Karolinska Institutet

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Cover: Japanese maple in my garden, *Acer palmatum atropurpureum* 'Bloodgood'

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"Death is not an event in life: we do not live to experience death.
If we take eternity to mean not infinite temporal duration but timelessness,
then eternal life belongs to those who live in the present."

Ludwig Wittgenstein, *Tractatus Logico-Philosophicus, 6.4311*, 1921

To my family

ABSTRACT

Background: The myelodysplastic syndromes (MDS) constitute a heterogeneous group of malignant bone marrow disorders, characterized by chronic anemia and increased risk of transformation to acute myeloid leukemia (AML). The first line therapy of anemia in MDS is erythropoietin (EPO) with or without granulocyte colony-stimulating factor (G-CSF). Recently, reports about adverse effects of EPO on survival in patients with solid tumors have resulted in questions about its role in MDS. Lenalidomide has a potent effect in 5q- syndrome, however, its mechanisms of action and long-term safety have not yet been studied sufficiently.
Aims: To assess the long-term efficacy and effects on outcome of treatment for anemia in MDS with EPO and G-CSF. To study the *in vitro* effects of lenalidomide on bone marrow progenitor cells from patients with low-risk MDS and del(5q). To investigate the presence of pre-treatment molecular lesions in patients with del(5q) low-risk MDS treated with lenalidomide, who subsequently underwent disease transformation.
Methods and results: We conducted a long-term follow-up of three studies of EPO and G-CSF treatment of anemia in MDS. The overall erythroid response rate was 39%, and the median response duration 23 months (range 3 to 116+ months). Patients with low-risk disease as well as complete erythroid responders had longer response duration. Most relapses were due to unknown factors; only 18% were attributable to a significant blast progression. Next, we evaluated the effect of treatment on survival and risk of leukemic evolution by comparing the treated cohort with untreated patients from two large datasets. In a multivariate analysis, we demonstrated that treatment with EPO and G-CSF was associated with improved survival in patients requiring <2 units of packed red blood cells (RBC) per month (hazard ratio [HR] 0.44; 95% confidence interval [CI], 0.29 to 0.66; $P < 0.001$). There was no association with the risk of AML evolution (HR, 0.89; 95% CI, 0.52 to 1.52; $P = 0.66$). We also studied the effects of lenalidomide on immature hematopoietic progenitor cells from patients with MDS and del(5q) in an erythroblast culture model. Lenalidomide inhibited the growth of malignant cells, while not affecting normal cells. Furthermore, lenalidomide affected gene expression of del(5q) progenitors, and up-regulated the tumor suppressor gene *SPARC*, located within the commonly deleted segment at 5q31. Finally, we describe two patients with 5q- syndrome, who initially responded well to lenalidomide but after two years unexpectedly developed progressive disease. Before treatment, we were able to demonstrate subclones of bone marrow cells with abnormal cytoplasmic nucleophosmin (NPMc+) and overexpression of p53, generally associated with high-risk MDS and AML. Both the NPMc+ and the p53 expressing subclones expanded at disease progression, and sequencing of *TP53* confirmed a pre-treatment heterozygous mutation and a homozygous mutation at disease transformation.
Conclusions: Treatment with EPO and G-CSF in MDS (a) leads to long-term responses, (b) is associated with improved survival in patients requiring <2 units of RBC per month, and (c) does not alter the risk of AML evolution. Lenalidomide specifically inhibits the malignant bone marrow progenitors from patients with MDS and del(5q), and up-regulates the tumor suppressor gene *SPARC* which may be an important aspect of lenalidomide's mechanisms of action of as well as of disease pathogenesis. Patients with 5q- syndrome responding to lenalidomide and subsequently undergoing disease progression may already before treatment have molecular lesions affecting the genomic stability. The presence of such abnormalities could play a role in a future pre-treatment risk-stratification.

