free of major disability. In standard- risk patients (i.e., those with a platelet count greater than 100.000 μl^{-1},de novo or quiescent type of onset) early treatment with prednisone alone significantly

improved outcome (21% mortality) compared to prednisone and azathioprine (40% mortality) (Sullivan et al. 1988). In high-risk patients (i.e., those with platelet counts less than $<100.000\ \mu l^{-1}$ or progressive type onset) survival after prednisone treatment was only 10 to 26%. Subsequently, the addition of cyclosporine to an alternating-day regimen of prednisone has improved the survival to 52% in high-risk patients (Sullivan et al. 1988). However, transplant-related mortality still continues to be higher (35%) in high-risk patients than in standard-risk patients (20%) due to increased rates of infection.

New treatment approaches include the use of FK506, thalidomide, mycophenolate mofetil and rapamycin (Vogelsang et al. 1992; Nash et al. 1997). Supportive care includes correction of hypogammaglobulinemia and administration of trimethoprim-sulfamethoxazole to reduce the risk of infection include the use of (Sullivan et al. 1996).

Bone disease

Bone disease is a well-known complication of solid organ transplantation, however the development of avascular necrosis, osteoporosis and fractures following stem cell transplantation is less well characterized (Kelly et al. 1990; Socie et al. 1994). Recently, adult patients treated with prednisone and cyclosporine for chronic GVHD and who were evaluated for biochemical factors associated with skeletal turnover at initiation of immunosuppressive therapy and 9 months later (Stern et al. 1996). Single and dual photon absorptiometry of the wrist and spine and dual energy x-ray absorptiometry (DEXA) were used to evaluate bone mineral density. Results showed a significant bone mineral density (greater than 2.5 times the test precision) decrease over 9 months in bone mineral density in three of five evaluable males and all three females who were receiving prednisone and cyclosporine treatment. The results of the study indicated increased collagen and bone turnover, increased urinary magnesium and calcium excretion, and a significant risk of osteoporosis in patients receiving corticosteroids for chronic GVHD.

Secondary malignancy

Secondary neoplasms may arise in the oncogenic milieu of genetically determined factors, infection, immunodeficiency and cytotoxic conditioning regimens, including TBI (Witherspoon et al. 1989; Witherspoon et al. 1992). The Seattle team reported the cumulative incidence of secondary cancers in 330 patients with aplastic anemia who received cyclophosphamide alone as pre-transplant conditioning (Witherspoon et al. 1992).The cumulative incidence at 5 years was 0.4% (95% confidence interval 0 to 1.1), at 10 years was 1.4% (0 to 3.4), and at 15 years was 4.2% (0.9 to 8.6). The rate was less than reported by the Paris team in patients with aplastic anemia given cyclophosphamide and thoraco-abdominal irradiation as conditioning (N = 147).

European studies confirm that pre-transplantation irradiation is a major determinant of late malignancies in patients with aplastic anemia (Socie et al. 1993). To further define these interactions, 700 patients with severe aplastic anemia treated with allogeneic marrow transplantation in Seattle or in Paris were reviewed (Deeg et al. 1996). A malignancy developed in 23 patients 1.4 to 221 (median 91) months after transplantation, for a Kaplan-Meir estimate of 14% (confidence interval 4 to 24%) at 20 years. Proportional hazards models indicated that azathioprine therapy ($P < 0.0001$) and the diagnosis of Fanconi's anemia ($P < 0.0001$) were significant factors for development of secondary malignancies for all patients. Irradiation was a significant factor ($P = 0.004$) only if the time - dependent variable azathioprine was not included in the analysis. If only non- Fanconi patients were considered, azathioprine ($P = 0.0043$), age ($P = 0.025$), and irradiation ($P = 0.042$) were independent risk factors for development of late secondary neoplasms.

In a recent report, a multi-institutional data base including 19.229 recipients of allogeneic marrow transplants was analyzed to determine the risk of developing late solid cancers (Curtis et al. 1997). The risk of new solid cancers was 8.3 times higher than expected for the general population among those who survived 10 or more years after transplantation. The cumulative incidence rate of solid cancers was 2.2% (95 percent confidence interval, 1.5% to 3.0%) at 10 years and 6.7% (3.7 to 9.6%) at 15 years. In this study, the risk of developing a new solid cancer was also found to be higher for recipients who were younger at the time of transplantation. Radiogenic tumors (especially of brain and thyroid) were noted in children, most of whom had cranial irradiation given before referral for transplantation.

Recurrent malignancy

Prior experience has indicated poor survival after recurrence of the original malignancy after marrow cell transplantation (Mortimer et al. 1989). In some patients recurrent leukemia has been successfully treated with second transplantations, but resistant disease and regimen -related toxicities contribute to high mortality (Radich et al. 1993). In recent years, the development of highly sensitive molecular biology techniques has helped detect minimal residual disease. For patients with CML, long-term monitoring includes cytogenetics for the Philadelphia chromosome and bone marrow and peripheral blood molecular determinations for the BCR/ABL transcripts every 6 months after transplantation through Year 3,

then annual evaluations through Year 5. Positive BCR/ABL studies six months or more after transplantation appears to predict risk of subsequent hematologic relapse (Radich et al. 1995). Patients with residual disease could be eligible for treatment with alpha-interferon during early molecular or cytogenetic relapse (Higano et al. 1992). Recurrent leukemia following stem cell transplantation may also be successfully treated with donor leukocyte infusions. This beneficial effect derives from an apparent graft-versus-leukemia (GVL) effect associated with allogeneic stem cells that recognize and destroy host histocompatibility antigens and/or tumor-associated antigens (Weiden et al. 1981; Sullivan et al. 1989). Donor leukocyte infusions have been used successfully to treat patients with recurrent leukemia and Epstein-barr virus- associated lymphoproliferative disorders (Kolb 1990; Papadopoulos et al. 1994).

Quality of life

Recovery from transplantation is a dynamic process blending physical and psychosocial aspects. Quality of life is a multi-dimensional construct composed of at least four domains: physical function, psychological function, social role function and disease and treatment symptoms. Recent studies examining the medical and psychosocial sequelae of stem cell or marrow transplantation have reported that most survivors do relatively well, while a smaller group continues to experience less than optimal quality of life (Wingard, Curbow, Baker and Piantadosi 1991; Chao et al. 1992; Schmidt et al. 1993). We conducted a prospective analysis of 67 adults with "Quality of Life" measures taken before and after allogeneic transplantation (Syrjala et al. 1993). Physical function was most impaired at 90 days post-transplant, with a return to pre-transplant levels of functioning in most areas by one year. By two years, 68% of patients had returned to full-time work and only 9% of 4-year survivors failed to return to full-time occupations. Before transplantation 27% of patients reported elevated anxiety. Mean levels of anxiety and depression did not change over the first year. In a multivariate analysis greater emotional distress at 1 year was predicted by pre- transplantation family conflict and non-married status. Impaired physical recovery at 1 year was predicted by more severe chronic GVHD, pre-transplant physical impairment and family conflict. Family relationships therefore appear to be important determinants of recovery.

A recent study used a cross-sectional analysis of 125 adults surviving a mean of 10 (range 6 to 18) years after allogeneic (87%) or autologous /syngeneic (13%) transplantation (Bush, Haberman,Donaldson, and Sullivan 1995). Seven wide-ranging tests measured physical, psychological, social functioning,and disease and treatment symptoms. Eighty percent of individuals rated their quality of life as good to excellent, and 5% rated it as poor. The most frequently cited problem during recovery was a perceived lack of social support from family and friends. Although complaints such as fatigue, sexual dysfunction and sleep disturbances were noted, most survivors judged these to be of low severity and 88% of the 125 patients said the benefits of transplantation outweighed the side-effects.

Summary

Bone marrow and peripheral blood stem cell transplantation is now considered the treatment of choice for a variety of non-malignant and malignant disorders. In some patients, the impact of late complications determines the success of the procedure and efforts directed at preventing rare events are vital. Knowledge of late complications and follow-up care by the specialist and general practioner will enhance the outcome of recipients of allogeneic and autologous hematopoietic cell transplantation.

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LATE EFFECTS OF HEMATOPETIC STEM CELL TRANSPLANTATION

Impact of chronic GVHD on late complications after hematopoietic cell transplantation

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Current results with transplantation of marrow or blood derived hemopoietic stem cells (HCT) in patients with aplastic anemia and patients who do not develop chronic graft-versus-host disease (GVHD) show life expectancies similar to age-matched controls. However, patients with advanced malignant diseases and patients who develop chronic GVHD after transplant are at risk of late disease recurrence and delayed, potentially fatal complications [1]. Major complications associated with chronic GVHD are listed in Table I.

Infections

Late infections due to bacterial, viral and fungal organisms occur most commonly in patients with chronic GVHD. Early post-transplant prophylaxis may result in an increased incidence of late infections (see e.g., acyclovir/ganciclovir and late CMV infections). It is standard practice to give prophylaxis for infections caused by Pneumocystis carinii, varicella zoster and encapsulated bacteria (and, more recently, fungal organisms) during the first year post-transplant, or longer, for patients with chronic GVHD.

Airway and pulmonary disease

The bronchial tree may be involved by GVHD [2], and immunosuppression related to GVHD or its therapy may enhance pulmonary infection. Late onset interstitial pneumonia usually occurs in patients with chronic GVHD [3]. Restrictive pulmonary changes do not appear to correlate with chronic GVHD.

The pathogenesis of air flow obstruction (AFO) after HCT is not fully understood [4], but recurrent aspirations, possibly associated with GVHD of the esophagus or purulent sinus drainage, contribute to airway inflammation and the development of obstructive lung disease. A recent study analyzed AFO in 1049 patients who received an allogeneic HCT at FHCRC [5].here were 257 patients (25%) with significant AFO as defined by a decline in pFEV1 by more than 5% per year. In multivariate logistic regression analysis, patients with quiescent (relative risk [RR] 1.5, 95% CI 1.2–1.7) or progressive onset (RR 2.5, 95% CI 1.4–3.1) of chronic GVHD, among other factors, were at an increased risk, and those with chronic GVHD and AFO had a higher risk of mortality (hazard ratio 1.9, $P=0.002$) than patients without AFO. Thus, AFO had a significant independent effect on long-term survival.

Progressive bronchiolitis obliterans has been reported to occur in 10% of all patients with chronic GVHD [6] from 3 months to 2 years after HCT. Clinical and pathological findings are similar to those seen after lung or heart-lung transplants [6]. Histological changes are thought to be due to a graft-versus-host reaction, possibly aggravated by infections. Pulmonary infections develop in more than 60% of allogeneic HCT recipients with GVHD compared to about 20% of patients without chronic GVHD.

A recent analysis of results in 6523 patients transplanted at FHCRC revealed 51 cases of bronchiolitis obliterans organizing pneumonia (BOOP), all but two after allogeneic transplants. BOOP was diagnosed at 5–2,819 (median 108) days after HCT. The disease was significantly associated with acute and chronic GVHD. The disease progressed in 22% of patients and resolved or was stable in the remaining patients [7].

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Table I. Chronic GVHD and delayed complications

Immunodeficiency and infections
Airway and pulmonary disease
Autoimmune disorders
Neuroendocrine dysfunction
Impairment of growth and development
Infertility
Cardiac disease
Ocular problems
Musculo-skeletal disease
Dental problems
Dysfunction of the genitourinary tract
Gastrointestinal and hepatic complications
Post-transplant malignancies
Central and peripheral nervous system impairment
Psychosocial effects

Dysregulation of immunity

All currently used conditioning regimens are associated with immunosuppression of the transplant recipient. The presence of thymic damage, due to transplant conditioning and GVHD, interferes with the negative selection of autoreactive cells and thereby facilitates the development of cellular and, via $CD4^+$ cells, humoral autoreactivity [8]. However, while autoantibodies occur frequently, particularly in patients with chronic GVHD, a correlation with GVHD activity is uncertain.

Hematologic problems

GVHD can also affect the marrow, and persistent thrombocytopenia is a poor prognostic factor in chronic GVHD [9]. Immunosuppressive treatment of GVHD is often successful [10].

Endocrine dysfunction

Adrenal glands

Many HCT patients receive glucocorticoid therapy in the pre-, peri- or post-transplant period and show the classic side effects of steroid therapy, including cushingoid features, myopathy, and bone loss. Endogenous cortisol production is suppressed, and any superimposed stress may cause a relative adrenal insufficiency. For the same reason, prolonged glucocorticoid therapy for GVHD should be given on alternate days and must be tapered very gradually.

Gonadal function, puberty and fertility

Impairment of gonadal function, fertility, and growth and development are primarily related to cytotoxic therapy. However, the presence of chronic GVHD and its treatment, in particular with glucocorticoids, may add to these problems. Local complications, such as vaginitis, may interfere with normal sexual relationships [11].

Pancreas

Prolonged therapy of GVHD with glucocorticoids, alone or combined with calcineurin inhibitors, may result in iatrogenic diabetes mellitus. It tends to resolve with discontinuation of steroid therapy.

Cardiovascular disease

Treatment of GVHD with calcineurin inhibitors, rapamycin and glucocorticoids may result in hyperlipidemia, hyperglycemia (see above), and hypertension, thereby compromising cardiovascular function. Coronary artery disease and thrombotic events have been reported at various time intervals after HCT [12].

Ocular problems

The most common problem affecting the eyes after HCT is ocular sicca, usually related to chronic GVHD, and treatment of GVHD with steroids carries the risk of capsular cataracts. The treatment of choice is lens extraction and implantation of an artificial lens. Contact lenses can be used but may be difficult to wear in patients with ocular sicca, which is often irreversible [13]. Chronic GVHD involving the eyes can result in scar formation (e.g., in the tarsus) and lead to synechiae, ectropion, corneal damage and, potentially, perforation if not treated meticulously and aggressively. Keratoconjunctivitis sicca also occurs in patients without chronic GVHD, although the possibility that it represents a sequel of prior GVHD or a form fruste of GVHD must be considered [14].

Obstruction of the nasolacrimal duct, related either to GVHD or conditioning-induced fibrosis, has been observed [15], but is infrequent.

Musculofascial problems

The most common muscular complication is corticosteroid-induced myopathy, most frequently in the context of chronic GVHD. Occasionally patients with chronic GVHD have involvement of muscle, fascia, and serous membranes, including the synovia [16]; joint effusions may occur in patients without any other sign of GVHD. Involvement of fascia or tendons by an eosinophilic infiltrate (early) or fibrosis (late) frequently preceded by edema and often resulting in joint contractures of the wrists (most common), fingers, shoulders, elbows, ankles and occasionally knees may be manifestations of chronic GVHD.

Skeletal complications

Osteoporosis/osteopenia

Bone loss after HCT is related to several factors, including irradiation, glucocorticoid therapy, inactivity, and iatrogenic hypogonadism. Monitoring and

management have been discussed extensively elsewhere [17]. In view of recent data on potential adverse effects of estrogen replacement in women, the overall risk and benefits of hormone replacement must be discussed individually before starting therapy and again at yearly intervals.

Avascular necrosis

Avascular necrosis, especially in weight-bearing joints, is a classic side effect of glucocorticoid therapy and has been reported in 4 to 10% of allogeneic HCT survivors at a median of 12 (range 2–132) months after transplant [18,19]. The hip is most frequently affected (two-thirds of all cases), and in most patients more than one joint is involved. A multi-institutional study from France, including 4,388 patients, found 77 patients with avascular necrosis for a 5-year incidence of 4.3% [18]. Symptoms developed at 2–132 months, and 1–7 (mean 1.9) joints were affected. The hip joint was affected in 88% of cases, and 48% of these patients required joint replacement. Type of GVHD prophylaxis, and acute or chronic GVHD, among other factors, were associated with an increased risk of avascular necrosis.

Dental problems

An oral sicca syndrome related to conditioning therapy or chronic GVHD may lead to poor oral hygiene with recurrent infection and periodontitis. Dental decay occurs because of a lack of cleansing by saliva, which is of altered consistency and reduced volume [20].

Genitourinary dysfunction

Patients receiving immunosuppressive therapy for chronic GVHD, particularly women with GVHD of the vagina, are at risk for recurrent urinary tract infections which require prompt antibiotic therapy.

Kidneys

There is very little evidence of renal involvement with GVHD, although proteinuria has been reported. Important, however, is the fact that several agents used for treatment of GVHD, in particular calcineurin inhibitors, may induce chronic renal failure. Renal failure associated with hemolytic uremic syndrome or microangiopathic hemolytic anemia can occur even in patients who are not heavily pre-treated and are not conditioned with TBI. The mechanism is not fully understood. The syndrome may become manifest either during or following discontinuation of therapy with CSP or FK506 [21].

Genital organs

Complications related to chronic GVHD may also involve the genital organs, in particular the glans penis and vagina. Vaginitis may be severe and cause considerable distress and dyspareunia. Prolonged treatment, topically with estrogens and systemically, e.g., with glucocorticoids, is indicated to prevent the development of atrophic vaginitis and adhesions. The vulva has also been a site of post-transplant malignancies (see below).

Gastrointestinal and hepatic complications

Gastrointestinal (GI) tract

The GI tract is a frequent target of acute transplant-related complications. Chronic problems are less common and generally related to GVHD. Involvement of the esophagus by chronic GVHD may lead to strictures and web formation [22,23]. Chronic GVHD of the small bowel may result in malabsorption.

Liver

Liver function abnormalities related to the conditioning regimen, and GVHD or infections, in particular viral infections, are frequent in the early post-transplant period. At 3 or more months after transplant, the most frequent cause for enzyme or bilirubin elevations is chronic GVHD. However, viral hepatitis has to be considered at any time after HCT. Some cases of hepatitis after HCT were diagnosed upon tapering of immunosuppressive drugs given for GVHD prophylaxis or therapy [24].

Post-transplant malignancies

Lymphoid malignancies

Lymphoproliferative disorders after HCT (post-transplant lymphoproliferative disorder [PTLD]), generally of B-cell lineage, occur mostly in allogeneic transplant recipients [25–27]; T-cell PTLD, non-Hodgkin lymphoma and Hodgkin disease have also been reported. Risk factors include the use of antithymocyte globulin (ATG) or anti-CD3 monoclonal antibodies for acute GVHD prophylaxis, treatment, or in the preparative regimen.

In a study of 18,531 transplant recipients (covering more than 42,000 patient years), 8 cases of Hodgkin disease were identified at 2.9 to 9.1 years after HCT (observed/expected ratio 6.2) [28]. Five cases (67%) showed mixed cellularity subtype, and 5 of 6 cases studied contained the EBV genome. Two patients were also positive for HIV. Patients who developed Hodgkin disease were more likely than matched

controls to have acute GVHD and to require therapy for chronic GVHD (RR in one study 4.0).

Hematologic malignancies

No association between GVHD, acute or chronic, and secondary hematologic malignancies has been reported.

Solid tumors

A spectrum of tumors, including glioblastoma, melanoma, squamous cell carcinoma, adenocarcinoma, hepatoma, and basal cell carcinoma, has been reported. A collaborative study analyzed results in 28,874 patients(<1–72 years of age, 74% with leukemia, 76% transplanted from an HLA identical sibling, 59% given TBI was part of the conditioning regimen) transplanted from 1964–1996. Among 10-year survivors, the O/E ratios for cancers were 26.5 for buccal cavity, 32.3 for liver, 18.3 for thyroid, 6.0 for melanoma, and 3.3 for breast. The rates of excess cancers/10,000 patients per year were highest in patients <17 years (16.06), and lowest for patients >40 years of age (2.42). A case control study based on the same cohort showed that the duration and severity of chronic GVHD were major risk factors, in particular for the development of squamous cell carcinomas of the skin and mucous membranes [29].

Nervous system

The possibility of CNS involvement by GVHD has been debated but generally rejected. However, HCT results in patients with Hurler disease show that the patient's microglia is being replaced by donor cells and, hence, donor/host interactions might take place in the CNS [30]. Others have described cerebellar and pyramidal signs correlating with GVHD activity [31]. Also, Padovan et al. [32] and Takatsuka et al. [33] described periventricular white matter lesions and vasculitis or an angiitis-like syndrome which they attributed to GVHD in several patients. Some patients improved on treatment with CY or glucocorticoids.

Several clearly documented cases of peripheral neuropathy with reduced nerve conduction velocity related to chronic GVHD have been reported [34]. Destruction of Schwann cells seems to be responsible for this phenomenon, and patients respond to glucocorticoid therapy.

Patients with chronic GVHD are also prone to develop septicemia and meningitis caused by encapsulated organisms and invasive fungal infections [35]. Patients with chronic GVHD who receive immunosuppressive therapy should, therefore, also be given prophylactic antibiotics.

Psychosocial effects and rehabilitation

An estimated 75% of patients are back to pretransplant physical function by one year post-transplant [36,37]. However, at least in one study, 20% of HCT recipients had failed to return to full-time employment 40 months after transplantation [38], whereas another group reported that only 9% of 4-year survivors had failed to return to full-time occupation [39]. The development of chronic GVHD after transplant predicts a delay in physical recovery.

Summary

Most patients who recover from the immediate post-transplant problems become healthy long-term survivors and return to normal activities of life. Some patients, however, develop chronic or delayed complications. Major factors contributing to these problems are pretransplant therapy, intensive conditioning regimens and chronic GVHD. Ongoing studies are expected to provide a better understanding of the psychosocial adjustment of patients. Effective therapy or preemptive treatment for some complications is available. Thus, systematic long-term follow-up is recommended for all post-transplant patients. Further refinement of conditioning regimens, prevention of GVHD, especially in its chronic form, and accelerated immunoreconstitution should reduce complications and improve quality of life of HCT recipients.

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How can hematopoietic cell transplant centers and referring physicians help each other during long-term follow-up?

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Late complications after HCT

Success with the use of HCT for treatment of leukemia and other malignancies has improved considerably during the past three decades. Much of this success has resulted from the use of HCT earlier in the natural history of these diseases and from improvements in preventing GHVD and infections. This success has enhanced the importance of long-term complications in determining the outcome after HCT. For example, in an early study describing results of marrow transplantation for treatment of acute leukemia, only 23 (28%) of the 83 reported deaths occurred beyond the first 120 days after the transplant [1]. In contrast, a 1991–1997 survey of allogeneic HCT for treatment of acute leukemia, CML, lymphoma, CLL or myeloma showed that mortality from 3 months to 3 years after transplant was equivalent to or higher than the mortality during the first 3 months after transplant [2]. With a further reduction in acute toxicity brought about by the use of nonmyeloablative pretransplant conditioning regimens, late complications have assumed even more importance in assessing the outcome of treatment.

Late complications after HCT not only affect survival but also cause considerable morbidity [3]. Most of the late morbidity is related to regimen-related toxicity, chronic GVHD, immunodeficiency and infections. These complications can impair function of virtually all organ systems, including the skin, eyes, gastrointestinal tract, liver, lungs, muscles, connective tissues, bones, teeth, endocrine glands, gonads, bladder, kidneys and central nervous system. More recently, secondary malignancies have been recognized as an important complication after HCT [4].

Mechanisms of long-term follow-up in a referral transplant center

Because survivors of HCT are at risk of therapy-related complications, a coordinated program of long-term follow-up is needed both for individuals and for the overall patient population. Such follow-up supports research that could lead to the development of preventive strategies and other interventions to reduce morbidity and mortality. The integrity of follow-up poses a major challenge in assessing long-term outcomes after HCT. Loss of cohort participants reduces study power and causes selection bias. Patients who participate in long-term follow-up may differ in systematic and important ways from those who do not [5–7].

Our center may differ from many others in the way we manage follow-up after HCT. Patients are referred for evaluation before treatment and remain under the direct care of physicians at our center until approximately 3 months after HCT. Direct responsibility for clinical care is then returned to the referring physician, but we remain involved in patient management through consultative services provided by the long-term follow-up (LTFU) program. The need for these support services is typically highest during the first 3 months after patients leave the transplant center and then declines gradually, unless patients develop chronic GVHD or other major complications.

The LTFU program provides a variety of services for patients and referring physicians, including education, telephone consultations, and formal medical evaluation of patients who return to our center. For patients who have had an allogeneic transplant, these services are provided by a group of physicians who rotate monthly as "LTFU attending," assisted by a mid-level nurse practitioner, a nurse, and a dedicated

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support staff. Interactions between these individuals, the patients and the referring physicians generate a large volume of letters, faxes, e-mail, office notes, telephone notes, and copies of medical records. In addition, approximately 400 patients return to Seattle each year for on-site medical evaluation. We encourage close collaboration with referring physicians both to ensure the quality and consistency of patient care and to maximize the amount of information we receive in documenting the outcome of our treatment.

Major efforts are made to provide educational support for patients and referring physicians. All patients attend classes taught by nurses before returning home. These classes focus on management of medications, recognition of side effects, nutrition, guidelines for activities, and manifestations of chronic GVHD. Referring physicians are given both generic and patient-specific guidelines whenever they resume primary responsibility for patient management. We have also designed an LTFU consult form as a mechanism to request clinical assistance from LTFU, to facilitate record keeping and to improve standardization of care. Responses are be made by sending standard guidelines or by a phone call from a member of the LTFU staff or from the LTFU attending physician.

LTFU also provides training for fellows, visiting physicians and physician assistants who are interested in developing expertise in the management of late complications after treatment. The LTFU functions to procure specimens and samples for laboratory tests. Because community pathologists might not have the expertise or familiarity needed to make the diagnosis of chronic GVHD, we make arrangements to have biopsy specimens reviewed by our pathologists. The clinical LTFU also assists in the procurement of samples for research studies.

The LTFU provides major support in guiding the diagnosis and treatment of chronic GVHD. This is accomplished by educating patients and referring physicians about the signs and symptoms that herald the onset of chronic GVHD, by recommending appropriate tests and biopsies to be obtained in patients who might have chronic GVHD, by recommending initial treatment after the diagnosis is made, and by guiding the subsequent management of immunosuppressive treatment. Whenever possible, patients are enrolled in prospective clinical trials for treatment of chronic GVHD. The LTFU plays a major role in the management of recurrent malignancy after HCT. Guidance and support services related to diagnosis, assessment of chimerism, follow-up monitoring and treatment are provided for patients and referring physicians.

Data collection for research studies

The LTFU was designed not only to serve the needs of patients and referring physicians but also to enable collection of data that could be used for research related to outcomes after HCT. Information about these outcomes originates from several sources. These sources included telephone notes from conversations with referring physicians or patients seeking medical advice, correspondence and medical records sent as part of a request for assistance, and records from evaluations of patients who have returned to Seattle. These sources of information are of particular value at early time-points after HCT when contacts between many referring physicians and the LTFU program are most frequent. As time from HCT lengthens, however, the information received becomes less structured and more selected. Other sources of information have included copies of medical records sent by referring physicians who were not requesting specific assistance.

To support research studies, all documents are made available as digitized images in a password-protected optical web library that is accessible to investigators via the Internet. Staff members in the LTFU also review all correspondence and medical records, and selected information is abstracted into a computer database. The LTFU database is organized into 14 sections and contains a total of 80 items addressing major complications after HCT. The list of 80 items was selected after extensive consultation with faculty members who were likely to use the information for research. The list include sites affected by chronic GVHD, complications due to chronic GVHD, infections, selected respiratory, cardiovascular, gastrointestinal, renal, genitourinary, musculoskeletal, endocrine, neuropsychiatric and hematologic complications, pregnancy, organ transplantation, recurrent or secondary malignancy or myelodysplasia, administration of immunosuppressive medications and performance score.

Patient questionnaires

Because we cannot conduct detailed clinical evaluations at frequent intervals, and because we cannot always obtain the relevant medical records, we have used patient self-reported data as one important source of information for long-term follow-up studies. Self-reported disease-specific and generic measures of health and quality of life have been used in questionnaires and telephone interviews to evaluate outcome after HCT [8–20].

In one study, a combination of 7 instruments was used to evaluate quality of life and health status at 6–18 years after marrow transplantation in a cohort of

117 patients [8]. Only 5% of the cohort reported poor health or health-related quality of life. The most common types of morbidity involved emotional and sexual dysfunction, fatigue, eye problems, sleep disorders, pain, and cognitive difficulties. In a cohort of 212 patients who had a marrow transplant for treatment of aplastic anemia, the most common types of long-term morbidity were skin abnormalities, cataracts, lung disease, bone and joint dysfunction, secondary malignancies and depression [9]. Chronic GVHD was a major risk factor for these complications.

Some attempts have been made to determine the validity and reliability of self-report data in HCT patients. Wingard et al. [14] found good agreement between patient and physician-rated Karnofsky scores in a cohort of 171 marrow transplant recipients. The presence of clinically significant illness correlated with self-reported health assessment, global health, pain and Karnofsky scores. In a study of health-related quality of life in 82 pediatric transplant survivors, Parsons et al. [15] found good correlation between health assessments reported by school age children and the disease severity rating reported by their physicians. Assessments by the parents did not correlate with the physicians' assessment, and parental ratings were consistently lower than ratings by the children. These results support the validity of self-reported data in children, when an appropriate instrument is utilized.

A more comprehensive validation study of self-reported medical complications in HCT survivors has been published [16]. One hundred survivors of bone marrow transplantation at the City of Hope National Medical Center were mailed a self-administered questionnaire that contained 225 items regarding medical health, reproductive function, health habits, physical activity, socio-demographic factors and family history. Validity and reliability of self-reported medical complications were assessed by comparing responses with data from medical records. High sensitivity, specificity, positive and negative predictive values and kappa statistics were found for musculoskeletal, cardiovascular, endocrine, gastrointestinal and pulmonary complications, and for GVHD. Overall accuracy in reporting new malignancies was low, mostly because skin cancers were included in the analysis and because recurrences were reported as new malignancies. Accuracy in reporting less well-defined ocular and neurological complications was also low, most likely because physicians did not address mild symptoms during routine follow-up visits. Data for poorly defined disorders with a fluctuating clinical course had the lowest validity and reliability.

We have had mixed success with the use of questionnaires as a method of obtaining self-report data from LTFU patients. The questionnaire that we have used during the past 5 years contained approximately 250 items organized into 6 sections. These sections included a self-assessed performance score, a symptom inventory, a list of complications procedures or medical interventions, a list of medications, a brief quality of life instrument, and a page for comments. Patients have been asked to complete the questionnaire when they return to the care of the referring physician, at 6 months and 1 year after HCT, and then at annual intervals. A postage paid return envelope is included with all mailings, and reminders are sent to patients who do not respond within 2 months.

One problem with the use of questionnaires is that not all patients respond. The overall response rate for our questionnaires has been approximately 50%. We have found that two variables influence the probability of response: age of the patient at the time of questionnaire and length of time from HCT. Older patients had much higher response rates than younger patients. Approximately 65% of patients respond to the questionnaire at 6 months after HCT, but only 50% respond to the questionnaire at 3 years. On the other hand, approximately 80% of patients responded to at least one of the questionnaires during the first 3 years after HCT. Patterns of response were highly variable for patients who were more than 5 years from HCT. Approximately 30% never responded to any of the first 4 questionnaires that they received, and approximately equal proportions of the remaining patients responded once, twice, three times or four times during the first 4 years.

Data from the patient questionnaires confirmed information from published reports, especially with regard to symptoms and attitudes about their experience after HCT. Shortness of breath with exertion, fatigue, muscle cramps, difficulty sleeping at night, and problems with memory and concentration were frequently acknowledged as problems for patients who had survived for more than 5 years after HCT. Comments from patients provided insights into the highly variable experiences of patients. As a way of improving response rates to the questionnaires, we have included a selection of anonymous quotations from interesting or eloquent comments with the questionnaire each year. We have also provided patients with summary data from the questionnaires. Many patients have found it useful to know that their problems are not unique.

Physician questionnaires

As part of our effort to collect LTFU data after HCT, we designed questionnaires and special notification forms to be completed by referring physicians. The questionnaire was designed to capture the same medical complications that are routinely abstracted into the database from medical records. The ques-

tionnaire was designed as a simple checklist contained entirely on one side of a single page. In addition, we designed an "Alert Fax" form that referring physicians could use to notify us after high-priority events such as death of a patient, diagnosis or change in therapy of chronic GVHD, diagnosis of recurrent malignancy, second malignancy or myelodysplasia, or any changes in address or other contact information for the patient or physician. The primary problem with the use of questionnaires is that very few referring physicians respond. The overall response rate to our physician questionnaires has been approximately 20%.

Conclusions and future directions

We are continuing our efforts to improve the quality of services to LTFU patients and their physicians, while at the same time enabling the collection of data for research purposes. We have found that the use of a single patient questionnaire for both clinical care and research purposes does not ideally suit both goals. In the future, we plan to use separate instruments for these different purposes. Two questionnaires will be sent to patients, one primarily for clinical care, and the other primarily for research.

The clinical care questionnaire will contain items related to activity level, review of symptoms, health habits, infections, hospitalization, diagnostic procedures, transfusions, medications and transfusions. Patients will be instructed that this questionnaire should be completed only if they are continuing to have problems related to HCT and if they anticipate that the transplant center might be involved in their care at some time in the foreseeable future. Patients who are not having problems related to HCT will be told that they should not complete this health care questionnaire. All completed questionnaires will be converted to digitized images and made available in the optical web library for use by providers when they are called upon to assist in the clinical care patients who have medical problems related to HCT.

A smaller research questionnaire will contain items related to self-assessed performance score, employment or school activity, marital status, some simple measures of health care utilization, such as number of hospitalizations, number of office or clinic visits, number of medications, and availability of insurance, together with a brief quality of life instrument such as the SF-12. All patients will be asked to complete this brief questionnaire. With a major decrease in the number of items in the questionnaire, we hope that the response rates will improve.