SAMMANFATTNING PÅ SVENSKA

Bakgrund: Benmärgscancern myelodysplastiskt syndrom (MDS) drabbar cirka 450 svenskar varje år. Snittåldern är 70 år och utmärkande är blodbrist (anemi) samt ökad risk att utveckla akut leukemi. En tredjedel drabbas av låga vita blodkroppar eller låga blodplättar, vilket leder till ökad infektionskänslighet respektive ökad blödningsbenägenhet. Prognosen varierar kraftigt; en lågriskpatient överlever i snitt fem år medan en högriskpatient ofta dör inom ett år. Den enda botande behandlingen är benmärgstransplantation, vilket endast kan erbjudas en minoritet av patienterna eftersom riskerna är alltför höga för äldre patienter. Övriga patienter behandlas främst för att korrigera blodvärdena och förbättra livskvaliteten. Förstahandsbehandlingen är tillväxtfaktorer som stimulerar bildandet av röda blodkroppar (erytropoetin; EPO), vilket även använts vid bloddopning. Lenalidomid är mycket effektivt vid en undergrupp av MDS som kallas 5q minus (5q-) syndrom. Lenalidomid är en vidareutveckling av thalidomid, som orsakade en våg av fosterskador i början av 60-talet när det gavs till havande kvinnor mot illamående och som rogivande (marknadsfördes under namnet Neurosedyn).
Syfte: Att utvärdera långtidseffekterna av behandling med tillväxtfaktorerna EPO och G-CSF (granulocyt kolonistimulerande faktor) vid MDS. Att i laboratoriet studera hur lenalidomid påverkar benmärgsceller från patienter med MDS av typen 5q- syndrom. Att undersöka förekomsten av molekylära avvikelser före behandlingsstart hos patienter med 5q- syndrom vilka under lenalidomidbehandling utvecklat akut leukemi.
Metoder och resultat: Vi utförde en långtidsuppföljning av tre studier där EPO och G-CSF utvärderades på patienter med anemi orsakad av MDS. Totalt 39% av patienterna förbättrade sina blodvärden under i snitt 23 månader. Vi utvärderade därefter om behandlingen påverkade överlevnaden eller risken att utveckla akut leukemi genom att jämföra de behandlade patienterna med obehandlade från två internationella databaser. Efter att ha justerat analysen för olika riskfaktorer visade vi att behandling med EPO och G-CSF var associerat med förbättrad överlevnad för patienter som inte hade ett tungt transfusionsbehov av röda blodkroppar. Vi fann ingen korrelation mellan behandling och risken att utveckla akut leukemi. Vi studerade också effekterna av lenalidomid på blodbildande stamceller från patienter med 5q- syndrom. Lenalidomid hämmade tillväxten av cancercellerna men inte av de friska cellerna. Vidare ökade lenalidomid nivån av den tumörhämmande genen *SPARC* som annars är låg vid 5q- syndrom. Till sist beskriver vi två patienter med lågrisk 5q- syndrom som svarade bra på lenalidomidbehandling men som efter två år oväntat utvecklade leukemi. Vi fann en liten andel celler i benmärgen före behandling som hade en mutation i den tumörhämmande genen *TP53* (den gen som mest frekvent är muterad vid cancer i allmänhet) och en onormal lokalisering av nukleofosmin, vilket vanligen är associerat med högrisk MDS eller akut leukemi.
Slutsatser: Behandling med EPO och G-CSF leder till förbättrade blodvärden under lång tid och förlänger överlevnaden hos patienter utan ett tungt transfusionsbehov. Lenalidomid hämmar specifikt tillväxten av cancerceller från patienter med 5q- syndrom, samt ökar nivån av den tumörhämmande genen *SPARC* vilken kan ha betydelse såväl för lenalidomids verkningsmekanism som för uppkomsten av sjukdomen. Patienter med 5q- syndrom som till en början svarar bra på lenalidomid men som sedan utvecklar leukemi kan ha ovanliga molekylära avvikelser redan före behandling. Detta kan utgöra en grund för riskbedömning innan behandlingsstart.

LIST OF PUBLICATIONS

I. **Jädersten, M**, Montgomery, SM, Dybedal, I, Porwit-MacDonald, A, and Hellström-Lindberg, E "Long-term outcome of treatment of anemia in MDS with erythropoietin and G-CSF" *Blood* 2005; 106(3): 803-11

II. **Jädersten, M**, Malcovati, L, Dybedal, I, Della Porta, MG, Invernizzi, R, Montgomery, SM, Pascutto, C, Porwit, A, Cazzola, M, and Hellström-Lindberg, E "Erythropoietin and G-CSF Treatment Associated with Improved Survival in Myelodysplastic Syndrome" *J Clin Oncol* 2008; 26(21): 3607-13

III. Pellagatti, A*, **Jädersten, M***, Forsblom, AM, Cattan, H, Christensson, B, Emanuelsson, EK, Merup, M, Nilsson, L, Samuelsson, J, Sander, B, Wainscoat, JS, Boultwood, J, and Hellström-Lindberg, E "Lenalidomide inhibits the malignant clone and up-regulates the SPARC gene mapping to the commonly deleted region in 5q-syndrome patients" *Proc Natl Acad Sci U S A* 2007; 104(27): 11406-11