We hope that a similar decrease in the number of items will improve response rates to physician questionnaires. Items of greatest interest to transplant centers include vital status with the date of last contact or death, presence or absence of chronic GVHD and immunosuppressive treatment at the date of last contact, dates of onset for any recurrent malignancy, secondary malignancy or myelodysplasia, involvement of the physician in care of the patient, and any change of physician or patient contact information.

For both the patient and physician questionnaire, we might find higher response rate though the use of a web-based system. Security and protection of privacy are major considerations in the development of such a system. Regardless of whether we use a paper-based system or an Internet-based system, it will be necessary to find the appropriate balance between too little and too much information, and we need to ensure that we are collecting the most useful information and making this information easily available to medical providers and research investigators.

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THALASSEMIA

Prospects for developing a molecular cure for thalassemia

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The delineation of the thalassemia syndromes started in the late 1950s with the distinction of alpha and beta thalassemias and the initial studies that pointed out to the considerable genetic heterogeneity of these disorders. Molecular investigations, however, had to wait for the development of recombinant DNA and molecular cloning techniques in the 1970s. The thalassemias were the first human disorders to be delineated at the DNA level and indeed, by the mid 1980s their molecular pathology had been determined. Investigators in this field always hoped that the molecular understanding of thalassemias would provide clues for the development of molecular therapies and perhaps cures. This hypothesis, however, was proven wrong; no clues for therapies have come from the molecular analysis of the large number of thalassemia mutations that have so far been characterized. Hopes for a molecular cure are now resting on the development of gene therapy. Special for beta thalassemia and the other beta chain hemoglobinopathies is a form of therapy that has been revealed to us by nature, i.e. the alleviation of the beta chain deficiency by the production of fetal hemoglobin in the patient's red cells.

Prospects for the development of gene therapy for beta thalassemia syndromes. Gene therapy for thalassemia has been an early goal of investigators working in this field. Indeed, we were thinking about gene therapy even before the molecular understanding of the control of globin genes had reached a stage that could make this goal realistic.

The ultimate goal of gene therapy of thalassemia is to correct the mutant globin gene in the patient' hemopoietic cells. Gene correction, however, requires technologies which are still at a very early stage of development. The current goal of gene therapy for thalassemia is to add in the stem cells of the patients a normal β globin gene or a fetal globin gene (gene addition). In order to be successful, gene additions require that the therapeutic beta or gamma globin gene (1) is transferred to a large number the patient's pluripotent repopulating stem cells; (2) it is regulated like a normal globin gene; (3) it is expressed at the very high level characteristic of the beta globin genes of normal individuals.

During the last 20 years a major effort has been directed towards the development of vectors that fulfill these requirements. For about 15 years retroviral vectors were used because of the very good understanding of the retroviral biology, and because they integrate into the cell's genome. Stable integration of therapeutic genes into the patient's stem cells is an absolute requirement for gene therapy of thalassemia. However, retroviral vectors (oncoretroviral vectors) require cell division for integration and this is a disadvantage because the vast majority of hemopoietic stem cells are at rest.

Systematic studies of globin gene transfer using retroviral vectors started in the mid 1980s but the results were disappointing because globin gene expression was inconsistent and very low. The discovery of the major regulatory element of the β locus, the locus control region (LCR) brought new impetus in the field and a large effort was made in several laboratories to produce new globin gene vectors containing the beta or gamma globin genes and components of the LCR that hopefully would have stimulated high globin gene expression. The initial excitement, however, was followed by new disappointment because these vectors were incapable of achieving therapeutic levels of globin gene expression; they were also unstable. The field was resurrected with the introduction of lentiviral vectors. These vectors are superior to oncoretroviral vectors because (1) they do not require cell division to enter the cell's nucleus and to integrate into the cell's genome. (2) They allow the incorporation in the vector of larger regulatory sequences from the locus control region;

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this in turn guarantees high levels of globin gene expression; (3) they are stable.

Lentiviral globin gene vectors have now been used by six groups for preclinical studies in murine beta thalassemia and sickle cell anemia models and have cured thalassemia and sickle cell anemia in these models. Lentiviral globin vectors completely correct the thalassemia hematological phenotype in cultures of erythroid cells from patients with Cooley's anemia. Although several improvements are expected to be implemented in the future, it is safe to state that the basic molecular tools for achieving curative levels of globin gene expression using gene therapy vectors are now available.

In order to translate these advances to gene therapy of our patients, two major challenges need to be met.

The first is safety. Patients with XSCID were cured after receiving gene therapy with retroviral vectors containing the γ^c gene (which is abnormal in this disease) but 3 of 10 patients developed leukemia. Subsequent studies of these patients and several other investigations established that integration of viral vectors is not random and can have unwarranted effects because of activation of protooncogenes. A goal of the gene therapy field is to develop approaches that decrease the probability of unwarranted interactions between the integrating viral vectors and the genes of the host.

The second challenge stems from the requirement for genetic modification of a large number of pluripotent stem cells; this is necessary in order to achieve therapeutic levels of globin gene expression in the patient's blood. It has been estimated that gene transfer to about 20 to 30% of the patients pluripotent hemopoietic stem cells is required to cure Cooley's anemia. Achieving this goal will necessitate bone marrow conditioning. It is expected that a moderate degree of conditioning will create enough spaces to allow homing by the genetically modified hemopoieitic stem cells.

Prospects for a molecular cure through activation of fetal globin genes

The possibility that Cooley's anemia can be cured by induction of synthesis of fetal hemoglobin was realized when the pathophysiology of the thalassemia syndromes started to be delineated in the late 1950s. It was then realized that the patients with Cooley's anemia survive beyond the period of the fetal to adult hemoglobin switch because synthesis of fetal hemoglobin continues in the patient's erythroid cells. The level of fetal hemoglobin in the patient's cells is, however, inadequate; hence the severe anemia with all its pathophysiological consequences.

The molecular control of globin gene switching was up to recently unknown, but this did not prevent us from attempting, in the 1970s, to investigate the control of fetal hemoglobin synthesis in adult individuals. A significant advance was the discovery that fetal hemoglobin can be readily induced in cultures of adult erythroid progenitors. Such studies in culture led to the concept that the adult erythroid cells have an inherent potential to produce fetal hemoglobin but that potential is lost during downstream differentiation. Another clue came from the discovery that fetal hemoglobin can be induced in primates and in humans under conditions of rapid erythroid regeneration. These observations led us test whether cytotoxic drugs which secondarily produce rapid erythroid regeneration can also activate fetal hemoglobin production. The outcome of these investigations was the introduction of hydroxyurea as an inducer of fetal hemoglobin in patients with beta chain hemoglobinopathies. Results of hydroxyurea treatment have been very encouraging in sickle cell disease and in sickle cell beta thalassemia but they have been rather poor in Cooley's anemia.

The understanding of the molecular control of globin gene switching started in the late 1980s when it was realized that globin gene expression reflects an interaction between globin genes and the locus control region. This interaction between fetal globin genes and the LCR activates the fetal globin genes. Inhibition of this interaction silences the fetal globin genes. A large research effort has been focused on understanding the specifics of these interactions. We now know that specific components of the locus control region and specific sequences of the fetal globin gene promoter are involved in the fetal globin gene activation. We also have started identifying the determinants of the fetal globin gene promoters which are involved in silencing. Both gene activation and gene silencing are mediated through transcriptional factors which interact with the globin genes and the locus control region. We hope that eventually specific molecular therapeutics for induction of fetal hemoglobin will be developed by modulating the action of these factors involved in fetal gene activation or silencing. However, in spite of considerable effort, the specific transcriptional factors participating in γ gene activation or silencing remain unknown.

It is however possible to affect fetal globin gene expression indirectly by manipulating the chromatin of the beta globin locus. Extensive evidence obtained from studies of loci of model organisms indicates that modifications of histones affect gene expression. Recently the histone code of the human fetal and adult erythroid cells has been defined in our laboratory and it is clear that globin gene switching is associated with substantial switches in acetylation of histones of the fetal globin genes; the gamma genes are highly acetylated in the erythroid cells of the fetus, where fetal globin gene expression is very high; but they are hypoacetylated in the adult erythropoiesis where fetal globin gene expression is very low. Since

fetal globin gene silencing is associated with histone deacetylation, it is expected that inhibition of histone deacetylation can lead to γ gene activation.

Practically, induction of fetal hemoglobin through inhibition of the enzymes responsible for histone deacetylation has been achieved before information on the globin histone code became available. Butyrate, a histone deacetylase inhibitor, has been shown to induce the γ genes in vitro, in animal models and in patients with sickle cell anemia and patients with thalassemia. Other short chain fatty acids and related compounds that are potent fetal globin gene inducers have been found. Certain fetal hemoglobin inducers also have erythropoietic effects and are active by oral administration. Clinical development of these compounds provide the best hope for production of a molecular therapeutic which will have an impact on the treatment of thalassemia patients all over the world.

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MYELODYSPLASTIC SYNDROMES

The myelodysplastic syndromes: Diagnosis, molecular biology and risk assessment

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Abstract
Myelodysplastic syndromes (MDS) are heterogeneous group of neoplastic clonal stem cell diseases characterized by dysplastic morphological features and clinical bone marrow failure. The FAB (French-American-British) system served as the gold standard for MDS classification for more than two decades. The WHO classification, built on the backbone of FAB classification, is an attempt to further improve the prognostic value of MDS classification as well as establish its clinical utility as a tool to select different treatments. In this article we review the epidemiology, pathogenesis, molecular biology, diagnosis and classification of MDS. We highlight the major differences between the FAB classification and the WHO MDS classification. We discuss in more detail the experience of using the new WHO classification since its publication and review the studies that tried to validate the prognostic value of the new classification or apply it to predict clinical responses to various treatments.

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of hematopoietic stem cells. The definition of MDS has two parts, as it is essentially a clinico-pathologic description. MDS can be defined as a clonal disease of the bone marrow with:

- The clinical manifestation of bone marrow failure as well as a tendency to transform into an acute leukemic phase (e.g. variable percentage of leukemic blast cells).
- The pathological manifestation of morphological abnormalities (termed "dysplasia", although it is a clonal disorder, and hence, neoplastic) of the peripheral blood and bone marrow cells such as ringed sideroblasts, megaloblastic erythroid precursors, hypogranulation/hyposegmentation of the granulocytes, and micromegakaryocytes.

In the early 1970s, a number of investigators gathered to form the French-American-British (FAB) Working Group. The goal was to provide uniform terminology for the myriad of different definitions for the leukemias and related diseases. At the time, new therapies and supportive care measures for hematologic disorders were evolving rapidly. Exciting new drugs were active and more were in clinical development. The group believed strongly that for international groups to be able to exchange information about these different entities, it was critical to agree on common definitions. The FAB Working Group developed a series of proposals and published its first article on the acute leukemias in 1976, which discussed two of the components of what are now called myelodysplastic syndromes (MDS)- refractory anemia with excess blasts (RAEB) and chronic myelomonocytic leukemia (CMML).

It was recognized that some patients could present with a disease that bore some resemblance to acute myeloid leukemia (AML), but that this entity, unlike AML, did not have many leukemic blasts in the bone marrow. It was associated with some alteration in maturation of the three major cell lines (granulocytes,

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erythroid precursors, and megakaryocytes), which resulted in pancytopenia and increased risk of infection and bleeding but did not necessarily progress to acute leukemia. Different terms were applied, including dysmyelopoietic anemias. The FAB Working Group applied the term MDS to these disorders to indicate that the common disease pathway began with a common neoplastic stem cell. The evolution from that stem cell could be highly variable: some patients never evolved to acute leukemia and others evolved quickly.

In 1980, a larger number of cases were reviewed with the intent to determine if specific morphologic abnormalities, singly or in groups, would predict for a different biologic outcome. This larger review of cases led to an expanded definition of the myelodysplastic syndromes into the well-known FAB five subgroups.

Epidemiology

MDS is primarily a disease of the elderly. It is more common than AML and appears to be increasing in incidence.

Most investigators believe MDS is at least twice as common as AML. Current projections are an annual incidence of approximately 12,000 cases in the United States, which makes it the most common leukemia observed, even more common than chronic lymphocytic leukemia (CLL).

One of the limitations in determining the true incidence and prevalence of MDS is the inability of tumor registries to record cases accurately. Most rely on tissue pathology, and many patients with MDS are diagnosed in a hematologist's office where a bone marrow aspirate may be performed without a biopsy, or the diagnosis is made accurately by the process of elimination without ever performing a bone marrow aspirate. This is not the case with AML or any other malignancy.

Addressing the question of whether MDS is increasing in incidence is equally if not more difficult. Older literature is unreliable because different disease classifications existed: idiopathic sideroblastic anemias, refractory anemias, preleukemias, dysmyelopoietic anemias, smoldering acute leukemias, and subacute myeloid leukemias. All of these entities presumably described a similar disease.

We suspect that the incidence and prevalence of MDS are rising, but there are no data to prove this. It makes sense, however, because people are living longer, and MDS is a disease of the aging population. Increasing numbers of people are also developing MDS as a result of exposure to the drugs used to treat patients with solid tumors, the acute leukemias, and autoimmune disorders, as well as in patients receiving bone marrow, liver, and cardiac transplantation.

The acceptance of the FAB classification has facilitated the determination of true age-specific incidences in confined populations, and the best estimates come from selected institutions, cities, and countries that are able to define the entire population at risk. Reports from England, Germany, France, and Thailand have been similar, and there is no evidence to suggest that the incidence of MDS varies worldwide. The approximate incidence is 6 to 10 cases per 100,000 individuals, with an increasing incidence above the age of 60. This compares with an incidence of AML of approximately 3 cases per 100,000. By age 80, the incidence of MDS may approach 65 to 100 per 100,000.

Despite similar incidences worldwide there is a difference in the median age and subtype classification in Asian vs. American/European countries. Lee et al. have published data on a higher incidence of trisomy 1q in MDS patients in Korea (15.2%). In addition there is a lower incidence of refractory anemia with ringed sideroblasts (RARS) in Korea and Japan and a younger median age (45–50 years).

Like AML, MDS can occur as a primary or de novo disease, or as a treatment-related or secondary event.

A number of retrospective studies suggest a correlation between MDS and occupational exposure to agents such as benzene. Although cigarette smoking has a slight but significant association with the development of AML, data suggesting an effect on the incidence of MDS are sparse.

Two types of secondary leukemias/MDS can occur following treatment with antineoplastic agents (Table I). The first type, initially recognized in survivors of Hodgkin's disease, generally presents 5 to 15 years after exposure to alkylating agents (e.g. mechlorethamine and procarbazine as part of the MOPP regimen). It shares many of the dysplastic features of MDS, and has a high incidence of chromosomal abnormalities, involving chromosomes 5 and 7 in particular. Patients have trilineage dysplasia and significant marrow fibrosis, and usually progress

Table I. Secondary Types of Leukemia/MDS

Characteristic	Class I	Class II
Leukemogen	Alkylating agent	Topoisomerase II Inhibitor
Onset	5–15 yr	<5 yr
Classification by FAB Group	No	Yes
Cytogenetic result	Unbalanced (chromosomes 5 and 7)	Balanced
MDS phase	Yes	No
Response to therapy	Variable	CR likely

CR = complete response; FAB = French-American-British; MDS = myelodysplastic syndromes.

rapidly to acute leukemia. These secondary leukemias are difficult to classify as one of the FAB subtypes.

The second, more recently recognized type of secondary leukemia is associated with administration of topoisomerase II inhibitors (e.g. etoposide, the anthracyclines, cisplatin). Interestingly, these leukemias are associated with the translocations present in de novo acute leukemia. For example, there are alterations involving chromosome 11 (11q23), translocations involving t(8;21), and translocations of t(15;17) and inv.16.

Survivors of testicular or lung cancer are now presenting with these type II secondary leukemias, and patients previously treated with alkylating agents and anthracyclines as adjuvant therapy for breast cancer are receiving diagnoses of a mix of type I and II secondary leukemias. Recent results of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-25, which evaluated high-dose cyclophosphamide combined with doxorubicin as adjuvant therapy in 2548 breast cancer patients, revealed 16 cases of AML (3 preceded by MDS) and 4 cases of MDS (4-year cumulative incidence of 0.87%), including a mix of both secondary leukemias associated with alkylating agents and topoisomerase II inhibitors (epipodophyllotoxins, anthracyclines). These results suggest a 60-times -higher incidence than would be expected in a control population.

Classification

FAB classification divides MDS into five subgroups according to the percentage of blasts in the marrow, percentage of ringed sideroblasts, presence of monocytes, and severity of dyspoiesis (Table II)

After publishing the FAB classification for MDS in 1982, investigators found that they could apply it reasonably well. Separations in survival curves, ranging from 5 to 6 years for the most favorable prognostic forms of MDS to less than 1 year for the least favorable forms, were demonstrated. However, the FAB classification has not been without its critics, and modifications have been suggested. For example, evidence suggests that patients with greater than 10% leukemic blasts in the bone marrow (11–19%) experience disease progression different from those with 5–9% blasts. Patients with 20–30% blasts had outcomes similar to AML. It was necessary to look at the natural survival of the FAB categories and to confirm that the percentage of blasts is an important factor for prognosis. It was also noted that the degree of dysplasia, whether uni-lineage or multilineage, played an important prognositic value not completely addressed by the FAB classification.

Because CMML contains "leukemia" in its name, critics often object to its inclusion with the preleukemic states and myelodysplasia. A similar objection was raised several years ago regarding atypical chronic myeloid leukemia (aCML). A group of patients with elevated white blood cell counts – usually greater than $12{,}000 ul^{-1}$ – have a disease resembling CML, but with many of the morphologic features of MDS. These patients have an outcome similar to that of RAEB patients, Investigators differ in referring to the diagnosis as proliferative, leukemic, or myelodysplastic; however, the important point is that patients with aCML whose WBC counts are only slightly elevated tend to resemble more closely patients with MDS. Their disease is unlikely to proliferate, and they can be treated successfully in the same way MDS patients are treated. Another small group of patients have an elevated monocyte count (proliferative CMML), dysplastic changes in their peripheral blood and bone marrow, do not have the Philadelphia chromosome or *BCR-ABL* gene rearrangement, and resemble patients with MDS, but have a proliferative illness. These patients may require CML-type treatment with drugs such as hydroxyurea, interferon-alfa, or busulfan.

There are also patients who meet the diagnostic criteria for MDS, but have only granulocytopenia and thrombocytopenia and no anemia. Some authorities justifiably question the diagnosis of RA when the patient is not anemic. A better term for these patients is “uncategorized MDS”. The original description of RA was intended to include patients with mild pancytopenias and dysplasia, but since there was no category in which to put other kinds of patients, it has become a catchall phrase.

In 1997 the World Health Organization (WHO) appointed a committee to revise and update the diagnostic categories of the Lymphomas and the

Table II. FAB Working Group Classification of MDS

FAB Type	Cases %	Bone Marrow Blasts %	Dyspoiesis	Ringed Sideroblasts, %	Monocytes	Progression to AML, %	Survival Rangers Yrs.	Survival Median Yrs.
RA	35	<5	+	<15	Rare	10	2–5	4
RARS	15	<5	+	>15	Rare	5	3–10	4
RAEB	20	5–20	++	Variable	Rare	45	0.5–2	1.5
CMML*	15	<20	++	Variable	Increased	20	1–5	2
RAEB-t	15	20–30	++	Variable	Variable	60	<1	0.5

*Blood monocyte counts must be $>1 \times 10^{-1}$.

Leukemias. One of us (John M. Bennett) was privileged to be appointed to the subcommittee for acute leukemias and MDS. Changes have been suggested that include the following:

1. Eliminate RAEB-t and establish AML when the percentage of marrow blasts is 20% or greater.
2. List CMML in a separate chapter entitled: myelodysplastic/myeloproliferative disorders. Subclassify CMML into CMML-1 and 2, based on the percentage of blasts in the marrow (1–10 vs. 11–19%).
3. List 2 types of RAEB: RAEB-I (5–10% blasts) and RAEB-II (11–19% blasts).
4. Include under RARS and RA a subtype for dysplasia in granulocytic and (or) megakaryocytic lineage. Dysplasia is defined as 10% or greater dysplastic progeny (granulocytes in peripheral blood and (or) granulocytes, megakaryocytes in bone marrow).
5. (5q -) Syndrome was recognized as a separate entity acknowledging the importance of Cytogenetics in MDS, recognizing this subgroup as unique clinical entity that have good prognosis, and may have more effective therapies geared to.
6. Add a new category for cases that do not fit the other subtypes, namely MDS, unclassified. (Table III)

Pathogenesis model

Occurrence of MDS is best viewed in the frame of a multi-hit theory. Hereditary and multiple environmental factors result in a neoplastic stem cell clone. The MDS clone is characterized by altered gene functions, the gene alterations result either from single gene mutations, chromosomal abnormalities (mostly deletions), or gene silencing. Many of those altered genes are suppressor genes that function in a recessive manner. The various gene alterations of the MDS clone result in an intrinsic increase in the susceptibility of the clone to apoptosis. The MDS clone is also recognized by the immune system leading, in some cases, to clonal T cell proliferation that leads to release of various cytokines including TNF-α. The cytokines not only lead to the apoptosis of the MDS clone but more so the normal hematopoietic cells. This intrinsic and immune mediated susceptibility to apoptosis are the hallmark of early MDS pathogenesis explaining the clinical findings of peripheral cytopenia in spite of hyper- cellular bone marrow. The changes that occur as the MDS progresses and transform to AML are not well understood yet but decrease in the apoptosis, clonal evolution as well as angiogeneisis are thought to be contributing factors.

Evidence of clonality

The neoplastic MDS clone arises from a pluripotent stem cell. The evidence of clonality was originally shown by evidence of G6PD mosaicism. Cytogenetic studies showed presence of two clones with and without trisomy eight in patients with sideroblastic anemia. The clonality is also supported by restriction fragment length polymorphisms (RFLPs) of the X-chromosome genes and by the use of fluorescence in situ hybridization (FISH). Several studies showed evidence of clonality in lymphoid lineage as well suggesting that the MDS clone arises from early pluripotent stem cell capable of myeloid and lymphoid differentiation. It is important to note that karyotypic evolution and complex karyotypic changes may occur with progression of MDS and transformation to AML.

Gene alterations

Loss or gain of gene function can result from single gene mutations, chromosomal translocations (unbalanced or balanced), and by epigenetic alterations such as silencing of the gene expression by hyper-

Table III. WHO Classification

Category	Peripheral blood	Bone marrow
1a. RA without dysplasia	Blasts <1%; monos <1000/cmm	Blasts <5% ringed sideroblasts <15%
1b. RA with dysplasia	Same +dysgranulo and or giant platelets	Same +dysgranulo and or dysmega.
2a. RARS without dysplasia	Blasts <1% monos <1000/cmm	Blasts <5%; =or >15% ringed sideroblasts
2b. RARS with dysplasia	Same +dysgranulo. and or giant platelets	Same +dysgranulo. and or dysmega.
3a. RAEB-1	Blasts 1–4 monos <1000/cmm	Blasts: 5–9%
3b. RAEB-2	Blasts 5–19% monos <1000/cmm	Blasts:10–19%
4. CMML*	Blasts <1–19% >1000 monos/cmm	Blasts: 0–19%

*list under myelodysplastic/myeloproliferative category.

methylation. The net result is either gain of an oncogene function or loss of tumor suppressor gene function. Tumor suppressor genes function in a recessive fashion that requires loss of both alleles function. Haploinsufficiency (loss of a single gene copy) can result in reduction of the gene products and predisposition to malignancies.

The RAS gene family is the most studied in MDS. Ten to forty percent of patients with MDS have RAS mutation. The most common mutation is single base change at codon 12 of the N-RAS family. The resultant mutated N-RAS protein retains active GTP form promoting continuous signaling to the nucleus. N-RAS mutation carries higher risk of AML transformation and worse prognosis. The farnesyl transferase inhibitors are a group of pharmaceutical agents that specifically targets RAS but have broader actions as well.

Other gene mutations described in MDS include P53 tumor suppression gene (5–10% of cases), FLT3 oncogene receptor kinase (5% of cases), P15 ink4b a tumor suppressor gene that is transcriptionally repressed through promoter silencing by hypermethylation (can be present in up to 50% of high risk cases-high grade MDS). The abnormality is seen in association with 7 q- syndrome and is associated with shorter survival.

Certain co-existing gene mutations may increase the individual susceptibility to develop MDS: for example NQO1 gene mutation increases the risk for t-MDS in both the homozygotic and heterozygotic states. NQO1 is a quinone oxireductase required for detoxifying benzene derivatives.

Microarray analysis in MDS

The introduction of microarray analysis revolutionized the analysis of gene profiles. Not only can thousands of genes be analyzed together, but the technique is also promising to identify gene profiles "molecular signatures" that can help identify the disease, categorize its subtypes, better predict the outcomes, and hopefully in the future deliver tailored treatments.

Microarray analysis studies in MDS identified new important genes, profiles that may help distinguish MDS from AML as well as low risk from high risk MDS. In one study investigators were able to discriminate between healthy control bone marrow samples and MDS patients bone marrow samples using the expression profile of 11 selected genes representing different gene classes. The gene expression profile was also able to discriminate between low risk and high risk MDS. The retinoic acid induced gene (RAI3), radiation-inducible immediate early response gene (IEX1) and the stress induced phosphoprotein 1 (STIP1) gene were among the genes down-regulated in low risk MDS reflecting that the CD34 MDS stem cells may lack the defensive proteins and thus is more susceptible to damage. In another study authors were able to distinguish between AML blasts and MDS blasts by certain gene profiles. Delta like gene (Dlk),Tec gene, and inositol 1,4,5-triphosphate receptor type 1 gene were among the genes highly specific for MDS. The Dlk 1 gene for example may be an important gene in cell proliferation and may allow stromal cells to support stem cells. Certain gene sets were identified for early stage MDS including the PIASy gene (PIAS family are group of signaling proteins) that function as tumor suppressor gene. As MDS progresses and transforms to AML those gene expressions are decreased.

Cytogenetics

Chromosomal abnormalities are described in 40–70% of all MDS cases. Chromosomal abnormalities are usually unbalanced loss, deletion or translocation. It may be surprising to find a normal karyotype in 30–60% of a clonal disease; however, this could be explained by technical failures as well as karyotypic evolution overtime. In spite of that, the normal karyotype carries a better prognosis similar to 5q-syndrome, 20q- or loss of chromosome Y. Complex karyotype is defined as presence of 3 or more different cytogenetic abnormalities. It is present in 10–20% of primary MDS and up to 90% of therapy-related MDS. More cytogenetic abnormalities occur in high risk MDS and therapy related MDS. The reported frequency of cytogenetic abnormalities under the new WHO classification are: refractory anemia 25%, refractory anemia with ring sideroblasts 10%, refractory cytopenia with multi-lineage dysplasia 50%, refractory anemia with excess blasts type I & II 30–50%. Cytogenentic abnormalities do not correlate with WHO subtypes except the 5-q syndrome that represents a separate entity.

Loss of chromosome 5/del (5q)

Loss of chromosome 5 or interstitial deletion of its long arm is one of the most common chromosomal abnormalities described in primary and therapy related MDS. This abnormality is associated with previous exposure to carcinogens including benzene, alkylating agents, and radiation. It is very important to distinguish this –5/(del 5q) abnormality from the 5q-syndrome that for the first time is recognized as a separate entity in the WHO classification. The 5-q syndrome occurs in the form of refractory macrocytic and often thrombocytosis; more commonly in upper middle age females. It carries the best prognosis of MDS subtypes. It seems that the deletion in 5-q syndrome breakpoint, which involves band 5q33, contains a different myeloid tumor suppressor gene

from 5q31 band that is commonly involved in –5/del (5 q).

Loss of chromosome 7/ del (7q)

Monosomy 7 or deletion of the long arm of chromosome 7 is well described in therapy related MDS and primary MDS. The breakpoint 7q22 seems to be involved in MDS cases. A monosomy 7 syndrome entity is described in the pediatric literature and common in juvenile myelomonocytic leukemia (JMML). Interestingly, -7/del (7q) is the most common abnormality described in patients with hereditary predispositions to MDS like Fanconi anemia (FA).

Loss of 7 q is associated with AML1 gene mutations. Monosomy 7 or deletion of the long arm of chromosome 7 is associated poor outcome in children and adults. Trisomy 8

Trisomy 8 is described in different hematological malignancies including MDS. Its significance is not well understood.

Loss of Y chromosome

Loss of chromsome Y is described in patients with hematological and non-hematological diseases so by itself does not represent a diagnostic evidence of a hematological process. Once present, however, in MDS it may carry a favorable prognosis.

Loss of chromosome 17 Short Arm

17 p- syndrome is associated morphologically with the classical pseudo-Pelger-Huët hypolobulation. P53 gene is located on 17 p 13.1 and is often involved in this syndrome.

Deletion of Long Arm of chromosome 20

Del (20q) carries a favorable prognosis by itself. It is more often seen in early MDS. Prominent erythrocytic and megakaryocytic dysplasia is often seen. Mature granulocytes from peripheral bone marrow may lack the abnormality suggesting increased propensity of apoptosis for the clone carrying this abnormality. Isochromosome 20 q with loss of interstitial material i (20q-) was described recently in six MDS patients out of 998 in a registry. This was seen in older patients and behaved different clinically from 20q- syndrome with rapid progression and shorter survival. The i (20q-) could represent a further evolution of the 20q karyotype, thus disease progression.

11q23 syndrome

Translocations involving 11q23 are classically described in therapy-related MDS secondary to topoisomerase II class drugs. The mixed lineage leukemia (MLL) gene is located on 11q23. The translocations involving 11q23 are described in acute leukemia with a biphenotypic phenotype that usually carries poor prognosis. The exact involvement of MLL gene in translocation in primary MDS is not well defined.

Apoptosis in MDS

A major advance toward understanding the pathogenesis of MDS has been the observation of apoptosis, programmed cell death, in MDS. The group of Raza/ Preisler et al. have carried out cell kinetic studies from MDS bone marrow biopsies using intravenous infusions of either iododeoxyuridine or bromodeoxyuridine, or both, and estimated the degree of apoptosis by in situ end-labeling of DNA. Virtually all marrows studied demonstrated increased rates of apoptosis as well as rapid cell proliferation.

Apoptosis may carry the explanation for the paradoxical observation of peripheral cytopenia and a normo- or hypercellular bone marrow in MDS. Evidence of apoptosis in MDS is supported by various techniques. Earlier on apoptosis was observed by electron microscopic examination of the bone marrow in MDS patients. Evidence of apoptosis is also shown by biochemical techniques, in situ methods and flowcytometric studies.

Apoptosis seems to be higher in early stages of MDS and decreases as MDS progresses and transforms to AML. Flowcytometric studies revealed that the proportion of CD 34+cells in G1-DNA phase are more in early MDS. Also, the ratio of C-Myc (pro-apoptotic gene) to BCL 2 (anti-apoptotic gene) is decreased as MDS progresses to AML. It is controversial whether apoptosis is restricted to CD34+ progenitors or it also includes mature cells.

Several mechanisms can explain the observation of excessive apoptosis in MDS. The MDS clone itself may carry an intrinsic liability for apoptosis due to altered gene functions and expression, however, there is lack of correlation between cytogenetics abnormalities and apoptosis suggesting that the phenomenon is not only restricted to MDS clone. Increased apoptosis in MDS could also be secondary to inhibitory cytokines mainly TNF-α that can induce apoptosis not only affecting the MDS clone but also the normal cells. Increased expression of FAS ligand (CD 95 cell surface protein) in MDS bone marrow cells could also be one of the mechanisms contributing to apoptosis. Other possible involved mechanisms include cell cycle abnormalities and mithochondrial abnormalities leading to increased apoptosis. Mutations of mitochondrial DNA may also impair iron metabolism contributing to sideroblastic anemia.

Targeting apoptosis could serve as a therapeutic strategy in MDS. In fact, treatment with erythropoie-

tin combined with G-CSF has been shown to decrease apoptosis in responders.

Diagnostic evaluation:

The diagnosis of MDS is based on routine laboratory and peripheral blood evaluation. Bone marrow aspiration and biopsy along with cytogenetic analyses should be performed

The laboratory diagnosis of MDS is prompted by detection of cytopenia or clinical symptoms, such as fatigue, bleeding or infection that indicate the presence of anemia, thrombocytopenia, or severe granulocytopenia. There are no clinical phenomena associated specifically with MDS versus other pancytopenic states, including mild to moderate forms of aplastic anemia, which are occasionally difficult to differentiate from MDS. Sometimes a patient presents with an acellular bone marrow, thereby fulfilling a criterion for aplastic anemia, but the patient also has significant dysplasia, slight macrocytosis, and an abnormal karyotype, such as monosomy 7 or trisomy 8. This patient will eventually develop MDS or acute leukemia if not treated with allogeneic bone marrow transplantation (BMT).

The diagnosis of MDS depends on the process of elimination for half of the patients we observe. For the other half, the diagnosis is not difficult if patients have more than 5% blasts. Having less than 5% blasts and normal cytogenetic and fluorescence in situ hybridization (FISH) results, even in the presence of mild to moderate dysplasia, makes most clinicians reluctant to assign a diagnosis of MDS until a few months elapse. This allows time to rule out a correctable hematologic process, such as pyridoxine-responsive anemia. If the pancytopenic state is not readily reversible by normal interventions within 3–4 months, the chances are overwhelming that the patient has MDS.