IV. **Jädersten, M**, Saft, L, Pellagatti, A, Göhring, G, Fernández-Santamaría, C, Wainscoat, JS, Boultwood, J, Porwit, A, Schlegelberger, B, and Hellström-Lindberg, E "Pre-treatment NPMc+ expressing and p53 mutated clones expand at disease progression in 5q- syndrome patients treated with lenalidomide" *Submitted*

*Co-first authors

CONTENTS

1 LIST OF ABBREVIATIONS

5-AZA	5-azacytidine
AML	acute myeloid leukemia
t-AML	therapy-related acute myeloid leukemia
BM	bone marrow
CDS	commonly deleted segment
CER	complete erythroid response
CI	confidence interval
CMML	chronic myelomonocytic leukemia
CR	complete remission
DAR	darbepoetin
EMEA	European Medicines Agency
ENL	erythema nodosum leprosum
EPO	erythropoietin
EPO-G	erythropoietin and G-CSF
EPO-R	EPO-receptor
FAB	French American British
FDA	Food and Drug Administration of the United States
FISH	fluorescence in situ hybridization
G-CSF	granulocyte colony-stimulating factor
HR	hazard ratio
HSC	hematopoietic stem cell
IL	interleukin
IMiD	immunomodulatory drug
IMRAW	International MDS Risk Analysis Workshop
JAK2	Janus kinase 2
MDS	myelodysplastic syndrome
t-MDS	therapy-related myelodysplastic syndrome
NPMc	nucleophosmin aberrantly expressed in the cytoplasm
NPM1	nucleophosmin gene
PER	partial erythroid response
PRCA	pure red cell aplasia
RA	refractory anemia
RAEB	refractory anemia with excess of blasts
RAEB-1	refractory anemia with excess of blasts type 1 (5-9% blasts)
RAEB-2	refractory anemia with excess of blasts type 2 (10-19% blasts)
RAEB-t	refractory anemia with excess of blasts in transformation
RARS	refractory anemia with ringed sideroblasts
RCMD	refractory cytopenia with multilineage dysplasia
RCMD-RS	refractory cytopenia with multilineage dysplasia, with ringed sideroblasts
rh	recombinant human
S	serum
SPARC	secreted protein acidic and rich in cystein (osteonectin, BM-40)
SPARC	SPARC gene
TNF-α	tumor necrosis factor α
U	units
WHO	World Health Organization

2 INTRODUCTION

2.1 THE MYELODYSPLASTIC SYNDROMES

2.1.1 History – a century of diagnostic challenges

At the beginning of the 20th century the first reports of a state of anemia preceding acute myeloid leukemia (AML) appeared.[1,2] During the following decades, a group of patients with anemia refractory to any supplements was identified and described as "pseudo-aplastic-anemia",[3] "achresthic anaemia",[4] and perhaps most suitably "refractory anemia".[5] The connection with leukemia was first described by Chevalier in 1942,[6] proposing the term "odo-leukemia" (odo meaning threshold in Greek), and subsequently in greater detail by Hamilton-Paterson in 1949 with the concept of "preleukemic anemia",[7] and by Block in 1953 coining the term "preleukemia",[8] incorporating patients with cytopenias and dysplasia of one or more hematopoietic lineages in the bone marrow, and with increased risk of leukemic evolution. A direct link between refractory anemia with ringed sideroblasts and acute myeloid leukemia was established by Björkman in 1956.[9] Various terms have since then been proposed for this patient category, "low-percentage",[10] "smouldering",[11] or "oligoblastic" leukemia.[12] Finally, in 1982 the French-American-British (FAB) cooperative group proposed criteria for the "myelodysplastic syndromes (MDS)",[13] greatly facilitating subsequent investigations of the prognosis and management of the disease. The history of MDS has been reviewed in depth by Layton & Mufti.[14]

2.1.2 Epidemiology

The crude incidence of MDS is reported to be around 5 per 100 000 individuals yearly, however, the incidence increases greatly with age (Figure 1).[15-18] The yearly incidence in people less than 30 years old is around 0.1-0.4 per 100 000, rising to 30 per 100 000 in the 80-90 year age stratum.[15-18] Several relatively weak risk factors have consistently been identified in epidemiological studies: smoking, exposure to organic solvents, ionizing radiation, male sex, and having a first degree relative affected by a hematopoietic malignancy.[19-21] In addition, exposure to cytotoxic drugs or therapeutic radiation is associated with greatly increased risk of MDS, which will be discussed in greater detail in section 2.1.6.7.