Classifying MDS continues to be valuable. We recommend performing a 500 cell differential, eliminating lymphocytes and plasma cells for the count. It involves performing a bone marrow biopsy and aspirate utilizing hematoxylin & eosin; reticulin; Romanowsky and iron stains and counting the number of blasts. The percentage of blasts is calculated, and patients are categorized according to this percentage (e.g. $<5\%$, 5–10%, 11–19%, >20–30%) or if they have CMML, which can be any percentage of blasts with a monocytosis of greater than $1{,}000 ul^{-1}$. (Figure 1)

If the absolute percentage of erythroid precursors is 50% or greater, the percentage blasts is based on the non-erythroid precursors (essentially granulocytes and monocytes). For example, a differential count of 6% blasts, 4% promyelocytes, 15% granulocytes, and 75% erythroid precursors would convert the percentage of blasts to "24% " blasts (6/25) changing the subgroup from RAEB to RAEB-t (in the FAB system).

Cytogenetic testing should be performed on every patient with MDS and cytogenetic and bone marrow evaluations repeated whenever there is a significant alteration in the peripheral blood parameters. Because chromosomal evolution frequently occurs in patients who become more pancytopenic, a different treatment category may be required. However, we are not suggesting a monthly bone marrow biopsy be performed in patients with MDS. Once a diagnosis is established, routine, repeated bone marrow testing is unnecessary, unless there is a valid indication or the patient is on a clinical trial that requires such studies.

Readable cytogenetic spreads can be obtained in approximately 75% of patients with MDS. Of these, 60% to 65% will be abnormal. The most common cytogenetic abnormalities occur with chromosomes 5, 7, and 8. There are abnormalities specific to MDS [e.g. 20q- or t/del (12p)] that are rare events in AML, specific translocations unique to AML not observed in primary MDS [e.g. t(15; 17)], and the same abnormalities seen in both. (Table IV)

Prognostic factors

A number of indexes have been proposed to aid in predicting clinical outcome for patients with MDS

Every city or country with adequate numbers of MDS patients has developed its own prognostic scoring system. Most of these systems separate patients into three groups, and they all have the same outcome: median survival times of 60, 30, and 15 months for the good-, intermediate-, and poor-prognosis groups, respectively.

Recently an improvement in the existing systems was proposed, referred to as the International Prognostic Scoring System (IPSS), which includes a fourth group of patients. Greenberg et al. colleagues performed an analysis of 816 patients with de novo MDS to determine the critical prognostic variables. Patient subgroups were classified according to cytogenetics, percentage of blasts in the bone marrow, and number of cytopenias.

In this system cytogenetic availability is important, as it enables one to predict survival and evolution to AML in the low-risk group. This will help to individualize strategies for treating the patients in whom karyotyping is available. (Table V)

Validation and application of WHO classification in MDS

Several studies evaluated the validity and clinical utility of the WHO classification since its publication. The majority of those studies confirm that the WHO classification was a refinement of the predictive

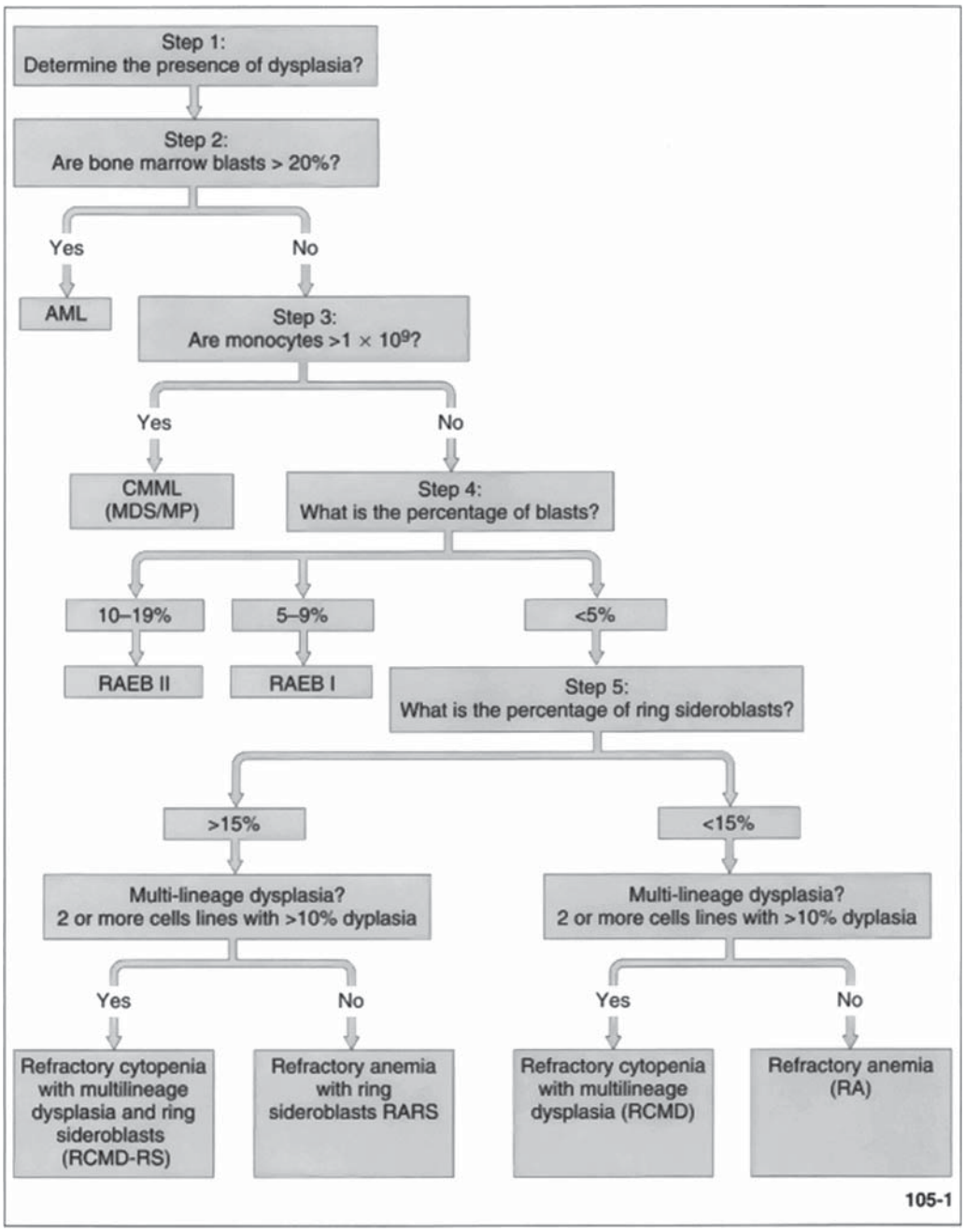

Figure 1. Algorithm for diagnosis and classification of MDS according to the WHO classification.

prognostic ability of the FAB classification where it clearly identifies more homogenous subgroups with similar outcomes. Also recent studies suggest that the WHO classification can be used as a tool to tailor treatment to its subgroups and that response rates to different treatments differ among those subgroups. Some studies, however, still raise the question whether the WHO classification is a better classification system than FAB or they call for a further refinement of WHO classification. In this section we review in more details some of the recently published above-mentioned studies.

Germing et al. conducted the largest retrospective validation study for the WHO classification so far. Their results verified the difference in outcome between RA/RARS and RCMD as well as between RAEB I and II. The WHO classification was retrospectively applied to about 1600 patients in the Dusseldorf MDS registry diagnosed between 1970 and 1999. In their original registry the following FAB subtypes were recorded: RA 26%, RARS 20%, RAEB 22%, RAEB-t 17% and CMML 15%. After reclassification according to WHO classification myeloproliferative CMML patients were excluded, dysplas-

Table IV. Karyotypic Changes Associated with Different Disease Subgroups

Disease Type	Most commonly associated Change
RA	5q-
RARS	+8, 5q-, −7, t/del(11), 20q-
RAEB and RAEB-t	5q-, −7, +8, +5, 7q-, +21, -Y
CMML	−7, +8, t/del(12p), +21, -Y, 7q-
AML (de novo)	t(8;21), t(15;17), t(9;11), inv(16), −7, +8

AML-acute myeloid leukemia; CMML-chronic myelomonocytic leukemia; RA-refractory anemia; RAEB-refractory anemia with excessive blasts; RAEB-t-RAEB in transformation; RARS-refractory anemia with ringed sideroblasts.

tic CMML cases were reclassified. RAEB-t cases were reviewed and only included if auer rods were present but bone marrow blasts were less than 20%. The following subtype frequencies were observed: RA 8.5%, RARS 11%, RCMD 24%, RCMD-RS 15%, RAEB-I 21%, RAEB-II 18.5%, and 5q syndrome 2.2%. The median survival in months for WHO subtypes were RA 69 months, RARS 69 months, RCMD 33 months, RCMD-RS 32 months, RAEB-I 18 months, RAEB-II 10 months, and 5 q- 116 months. The frequency of AML progression was: RA 7.5%, RARS 1.4%, RCMD10%, RCMD-RS 13%, RAEB-I 21%, RAEB-II 34.5%, and 5 q- 8%. Various scoring systems were applied to the reclassified group (IPSS, Dusseldorf, Bournemouth, and Spanish). All scoring systems were applicable and valid. RAEB-II subgroup had more patients in the high risk-IPSS group compared to RAEB-I, probably due to higher score denoted for blast counts.

A similar study was conducted in Brazilian population. The study again verified the difference in outcome between RA/RARS and RCMD and also suggested that the WHO classification correlated better with survival compared to FAB. One hundred fifty patients diagnosed between 1996 and 2002 were retrospectively re-classified according the WHO classification. In the original FAB classification they had the following subtypes: RA 90 patients, RARS 18 patients, RAEB 34 patients, CMML 5 patients, and RAEB-t 3 patients. Upon re-classification by the standard WHO criteria 47 patients were RA, 12 patients were RARS, 25 patients were RCMD, 34 patients were RAEB, 23 patients were unclassified MDS and one patient was 5-q syndrome. The median survival for RCMD was in between RA/RARS and RAEB. No break between RAEB I and II was presented. In a regression model WHO classification better correlated with survival compared to FAB with and without IPSS in the model.

Some studies, however, did not confirm the superiority of WHO classification. Nosslinger et al. applied the new WHO classification again in a retrospective fashion. Four hundred and thirty one patients diagnosed with primary MDS between 1976 and 1999 at Hanusch Hospital in Vienna were included. In the original FAB classification 33% were RA, 11% RARS, 21% RAEB, 12% RAEB-t and 23% CMML. Only 281 patients were reclassified, 150 patients with RAEB-t and CMML were excluded. 43 patients were classified as RA, 4 as RARS, one patient as 5 q syndrome, 91 were RCMD, 50 were RAEB-I, 42 were RAEB II, and 50 were unclassified. RCMD had better median survival than RA (RARS patients were excluded). No significant difference between RAEB-I and RAEB-II was seen. This study was criticized for a major limitation. The authors used 50% as cutoff to define presence of dysplasia in a cell line. This is clearly not what the WHO set as cut off where 10% or more dysplasia in two or more cell line defined RCMD. The whole reclassification would have been different if the WHO cutoff was used. The degree of dysplasia in a cell line is not a yes or no question but obviously a continuum and a certain threshold should be uniformly adapted to unify the classification.

Table V. Risk Analysis (IPSS)

Risk Subgroup	Score	Median survival (years)	Percent AML risk
Low	0	5.7	9.4
Intermediate-1	0.5–1.0	3.5	3.3
Intermediate-2	1.5–2.0	1.2	1.1
High	>2.5	0.4	0.2

The score is based on the following parameters: Score					
Prognostic Variable	0	0.5	1.0	1.5	2.0
BM blasts (%)	<5	5–10	–	11–20	21–30
Karyotype	Good (normal or 5q- or 20q- or -Y)	Intermediate	Poor (>3 abnormalities or monosomy 7)	11–20	21–30
Cytopenias	0/1	2/3			

(Hemoglobin <10 g%, absolute neutrophil count (ANC <1,800ul^{-1}, platelet count <100,000ul^{-1}).

Two recent studies addressed specifically the difference between uni-lineage and multi-lineage dysplasia. In the first study, 103 patients with low risk primary MDS RA subtype by FAB were reclassified according to WHO classification. 56 were labeled RCMD, 43 were labeled RA and 4 as 5q-. Using FISH for cytogenetic determination the authors reclassified the patients into 37 with pure RA, 37 with RCMD and 29 with 5 q deletion. Patients with RCMD had shorter median survival compared to RA (47 month versus 85.2 month). The outcome of 5q- patients was the best if it was the sole cytogenetic abnormality and worse especially if associated with other chromosome 5 cytogenetic abnormalities. In another very interesting study Cermak et al. showed the presence of clonal cell subpopulations in the peripheral blood and bone marrow of RCMD but not in RA/ RARS or 5q- patients reflecting multistep pathogenesis of MDS. They studied clonality in 36 females with MDS using XCIP (X chromosome inactivation patterns) as well as FISH. The patients were classified according to FAB and WHO classifications. Patients with advanced MDS had clonal granulocyte subpopulation and simultaneously clonal CD14+ cells in both peripheral blood and bone marrow. When comparing RCMD versus RA, 12 out 14 RCMD patients had clonal peripheral granulocyte population with 8 of them exhibiting CD 14+ clonal cells. In the bone marrow 10 and 8 of RCMD patients had clonal granulocytes and CD14+ cells respectively. Only 2 out of 11 patients with RA/RARS or 5q- syndrome had clonal granulocytes or CD14+ cells in the peripheral blood. For all patients, median survival for patients with clonal subpopulation in peripheral blood was shorter although not statistically significant.

Focusing more on RAEB-t subgroup in the FAB classification and its elimination from the WHO classification Strupp et al. examined this subset of patients in the Dusseldorf registry. They analyzed 310 patients with RAEB-t. They divided the patients into 3 subgroups: (1) patients diagnosed as RAEB-t with medullary blasts $>20\%$ (2) patients diagnosed as RAEB-t with medullary blasts $<20\%$ but peripheral blasts $\geq 5\%$ and (3) patient diagnosed with RAEB-t based on presence of Auer rods with $<20\%$ medullary blasts. The median survival was 5,3, and 11 month respectively. In group 3 the survival and AML progression was similar to RAEB II but not in group 2. The authors concluded that their results support the WHO classification in eliminating RAEB-t and that presence of Auer rods by itself doesn't carry a prognostic value, however, they cautioned that patients with peripheral blasts $\geq 5\%$ carry a worse prognosis than what is classified as RAEB in WHO and may be should be addressed separately. Similar results were also presented in a Japanese study re-evaluating 113 patients with RAEB-t where they were subdivided into 2 groups (1) RAEB-t with medullary blasts $>20\%$ and (2) RAEB-t with medullary blasts $<20\%$ but peripheral blasts $\geq 5\%$. Interestingly, the median survival was shorter for the second group and when compared to RAEB II this group had more complex cytogenetics and poor prognosis.

The Nordic MDS group is the first to address the clinical utility of WHO classification in predicting clinical response. They re-classified 103 low risk MDS patients from FAB to WHO. Among 64 patients treated with G-CSF and EPO (erythropoietin) on previous Nordic group trials the WHO reliably predicted response rates. The response rate was 67% in RA versus 50% in RCMD. The difference was more pronounced between RARS (75%) and RCMD-RS (9%) (p value 0.003). All over response rate was 73% among patients with uni-lineage dysplasia and 35% among patients with multi-lineage dysplasia. More over, patients with uni-lineage dysplasia had better survival: 51% were alive at 62 month versus 50% at 28.5 month in the multi-lineage dysplasia group (P value $=0.03$). This study is a testimony that the WHO classification does make a difference from a therapeutic point of view. This study also addressed the inter-observer variation in MDS classification. Three blinded and separate reviewers classified the MDS. There was 92% agreement on the WHO subtype. Most discrepancies were on dysplastic features of neutrophils and megakaryocytes, the 10% cutoff of dysplasia in a cell line was sometimes difficult to apply, and the RAEB I and II borderline blast percentage was an issue in few cases.

Conclusion

The experience using the new WHO classification for MDS after its publication confirms its utility as a better prognostic classification system and as a tool able to predict clinical responses. The WHO should be used in adjunct with the IPSS. Future application will hopefully further validate its prognostic value and potentially better identify homogenous subsets of patients who will respond to different therapeutic options. The classification of MDS should be viewed as a continuous process that is refined as we learn more about the disease and its biology. A particular area that may undergo further evolution in the MDS classification includes the cutoff of dysplasia required in each cell line and the impact of peripheral blood blast counts.

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ACUTE MYELOID LEUKEMIA

Acute promyelocytic leukemia: A model of molecular target based therapy

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Abstract
Leukemia, a group of hematological malignancies characterized by clonal expansion of hematopoietic cells with uncontrolled proliferation, decreased apoptosis and blocked differentiation, is one of the most notorious enemies of mankind which accounts for some 300,000 new cases and 222,000 deaths each year worldwide [1–4]. Leukemia can be divided into acute or chronic, lymphoid or myeloid types, based on the disease progression and hematopoietic lineages involved [5]. The responses of leukemia to therapies differ from one type or subtype to another. Hence, to improve the clinical outcome, the therapeutic strategies should be disease pathogenesis-based and individualized. The close collaboration between bench and bedside may not only shed new lights on leukemogenesis, gain insights into therapeutic mechanisms, but also provide opportunities for designing more rational therapies. The development of curative approaches for acute promyelocytic leukemia (APL) may serve as a paradigm [6].

Unveiling APL, a unique subtype of leukemia with ugly head

It took a long time for doctors to know and designate the disease, APL. In 1935, Risak [7] reported a patient with a 'rapid down hill course and the coincident rise of myelocytes in the peripheral blood.' In 1955, Cooperberg and Neiman [8] described a case of acute myelogenous leukemia (AML) with fibrinolytic purpura. A similar patient was also described by Pisciotta and Schultz [9]. In 1957, the Swedish author Leif Hillestad [10] reported three AML patients characterized by a very rapid fatal course of only a few weeks duration, a white blood cell picture dominated by promyelocytes, a severe bleeding tendency due to fibrinolysis and thrombocytopenia, and a normal erythrocyte sedimentation rate probably caused by the reduced fibrinogen concentration in the plasma. He mentioned that his cases were identical to those described by Risak [7], Cooperberg and Neiman [8] and Pisciotta and Schultz [9]. He designated this type of AML as acute promyelocytic leukemia (APL) (Box 1), and he concluded that APL 'seems to be the most malignant form of acute leukaemia'. More detailed features of APL were then described by Bernard et al. [11] and Caen et al. [12].

In 1976, the well characterized morphology of the promyelocytes led the French–American–British (FAB) Nomenclature Committee to assign them the specific classification of M3 cells, and APL was named the M3 type AML (AML M3) [13]. Thereafter, two variants of APL, the hypogranular variant [14] and hyperbasophilic microgranular variant [15], were reported sequentially.

Also in 1976, a consistent chromosomal change, the balanced reciprocal translocation between the long arms of chromosomes 15 and 17 [t(15;17)(q22;q21)], was reported by Rowley et al. [16]. Intriguingly, 17q21 was shown to be involved in variants of t(15;17), the t(11;17)(q23;q21) [17], t(5;17)(q35;q21) [18], t(11;17)(q13;q21) [19], and dup(17)(q11;q21) [20] (Figure 1), suggesting 17q21 is important for normal hematopoiesis and disruption of 17q21 is crucial for disease pathogenesis of APL.

So far, APL has been shown to be characterized by three features [6]: the presence of an accumulation of

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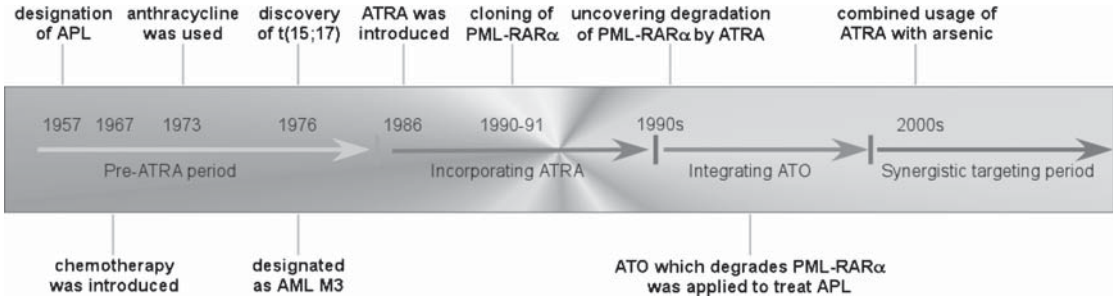

Box 1. Milestones in development of curative approaches for APL.

abnormal promyelocytes in bone marrow; the occurrence of fibrinogenopenia and disseminated intravascular coagulation that is often worsened by chemotherapy; and the presence of the chromosomal translocation t(15;17)(q22;q21) or variants.

Development of curative therapeutic approaches for APL: From highly fatal to highly curable

The past half century has seen great advances in evolving therapeutic approaches for APL. In the first three decades after being recognized, APL was treated with chemotherapy and was once considered the most devastating subtype of AML. The introduction of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) by Chinese hematologists since mid 1980s opened a new page in the history of leukemia therapy. ATRA is a derivative of vitamin A which has dramatically improved the complete remission (CR) rate and long-term survival of APL patients [21]. On the other hand, the application of arsenic trioxide (ATO) further improved the clinical outcome of refractory or relapsed APL [22,23]. Intriguingly, a higher quality remission and survival in newly diagnosed APL were achieved when ATRA was combined with ATO as compared to either monotherapy, making APL a curable disease [24]. Thus, the history of APL could be subdivided into four periods: pre-ATRA period, incorporating ATRA, integrating ATO, and synergistic targeting periods (Box 1).

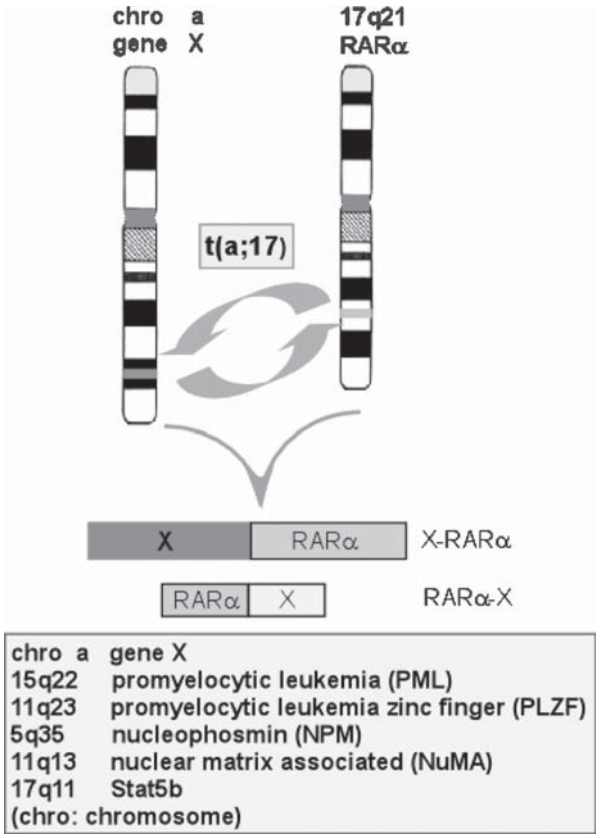

Figure 1. Chromosomal translocations in APL.

The pre-ATRA period (1957–1986) [25]

APL was once considered "the most malignant form of acute leukemia" [10] and the clinical management of the disease at the first decade remained a nightmare for physicians as a result of the unpredictable onset of life-threatening bleeding disorders [25]. Chemotherapy was used unsuccessfully against APL in 1967. At that time proper supportive care was in shortage and standard cytotoxic chemotherapy as induction treatment exacerbated coagulopathy, with approximately 10–30% of patients with APL died of hemorrhage [26].

Particular sensitivity of APL to anthracyclines was reported by Bernard in 1973 [27], and the use of anthracyclines represented the first step forward in taming APL prognosis. Daunorubicin (DNR) and appropriate management of the APL-related coagulopathy improved the CR rate to between 55% and 80% during the 1980s [25,28,29]. However, even with consolidation and maintenance therapy, the median duration of CR was no more than 1–2 years, with only 20–35% of patients cured with chemotherapy alone and the remainder dying from hemorrhage or relapsed or refractory disease [30–33].

The second period: Incorporating ATRA gives APL patients new lease on life

APL as the first paradigm for cancer differentiation therapy

Accumulation of abnormal promyelocytes within bone marrow demonstrates a blockage of normal differentiation program in APL. Whether the induction of differentiation could be a treatment strategy for APL was an intriguing question.

In 1970s, Sachs et al. [34,35] reported that myeloid leukemic cells could be reprogrammed to resume normal differentiation and to become non-dividing mature granulocytes or macrophages as a result of stimulation by various cytokines. Based on this discovery, Leo Sachs hypothesized in 1978 [36,37] that treatment with agents that induce leukemic cells to complete differentiation could be a potential therapeutic option for leukemia patients. In the early 1980s, Breitman et al. [38,39] showed that retinoic acid (RA) could induce terminal differentiation of human APL cells in vitro. But the first clinical reports of using RA showed conflicting results. Some case reports showed beneficial effects of 13-cis RA in people with refractory or relapsed APL [40–42], while other reports showed that 13-cis RA was ineffective in treating APL [43,44].

Beginning in the early 1980s, the Shanghai Institute of Hematology (SIH) conducted a series of experiments on differentiation therapy for APL. These experiments showed that ATRA could induce terminal differentiation of HL-60, a cell line with promyelocytic features, and fresh leukemic cells from patients with APL. These intriguing results were the impetus for a clinical trial which was carried out in 1986. Twenty four patients with APL were treated with ATRA at a dose of 45 to 100 mg m^2 per day. The result was dramatic: 23 patients (95.8%) went into CR without developing bone marrow hypoplasia or abnormalities of clotting. The remaining one patient achieved CR when chemotherapy was added. The most striking feature was the gradual terminal differentiation of malignant cells in the bone marrow, sometimes combined with the presence of Auer rods in mature granulocytes. Hence, the hypothesis-oriented cancer differentiation therapy was brought for the first time into practice.

The efficacy of ATRA on APL was later confirmed by many randomized studies in centers around the world. Further trials showed improved rates of CR, a decrease in severe adverse effects, and lengthening of the duration of remission [6,44–47]. Trials combining ATRA with intensive chemotherapy were soon initiated and the results showed that ATRA combined with anthracycline-based chemotherapy could achieve CR in 90–95% of patients with APL and cure the disease in 70–75% of the cases [6,25,28,29,47]. These data suggest that treatment regimens incorporating agents with different performance can result in a better outcome, hence a global view at systems level should be consider when choosing a therapeutic protocol.

Mechanism of action at the cellular level

Treatment with retinoic acid appears to act on at least two stages of myeloid cell development, e.g. promyelocytes and earlier neoplastic progenitor cells that have retained a capacity for self-renewal but are nonetheless already committed along a myeloid differentiation pathway [44]. The absence of bone marrow hypoplasia during induction, the appearance of immunophenotypically unique "intermediate cells" that express both mature and immature cell-surface antigens and the persistence in morphologically mature granulocytes of both Auer rods and the t(15;17) until a late stage of induction [21,48–50], indicate that the induction of remission by ATRA is associated with the differentiation of immature neoplastic cells into mature granulocytes, followed by the emergence of normal hematopoietic cells as the patient achieves remission. After inducing an irreversible commitment to differentiation, ATRA may trigger apoptosis in the maturing cells [51–53].

Mechanism of action at the molecular level

The molecular mechanism underlying ATRA induced APL cell differentiation was uncovered nearly 10 years after the introduction of ATRA to the clinic. It is of the first important to understand the disease pathogenesis of APL.

Complex leukemogenesis with PML-RARα as a key player. The fact that t(15;17) was detected in 98% of patients with APL [54] suggested the key player of APL leukemogenesis might reside in this chromosomal abnormality. Great efforts had been made to unveil the gene generated by the reciprocal translocation. In 1990, Hugues de The et al. [55] reported that the retinoic acid receptor alpha (RARα) gene on 17q21 has been translocated to a locus, myl (renamed PML later), on chromosome 15, resulting in the synthesis of a myl/RARα fusion messenger RNA. In 1991, Kakizuka et al. [56] demonstrated that RARα was fused to promyelocytic leukemia (PML) gene on 15q22 with generation of a fusion gene, PML-RARα. PML-RARα was soon considered a molecular marker for diagnosis and monitoring the minimal residual disease. Fusion genes produced in t(11;17) and other variants were cloned subsequently [17–20,57].

The roles for PML-RARα to play in APL leukemogenesis were investigated. It has been well established that retinoids that are crucial for normal myeloid differentiation act via RA receptors (RARs)

and retinoid X receptors (RXRs). They belong to the steroid/thyroid/retinoid nuclear receptor superfamily of ligand-inducible transcription factors. Both RAR and RXR families consist of three subtypes: α, β, and γ [54]. RARα forms a heterodimer with RXR and binds to RA response element to control the expression of target genes in the presence of physiological concentrations (10^{-9}–10^{-8} M) of retinoids. The PML-RARα chimeric protein acts as a dominant negative mutant over wild-type RARα. The chimeric protein prevents activation of key target genes required for normal myeloid differentiation by sequestering RXR and other RARα cofactors and inhibiting normal RARα functions. The PML-RARα oncoprotein binds to RAR target genes either on its own or with RXR and then recruits histone deacetlyase complexes, which act as repressors of transcription [5,54]. PML-RARα may affect transcription in other pathways including those in which the transcription factor AP1 and interferon-responsive factors are involved. PML-RARα also binds to promyelocytic leukemia zinc finger (PLZF) protein and potentially affects its functions (e.g. growth suppression and transcription repression; control of developmental programs and differentiation) [5,54]. In addition, PML-RARα prevents apoptosis through delocalization of PML and antagonizes cytoplasmic PML function that is essential for TGFβ signaling [58]. PML-RARα may cooperate with activated mutations in protein tyrosine kinases, such as FLT3 [59], which confer proliferative and/or survival advantage to hematopoietic stem/progenitor cells. Recently Lane and Ley [60] reported that PML-RARα was cleaved in several positions by neutrophil elastase (NE) which was produced at maximal levels in promyelocytes. Interestingly, NE-mediated cleavage of PML-RARα may alter its activity and is important for the development of APL in mice. Zhu et al. [61] reported that the K160 sumoylation site in PML/PML-RARα allowed the recruitment of a potent repressor, Daxx, and was absolutely required for PML-RARα transformation activity ex vivo. PML-RARα causes APL in transgenic mice [62–64], while PML-RARα K160R transgenic mice develop myeloproliferative syndromes, but never APL. The Daxx repressor no longer binds PML-RARα K160R, but fusion of these two proteins restores the differentiation block ex vivo. These results identify a repression domain in PML that controls the APL-specific differentiation block, which could explain why PML is the most common fusion partner of RARα in APL.

Catabolism of PML-RARα underlies ATRA-induced APL cell differentiation. PML-RARα is a direct target of ATRA [65]. ATRA triggers a caspases-mediated cleavage of the PML-RARα fusion protein at residue D522 within the a-helix region of the PML component of the fusion protein [66]. Further dissecting of the pathways involved in PML-RARα catabolism led to the discovery of ubiquitin/proteasome- mediated degradation of PML-RARα and RARα, which was dependent on the binding of SUG-1 in the AF2 transactivation domain of RARα with 19S proteasome [62,67]. Intriguingly, the drug ATO which induces a high CR rate in APL relapsed or refractory to ATRA and/or chemotherapy, also induces a degradation of PML-RARα oncoprotein (described below). The catabolism of PML-RARα results in restoration of normal retinoid signaling. RXR and PML sequestration is abrogated, and PML nuclear body is restored. The corepressor is released and the coactivator is recruited and bound with RARα, relieving the transcriptional repression of target genes. ATRA also induces cyclic AMP, a differentiation enhancer that boosts transcriptional activation, reverses the silencing of the transactivating function of RXR, and restores ATRA-triggered differentiation in mutant ATRA-resistant APL cells [68]. Additionally, ATRA induces the expression of RA-induced genes (RIGs, such as RIG-G, E, K and I) [69], and cyclooxygenase 1 [70], inhibits angiogenesis [71], downregulates the expression of tissue factor [72], and restores other signal pathways (e.g. the interferon pathway). Systems analysis of transcriptome and proteome in ATRA induced APL cell differentiation reveals an array of transcription factors and cofactors, activation of calcium signaling, stimulation of the IFN pathway, activation of the proteasome system, degradation of the PML-RARα oncoprotein, restoration of the nuclear body, cell-cycle arrest, and gain of apoptotic potential [73]. Consequently, the abnormal promyelocytes differentiate and die through programmed cell death.

PLZF-RARα fusion transcript resulted from t(11;17)(q23;q21) [17,57] can also bind as homodimers to retinoic acid response elements (RAREs) [54] and acts as a dominant negative manner to inhibit the activity of wild-type RARα [54]. It is noteworthy that PLZF-RARα homodimers bound to a direct repeat of the sequence GGG TCA separated by 5 bp (DR5G) with equal avidity as PML-RARα but bound more strongly than PML-RARα to a repeat of the sequence GGT TCA (Dr5T) [54,74]. Although it is possible that PLZF-RARα homodimers might display altered target gene specificity, in the presence of RXR, the PLZF-RARα/RXR heterodimer binds to RAREs in vitro with higher affinity than PLZF-RARα homodimers [75]. PLZF-RARα interacts aberrantly with the SMRT and NCoR corepressors, Sin3A and histone deacetylase 1 (HDAC1), both in vitro and in vivo. In the presence of 10^{-6} mol l^{-1} ATRA, PML-RARa was able to release the corepressors and HDAC1 whereas PLZF-RARa retained corepressors and HDAC1 even under these high ligand concentrations. As a result, ATRA alone can

not induce maturation of t(11;17)-harboring cells. HDAC inhibitor is required to cooperate with ATRA to induce t(11;17)-bearing cell differentiation [54].

The third period: Incorporating ATO presents benefit to APL especially relapsed ones

Clinical outcome

One of the limitations of ATRA in treating APL is its inefficiency for relapsed or refractory patients. Fortunately, great benefit was brought to patients of this proportion as well as those newly diagnosed by application of ATO which was also firstly reported in China. Arsenic is a common, naturally occurring substance that exists in organic and inorganic forms. The organic arsenicals consist of an arsenic atom in its trivalent or pentavalent state linked covalently to a carbon atom. There are three inorganic forms of arsenic: red arsenic (As_4S_4, also known as "realgar"), yellow arsenic (As_2S_3, also known as "orpiment"), and white arsenic, or arsenic trioxide (As_2O_3) which is made by burning realgar or orpiment [76].

Arsenic was used to treat chronic myelogenous leukemia (CML) in the 18th and 19th centuries, but was discarded as a treatment in the early 20th century because of its toxicity and the advent of radiation and cytotoxic chemotherapy. In the 1990s, Sun et al. [23] showed that intravenous infusions of Ailing-1, a crude solution composed of 1% arsenic trioxide with a trace amount of mercury chloride, induced CR in two-thirds of patients with APL. There was an impressive 30% survival rate after 10 years. In 1997, SIH reported [22,77,78] their striking results of pure ATO in treating relapsed APL. Fifteen APL patients at relapse after ATRA induced and chemotherapy maintained CR received intravenously administration of ATO at a dose of 0.16 mg kg^{-1} per day for 28–54 days. Clinical CR was achieved in nine of 10 (90%) patients treated with ATO alone and in the remaining five patients treated by the combination of ATO and low-dose chemotherapeutic drugs or ATRA. During the treatment with ATO, there was no bone marrow depression and only limited side effects were encountered. These results showed that ATO is an effective and relatively safe drug for APL patients refractory to ATRA and conventional chemotherapy.

Since 1996, a large number of reports have shown that arsenic compounds induce a CR in 85% to 90% of patients with both newly diagnosed and relapsed APL [47]. Tetra-arsenic tetra-sulfide was also reported to be effective in APL treatment [79]. Furthermore, after CR is achieved by arsenic compounds, a molecular remission (i.e. negative for PML-RARα transcript detected by reverse transcriptase polymerase chain reaction) is obtainable either with arsenic compounds or with ATRA and chemotherapy as consolidation treatment. It seems likely that arsenic compounds appropriately used in post-remission therapy could prevent recurrence and achieve a longer survival time [47,79,80].

Mechanism of action

Unlike ATRA which induces terminal differentiation, ATO at cellular level exerts dose-dependent dual effects on APL cells including NB4 cell line and APL primary cells: inducing preferentially apoptosis at relatively high concentrations (0.5 to 2 μmol l^{-1}) and inducing partial differentiation at low concentrations (0.1 to 0.5 μmol l^{-1}) [78]. The clinical response of APL to ATO is also associated with incomplete cytodifferentiation and the induction of apoptosis with caspase activation in leukemic cells [80].

Sequence analysis of the PML gene has indicated the presence of a cysteine-rich region that may be a principal candidate for interaction with trivalent arsenic. Similar to ATRA, ATO at 0.1 to 2 μmol l^{-1} induces a rapid modulation and degradation of PML-RARa proteins [77,78]. ATO targets the PML moiety of PML-RARα through a still unclear mechanism, and causes PML to localize to the nuclear matrix and become sumoylated. Sumoylation at K160 is necessary for 11S proteasome recruitment and ATO-induced degradation, whereas sumoylation at K490 is needed for nuclear localization [81,82]. Since ATRA-induced degradation of the PML-RARa leads to terminal differentiation, a question should be answered here: why ATO-triggered PML-RARα catabolism only results in partial differentiation? A two-step model in induction of APL cell differentiation was developed [83,84]. This model suggests that there are two discrete steps in the maturation process: an RA-dependent priming step that maintains proliferation while cells become competent to undergo maturation in response to retinoids and a cAMP-dependent step that triggers RA-primed cells to undergo terminal maturation. The first event, priming, corresponds to the derepression of a critical target gene repressed by PML-RARα. If the expression of this gene is high enough, differentiation will ensue. If this gene is expressed at intermediate levels, then additional signaling is required, for example by cAMP, G-CSF or histone desacetylase inhibitors. This model clearly accounts for the fact that either full dose arsenic or low dose RA triggered differentiations are non-terminal and that adding cAMP or G-CSF then promotes terminal differentiation, strongly supporting a model where derepression of a first set of genes has a permissive role on differentiation [83,84]. Indeed, ATO in combination with cAMP does fully induce APL cell differentiation [85].

Mechanisms underlying ATO-triggered APL cell apoptosis have been broadly studied. The apoptosis-inducing effect is associated with the downregulation of Bcl-2 [77] which cooperates with PML-RARα to

block neutrophil differentiation and initiate APL [86], collapse of mitochondrial transmembrane potentials (MTP) in a thiol-dependent manner [87,88], activation of caspases [89,90], and modulation of tumor suppressor PML [91] (Figure 2). PML has drawn intense attention recently for its role in growth suppression and apoptosis. PML is a tumor suppressor which normally epitomizes a multiprotein nuclear structure, the PML-nuclear body (PML-NB) that is a macromolecular structure of doughnut shape and approximately 0.2–1.0 micrometer in size. Cells typically contain 10–30 of these macromolecular structures. Cytoplasmic PML is a critical TGFβ regulator [58]. It is becoming apparent that PML and the PML-NB act as molecular hubs for controlling apoptosis [92–94]. At lower levels, PML is essential for the proper function of proapoptotic transcription factors, ultimately leading to caspase activation, while at higher levels PML might trigger apoptosis independently of transcription or caspase activation through protein sequestration into the PML-NB [94,95]. PML also regulates cell proliferation and senescence. In the APL blasts, PML-RARα causes the delocalization of PML into microspeckled nuclear structures through physical association leading to disruption of the PML-NB [54], and antagonizes cytoplasmic PML function [58]. ATO induces the reaggregation of NB antigens, recruits PML proteins onto NBs and induces degradation of PML and PML-RARα [91]. Recently Hayakawa and Privalsky [96] reported that ATO treatment induced phosphorylation of the PML protein through a mitogen-activated protein (MAP) kinase pathway. Increased PML phosphorylation is associated with increased sumoylation of PML and increased PML-mediated apoptosis. Conversely, MAP kinase cascade inhibitors, or the introduction of phosphorylation or sumoylation- defective mutations of PML, impair As_2O_3-mediated apoptosis by PML. Thus phosphorylation by MAP kinase cascades potentiates the antiproliferative functions of PML and helps mediate the proapoptotic effects of ATO.

Recently systems analysis of transcriptome and proteome in ATO-induced APL cell apoptosis [73] showed that at transcriptome level many ATO-regulated genes were also regulated by RA. Unlike RA, ATO mainly induces the degradation rather than the activation of PML-RARα. ATO may target or interact with many other proteins which may underlie the synergistic effect with RA. For example, several ubiquitin/proteasome genes appear to be specifically regulated by ATO, which may consequently contribute to a more effective and efficient protein-degradation system in RA plus ATO-treated cells than in RA-alone- or ATO-alone-treated cells, suggesting a possible synergic effect for ATRA plus ATO in treating APL. At proteome level, ATO may particularly enhance mechanisms of post-transcriptional/translational modifications.

The fourth period: Systems biology-based synergistic targeting makes APL a curable disease

As mentioned above, APL pathogenesis is complex with PML-RARα acting as a key player, suggesting the treatment regimen containing drugs against different targets or mechanisms might confer a superior outcome. Moreover, the two step model for differentiation induction (Figure 2) suggests that combined use of cAMP/cytokine with ATO or low dose ATRA may cause APL terminal differentiation. Indeed,

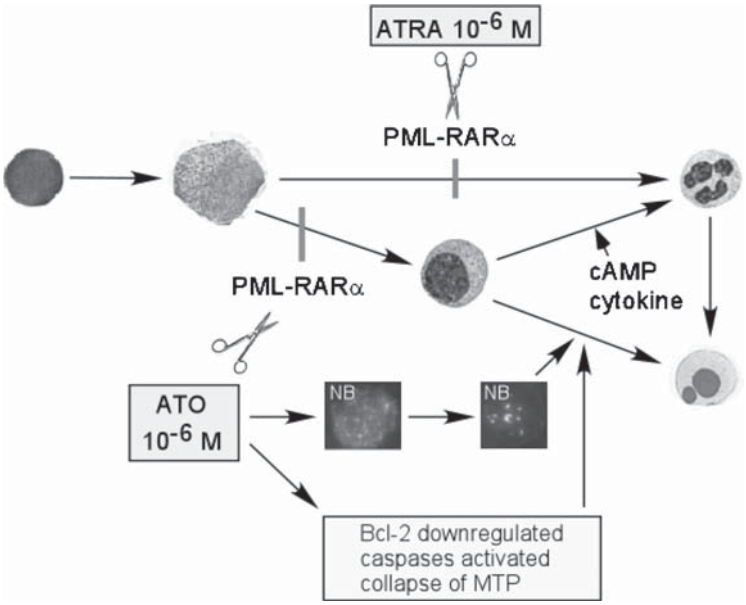

Figure 2. Induction of APL cell differentiation and apoptosis.

ATRA plus chemotherapy yield a higher CR rate and a longer overall survival [25,28,29,47]. Hence, understanding the complexity of APL and drug mechanism at systems level may be of the first importance for proficient option of therapeutic protocol.

ATRA combined with ATO: Two hits on one molecule accelerate APL clearance

A striking similarity in the effect of the two otherwise unrelated agents, ATRA and ATO, is the degradation of PML-RARα oncoprotein through distinct pathways [61,84]. An intriguing question is thus to be answered: will synergistic effects be attained when ATRA is combined with ATO in treating APL? Gianni et al. [97] reported that Arsenic-resistant NB4 cells treated with the combination of ATO and RA showed accelerated differentiation and/or a dramatic induction of apoptosis. Using syngenic grafts of leukemic blasts from PML-RARα transgenic mice as a model for APL, Lallemand-Breitenbach et al. [98] demonstrate that arsenic induces apoptosis and modest differentiation, and prolongs mouse survival. Furthermore, combining arsenic with RA accelerates tumor regression through enhanced differentiation and apoptosis. Although RA or arsenic alone only prolongs survival 2 to 3-fold, associating the two drugs leads to tumor clearance after a 9-mo relapse-free period. These results were in consistence with those reported by Jing et al. [99] and Rego et al. [100].

Trials had been conducted using ATRA plus ATO in treating relapsed APL. Though Raffoux et al. [101] reported that ATRA did not significantly improve the response to ATO in patients relapsing from APL, results of Shen et al. [24] using ATRA plus ATO in treating newly diagnosed APL were dramatic. In Shanghai Institute of Hematology, 61 newly diagnosed APL subjects were randomized into three treatment groups, namely by ATRA, ATO, and the combination of the two drugs. CR was determined by hematological analysis, tumor burden was examined with real-time quantitative RT-PCR of the PML-RARα fusion transcripts, and side effects were evaluated by means of clinical examinations. Although CR rates in three groups were all high ($>$ or $=90\%$), the time to achieve CR differed significantly, with that of the combination group being the shortest one. Earlier recovery of platelet count was also found in this group. The disease burden as reflected by fold change of PML-RARα transcripts at CR decreased more significantly in combined therapy as compared with ATRA or ATO mono-therapy ($P<0.01$). This difference persisted after consolidation ($P<0.05$). Importantly, all 20 cases in the combination group remained in CR whereas 7 of 37 cases treated with mono-therapy relapsed ($P<0.05$) after a follow-up of 8–30 months (median: 18 months). Synergism of ATRA and ATO on apoptosis and degradation of PML-RARα oncoprotein might provide a plausible explanation for superior efficacy of combination therapy in clinic. Thus, the ATRA/ATO combination for remission/maintenance therapy of APL brings much better results than either of the two drugs used alone in terms of the quality of CR and the status of the disease-free survival.

Zheng et al. [73] also reported that at an early state (within 6 h), ATRA plus ATO modulated transcription factors/cofactors associated with myeloid-specific gene expression, nuclear receptor signaling molecules, interferon pathway members, and factors involved in some other cascades. At the time point of 12–24 h, ATRA/ATO regulated genes/proteins seemed to be an amplification of RA signaling and a strong activation of the ubiquitin/proteasome system which might facilitate degradation of PML-RAR. After 48–72 h of treatment with RA/ATO, the expression of differentiation markers and functional molecules reached a maximum, while genes/proteins promoting cell cycle or enhancing cell proliferation were significantly repressed. As the cells approached terminal differentiation, the expression of apoptosis agonists increased gradually. These might contribute to the mechanisms of ATRA/ATO-induced differentiation/apoptosis of APL cells.

Synergistic effects of ATRA/ATO and cAMP signaling in APL cell differentiation: Crosstalk promise

Cyclic AMP was shown to be capable of inducing differentiation in AML cell lines [102]. cAMP boosts transcriptional activation by RA and activates PML-RARA targets [68]. In ATRA-resistant NB4-R1 cells, cAMP-elevating agents or stable agonistic cAMP analogs can induce maturation of these cells at the presence of ATRA [83], while a sustained increase in the endogenous level of cAMP reduces the ATRA concentration required for APL cell maturation to near physiological levels [103]. Furthermore, cAMP also strongly synergizes with low concentration of ATO (0.25 μM) to fully induce differentiation of NB4, NB4-R1, and fresh APL cells, and facilitates the ATO-mediated PML-RARα catabolism. cAMP significantly inhibits cell growth by modulating several major players in G(1)/S transition regulation. An antagonist of protein kinase A, H89, could block the differentiation-inducing effect of ATO potentiated by cAMP. In RA-sensitive or RA-resistant mouse models of APL, continuous infusions of 8-chloro-cAMP triggers major growth arrest, greatly enhanced both spontaneous and RA- or ATO-induced differentiation and accelerated the restoration of normal hematopoiesis. Theophylline, a well-tolerated phosphodiesterase inhibitor which stabilizes endogenous cAMP, also impaired APL growth and enhanced spontaneous or ATO-triggered cell differentiation in vivo. Remark-

ably, in an APL patient resistant to combined ATRA-ATO therapy, theophylline induced blast clearance and restored normal hematopoiesis [104]. These results suggest that cAMP signaling is essential for the intricate cell differentiation process, activation of cAMP pathway provides an alternative option not only for APL synergistic differentiation therapy, but also for other subtypes of myeloid leukemias.

The 50-year history of APL has seen tremendous advances in developing curative approaches, turning APL from once considered "the most malignant form" to currently the most curable form of AML. Of note, the introduction of ATRA in initial therapy represents one of the most spectacular advances in the treatment of human cancer [105] and the first example for oncoprotein-targeting therapy [25]. Benefits gained from ATO which also targeting PML-RARα confirm the philosophy of an old Chinese saying "treating an evil with a toxic" in modern medicine. Moreover, systems biology-based synergistic targeting therapies bring a superior clinical outcome as compared with monotherapy. The experience in taming APL indicates that understanding the disease pathogenesis of other subtypes of leukemia at systems level may be helpful in developing curative approaches for these leukemias.

Possible synergistic targeting treatment cocktail for other leukemias

APL is the first model of a malignant disease that can be treated by drugs targeting an oncogenic event which alters the biological process of the diseased cells [25]. Recently Imatinib Mesylate (Gleevec, STI-571), which competes the adenosine triphosphate (ATP) binding site of the kinase domain of ABL [106], has been shown to have significant antileukemic activity in patients with CML [107–110], establishing another paradigm for leukemia targeted therapy. However, its effects on patients with accelerated phase or blast phase (advanced phase, AP) are unsatisfactory [111–113]. In addition, even among patients at chronic phase (CP), Imatinib seems unable to eradicate the malignant progenitors and a significant portion of patients develops drug resistance after long-time use [114–117]. Li et al. [118] at Shanghai Institute of Hematology showed that arsenic sulfide (As_4S_4) induced apoptosis of CML cells, then the synergic effects of Imatinib and As_4S_4 on CML cells were investigated. The results showed that As_4S_4 induced G2/M arrest whereas imatinib induced G1 arrest. Imatinib plus As_4S_4 induced a much higher ratio of apoptotic cells than that triggered by either Imatinib or As_4S_4 alone. Moreover, the 2 drugs exhibited a synergistic effect in targeting BCR-ABL protein. While As_4S_4 triggered its degradation and imatinib inhibited its tyrosine kinase activity, combined use of the two led to lower protein/enzymatic activity levels of BCR-ABL [119]. Clinical trial using Imatinib plus As_4S_4 to treat CML is undergoing in Shanghai Institute of Hematology and the phase I results in a group of CML AP patients confirmed the safety of the combination protocol.

In AML M2 with t(8;21), the resultant AML1-ETO fusion protein plays a crucial role in pathogenesis of t(8;21) leukemia [120,121]. Though Grimwade et al. [122] showed that t(8;21) was a favorable prognostic factor for AML with a 5-year overall survival rate of 69%, others demonstrated that the median survival time of t(8;21) AML was less than 2 years with a 5-year survival rate of no more than 40% [123–128]. Recently to explore the genetic abnormalities that cooperate with AML1-ETO fusion gene to cause t(8;21) leukemia, SIH screened a number of candidate genes and identified 11 types of mutations in C-KIT gene, including 6 previously undescribed ones among 26 of 54 (48.1%) cases with t(8;21). To address a possible chronological order between AML1-ETO and mutant C-KIT, it has been showed that, among patients with AE and mutant C-KIT, most leukemic cells at disease presentation harbored both genetic alteration, whereas in three such cases investigated during complete remission, only AML1-ETO, but not mutant C-KIT, could be detected by allele-specific PCR. Therefore, mutant C-KIT should be a subsequent event on the basis of t(8;21). Furthermore, induced expression of AML1-ETO in U937 cells significantly up-regulated mRNA and protein levels of C-KIT. This may lead to an alternative way of C-KIT activation and may explain the significantly higher C-KIT expression in 81.3% of patients with t(8;21) than in patients with other leukemias. Additionally, Gleevec suppressed the C-KIT activity and induced proliferation inhibition and apoptosis in cells bearing C-KIT N822K mutation or overexpression, but not in cells with D816 mC-KIT. Gleevec also exerted a synergic effect in apoptosis induction with cytarabine, thus providing a potential therapeutic for t(8;21) leukemia. Since AML1-ETO is crucial for leukemogenesis of AML M2, it is reasonable to develop AML1-ETO targeted therapies for t(8;21) leukemia, as several lines of evidence [120,121,129–133] have proved this hypothesis. Thus AML1-ETO-targeting agents and C-KIT kinase inhibitors together with cytarabine should be an attractive treatment cocktail for AML M2 with t(8;21).

Conclusion and perspectives

The story of successfully developing curative approaches for APL shows that by targeting the molecules critical to the pathogenesis of certain diseases, cells can be induced to return to normal or dead by programmed cell death. The close collaboration between bench and bedside is thus important not

only for unraveling leukemia pathogenesis, designing targeted therapy, elucidating drug mechanism, but also for developing systems biology-based synergistic targeted therapy which may in turn greatly improves clinical outcome. The sequencing of the human genome and ongoing functional genomic research are now accelerating the dissection of disease mechanisms and identification of therapeutic targets. This in turn may facilitate the screening of promising treatments. On the other hand, the history of APL has not come to an end. By extending the model of APL, there is reason to hope that several forms of AML and CML can eventually be cured by specifically tailored cell-modifying treatments.

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ACUTE MYELOID LEUKEMIA

Treatment of AML in biological subgroups

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Risk-adapted therapy summarizes new attempts to further improve the therapeutic outcome in various biologic or prognostic subgroups and thus improve the outcome in AML as a whole. This rises the questions (1) about the present status of therapeutic results in AML overall, (2) data available on therapeutic effects in specific risk groups, (3) present knowledge about risk factors and prognostic groups, (4) an approach to subgroup specific treatment effects, and (5) a more differentiated use of therapeutic options in different risk groups. Since acute promyelocytic leukemia is addressed by an own article in this issue, present report concentrates on the remaining subclasses affecting 80–90% of AML patients.

Present status of therapeutic results

The therapeutic progress in AML is best exemplified by the multicenter randomized trials published since 1981 (overview, 1). Table I shows the cumulative data on complete remissions (CR) and long-term continuous remissions in a total of 16061 patients treated in 28 trials according to two age groups and two time periods of publication. Accepting differences in therapeutic endpoints and patient selection, about 2/3 of patients go into remission and ¼ of them remain relapse-free. Major progress over time is seen in the continuous remission of younger patients while older patients stay far behind this benefit. Thus, overall AML remains a disease mostly responding to chemotherapy by a remission but not continuing relapse-free in more than a minority of patients.

Therapeutic effects in prognostic groups

Improved outcomes by more intensive therapy were first observed in good prognostic groups. Thus, postremission high versus standard dose araC produced longer remissions in CBF AML (t(8;21), inv[16], t(16;16)) and not in other abnormal karyotypes [2]. Autologous stem cell transplantation (SCT) versus chemotherapy alone prolonged the relapse-free survival in the good and not in the poor risk group according to karyotype and early blast clearance from bone marrow [3].

In contrast, the group responding with an improved relapse-free survival to prolonged maintenance treat-

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ment versus intensive consolidation were poor risk patients according to unfavorable karyotype or high LDH or high day 16 bone marrow blasts or age ≥ 60 years, and not the respective good risk patients [4].

Since these trends in trials using different design and risk criteria are contradictory, the role of multiple patient characteristics related to age had to be requestioned [5].

Present knowledge about risk factors and prognostic groups

A new opportunity to analyze the role of multiple risk factors was provided by two consecutive trials of the German AML Cooperative Group (AMLCG) where 1084 patients of <60 years and 750 patients of 60^+ years were treated concurrently for de-novo AML. Chemotherapy was TAD–HAM (HAM, high-dose araC/mitox) or HAM–HAM for induction, TAD for consolidation, and reduced TAD for maintenance randomly compared with intensified consolidation [4].

By multivariate analysis and confirming published results we identified cytogenetic groups [6,7], age [4,8], LDH [4,8,9], WBC [10] and day 16 marrow blasts [9,11] as independent prognostic factors, while the treatment modifications did not reach significance.

The only difference between the younger and the older age group was in favorable (14% vs. 7%) and unfavorable (20% vs. 24%) karyotypes. The overall survival at 4 years was 35% in the younger vs. 13% in the older patients. Table II shows the cumulative incidences of ongoing remission in both age groups and in the different prognostic groups [5].

As results of this analysis, age over 60 years as a whole (with the only exception of the 7% favorable karyotype) must be considered poor risk, and there are unknown biological variables such as the age factor essentially determining the outcome of patients beyond the defined risk factors. This awareness gains great importance since 2/3 of patients with AML are 60 years of age or older (Figure 1).

Additional risk factors have been contributed or discussed by others. A history of myelodysplastic syndrome (MDS) or cytotoxic treatment [12,13] is pertinent if these patients are included. Patients at all ages with secondary AML have half the cure rate of those with de-novo AML (AMLCG updates). However, the expression of multidrug resistance has been found to predict for response but not for long-term prognosis [14,15]. AML with morphologic dysplasia was found being related to unfavorable karyotype and not an independent risk factor [8].

Among other abnormalities associated with poor prognosis MLL tandem duplications with 5% [16] and MLL rearrangements by translocation with 2.8% [17] are unfrequent. The recently described unfavorable EVI1 [18] and BAALC expressions [19] require further testing in trials. The frequent Flt3 mutations occurring in 23–32% of AML and predicting poor long-term prognosis [20,21] should become part of the routine risk assessment.

An approach to subgroup- specific effects

In 1999 the German AMLCG started a new trial on major treatment alternatives in de-novo and secondary AML and high-risk MDS, where patients at all ages were up-front randomized to TAD–HAM versus HAM–HAM induction, G-CSF priming versus no G-CSF, and autologous stem cell transplantation (SCT) versus maintenance. Randomizations were balanced against each other and were stratified for age <60 versus 60^+ years, cytogenetic groups, LDH ≤ 700 versus >700U ml^{-1}, and de-novo vs. secondary AML vs. MDS (Figure 2). More than 2000 patients entered the trial so far, and new insights into the relative value of major treatment options in major prognostic subgroups are expected which can contribute to risk-adapted treatment strategies.

Differentiated use of therapeutic options in different risk groups

For the time being and with the limited knowledge about specific treatment effects in prognostic groups the following target groups and their differentiated management may be distinguished.

A. The entire patients 60 years or older (except for the few patients with favorable karyotypes) are considered poor risk. With standard dose induction or intermediate dose araC half of them go

Table I. Combined data on complete remissions (CR) after induction treatment, and continuous complete remissions (CCR) at 4–5 years in 28 randomized multicenter trials in patients with AML (Overview, 1)

	1980–1990		1991–2004		Total	
Publication Year	2686		13375		16061	
Patients entering	CR	CCR	CR	CCR	CR	CCR
Age <60 years	69%	17%	72%	32%	72%	30%
Age $60+$years	45%	11%	50%	14%	50%	14%
All ages	64%	15%	65%	27%	64%	25%

Table II. Probability of ongoing complete remission at 4 years in 2 age groups and multiple prognostic subgroups. Data from German AMLCG enrolling 1834 patients at all ages with de-novo AML concurrently treated and evaluated according to intention-to-treat (Reference 5)

	Age <60 years		Age ≥60 years		
	Patients	4 Y%	Patients	4 Y%	P
Favorable karyotype	97	69	30	39	0.14
Intermed. karyotype	423	44	270	24	<0.001
Unfavor. karyotype	92	12	59	8	0.10
LDH ≤700 U/l	528	45	304	23	<0.001
LDH >700 U/l	201	39	96	0	0.006
WBC ≤20000 /µl	401	47	247	24	<0.001
WBC >20000/µl	347	39	176	20	0.002
Day 16 blasts ≤40%	591	44	338	24	<0.001
Day 16 blasts >40%	76	28	42	9	0.39

into remission but their long-term survival and remission with maintenance or repeated consolidations is with 15%. This justifies novel approaches using targeted agents such as tyrosine kinase inhibitors [22], farnesyl transferase inhibitors [23] or immunotoxins [24]. Dependent on their toxicity, these options can be used either alternatively or additionally to the standard chemotherapy (overview, 25). The older patients are also candidates for allogeneic SCT at optimized conditioning including matched unrelated donors [26,27]. Older patients with contraindications against standard chemotherapy may be given a chance by experimental low toxicity agents.

B. Patients under age 60 with unfavorable karyotype: Half of them are brought into remission by standard or double induction, but their long-term survival and ongoing remission is only 10–15%. A high priority allogeneic SCT is indicated in these patients and may be even approached early and without attaining a complete remission.
C. Patients at all ages with favorable karyotypes (CBF leukemias): In the current 1999 trial of the German AMLCG enrolling patients at all ages with de-novo AML, secondary AML, and high-risk MDS, CBF leukemias achieve a CR rate of 71%, an overall survival of 50%, and a long-term remission rate of 68%. A meta-analysis by the German AML Intergroup in 392 patients of 16–60 years with CBF AML the CR rate is 86%, the overall survival is 65% and the relapse-free survival is 60% [28]. The Cancer and Leukemia Group B first demonstrated the advantageous long-term results in CBF leukemias which were found to depend on repetitive cycles of post-remission high-dose araC [2,29,30]. By the German data [28] resulting from different strategies with high-dose araC induction and partly prolonged maintenance the US data seem to be reproduced.

As from the above and other international results (overview, 28) CBF- AML represents a subgroup with an outcome superior to that in other AML patients receiving the same state of the art chemotherapy. Considering the ongoing mortality and morbidity associated with allogeneic SCT

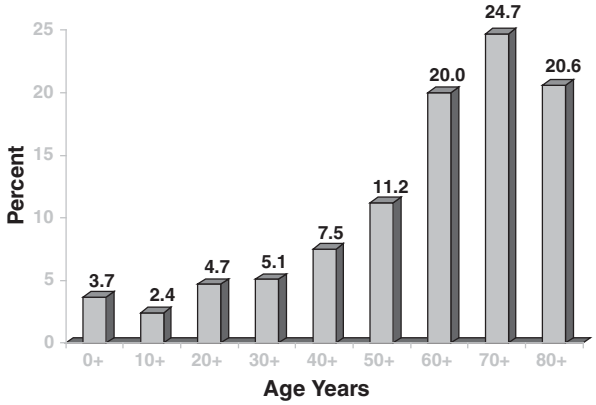

Figure 1. Age structure of the total population of patients with acute myeloid leukemia according to National Cancer Institute SEER Cancer Statistics Review 1975–2000 (Overview see 1)

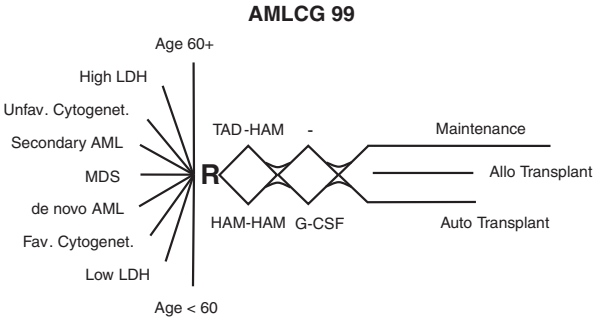

Figure 2. Design of the 1999 trial by the German AMLCG as an approach to subgroup-specific treatment effects. All 3 randomizations between TAD–HAM and HAM–HAM induction, G-CSF-priming and no G-CSF and autologous transplantation and maintenance chemotherapy are done in one step up-front and are balanced against each other. In addition, randomizations are stratified for age, LDH, cytogenetics, de-novo or secondary AML or high-risk MDS.

there are good reasons to avoid this procedure in the first-line therapy and postpone it to the event of relapse in this group of patients.

D. Remaining from the categories A–C patients under age 60 with intermediate karyotype represent a large population with average prognosis. Their CR rate is 69%, their 4 years survival is 38% with 44% ongoing remission. This group also includes a part of secondary AML and high-risk MDS achieving 50–60% CR, about 25% survival and 30% ongoing remission (updates AMLCG 1999 trial). Within patients younger than 60 years with intermediate karyotype there is no evidence for a meaningful risk adaption of treatment. The option of allogeneic SCT in first CR mainly with family donors is justified even if investigational.

Conclusions for present and future risk adaption in AML

Based on adequate trial results the group of patients younger than 60 years with intermediate karyotype appears to best representing present therapeutic standard and progress, also including allogeneic SCT in first CR. Even more successful in patients with favorable karyotypes, the state of the art strategy should avoid the risk of allogeneic SCT as first line therapy in this good prognostic group. High priority, early allogeneic including matched unrelated donor SCT gives almost the only chance of cure to younger patients with unfavorable karyotypes. New comparative data suggest patients over age 60 as a largely homogeneous group of poor prognosis. Novel approaches including allogeneic SCT at optimized conditioning are required and justified in this group, actually contributing the majority to the AML population. New target groups for more specific treatment options may result from ongoing prospective and stratified clinical investigation.

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ACUTE MYELOID LEUKEMIA

Current approaches in refractory AML

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Keyword: *Salvage therapy*

The most important factor to consider in planning initial treatment for a patient with relapsed or refractory AML is the length of the first CR. For practical purpose, patients can be divided into those with first CRs >1 year and those with shorter first CRs (or no first CR, i.e. primary refractory). Three types of therapies are available: allogeneic transplant (allotrans), high-dose ara-C-containing regimens (HDAC), and regimens including various drugs other than ara-C and an anthracycline (non a-a). Comparison of M.D. Anderson data (chemotherapy) with IBMTR data (transplant) suggest that the best option is an allotrans, particularly if the patient had a short or no first CR; however, the degree to which the data are influenced by selection bias is unknown. If an allotrans is not feasible, patients with longer 1st CRs should receive HDAC-containing therapy. Our data suggest that such therapy not only produces higher CR rates, but, more importantly, improves survival relative to non a-a regimens; obviously this conclusion could change were a very successful new non a-a regimen to appear. In contrast, the data indicate that, although HDAC also produces higher CR rates than non a-a in patients with shorter first CRs, or who are primary refractory, this higher CR rate is not associated with a survival advantage, probably because the achieved CRs are very brief and because of higher early death rates with HDAC. As a result, such patients are ideal for investigation of new non a-a regimens. The same is true for virtually all second salvage patients (patients who have received initial induction therapy$+1$ additional therapy to produce a CR). At any time several new non a-a regimens are under investigation at M.D. Anderson. Currently, these include a new anti-fol, new histone acetylase inhibitors, a combination of the latter with decitabine, and other drugs whose mechanism of action is unclear, although they are active in, admittedly very imperfect, model systems. A problem with evaluation of these agents has been a focus on assessing the activity of a given regimen, rather than on assessing whether that regimen is more active than another regimen; this latter question is of more interest to patients. To address this problem it might be a good idea to randomize patients among various protocols, and methods for doing this will be discussed.

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