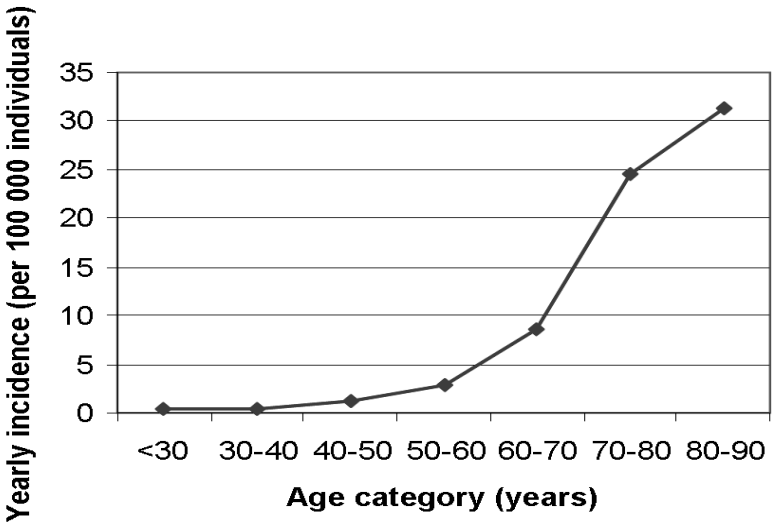

Figure 1. The incidence of MDS increases with age.
Based on tabulated data from Germing *et al* Haematologica 2004; 89:905-10.

2.1.3 Clinical and morphological diagnosis

The typical MDS patient presents with unexplained anemia, with or without other cytopenias.[22] A minority of patients may also present with infections, bleedings, or autoimmune symptoms. The bone marrow is usually hyper- or normocellular, although a minority are hypocellular. The bone marrow smear shows uni- or multilineage dysplasia with or without elevated bone marrow blast counts.[23] A significant dysplasia is defined as presence of dysplastic features in at least 10% of the precursors of a particular lineage, with at least 500 nucleated cells and 20 megakaryocytes assessed.[24,25] Comprehensive diagnostic criteria for MDS were recently proposed at an international Working Conference on MDS in 2006 (Table 1).[25] A proportion of the patients assessed for suspected MDS do not fulfill the criteria, and in order to incorporate the majority of these Dr. Ghulam Mufti proposed the term idiopathic cytopenia of uncertain significance (ICUS) at the 8th International MDS Symposium in Nagasaki, Japan, 2005.

Table 1. Minimal diagnostic criteria in MDS proposed at an international Working Conference on MDS in 2006.[25]

Minimal diagnostic criteria in MDS
A. Prerequisite criteria
1. Constant cytopenia in one or more of the following cell lineages: erythroid (hemoglobin <110 g/L), neutrophilic (ANC <1.5 x 10^9/L), or megakaryocytic (platelets <100 x 10^9/L) **2. Exclusion of all other hematopoietic or non-hematopoietic disorders as primary reason for cytopenia/dysplasia**
B. MDS-related (decisive) criteria
1. Dysplasia in at least 10% of all cells of the erythroid, neutrophilic, or megakaryocytic lineages in the bone marrow smear, or presence of >15% ringed sideroblasts (iron staining) **2. 5-19% blast cells in the bone marrow smear** **3. Typical chromosomal abnormality (by conventional karyotyping or FISH)**
The diagnosis of MDS can be established when *both* prerequisite criteria and at least one decisive criterion are fulfilled.

2.1.4 Classification

The FAB criteria for the classification of MDS proposed in 1982 have been of tremendous importance for subsequent studies.[13] Based mainly on percentage of bone marrow blasts and percentage of ringed-sideroblasts, five morphological groups were defined, refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess of blasts (RAEB), chronic myelomonocytic leukemia (CMML), and RAEB in transformation (RAEB-t). The FAB cooperative group also proposed unified morphological criteria for the assessment of blasts, and proposed a cut-off level of 30% bone marrow blasts for transition to AML.

The World Health Organization (WHO) published a new morphological classification of MDS in 2001 (Table 2).[26] Patients with refractory anemias were subdivided based on presence or absence of multilineage dysplasia. Also, RAEB patients were divided in two categories based on the percentage of bone marrow blasts. MDS patients with >20% blasts were classified as AML. RA patients with an isolated deletion of the long arm of chromosome 5 (del[5q]) were categorized as 5q- syndrome. CMML was moved to a novel category of diseases named mixed myelproliferative/myelodysplastic syndromes. Finally, a new category called MDS-unclassifiable (MDS-u) was introduced, incorporating patients with myelodysplastic features in the bone marrow but not fulfilling the other criteria. The WHO 2001 classification is currently the gold standard for prospective studies

in MDS. There will be a minor revision of the WHO criteria in the updated version of 2009, aiming to further reduce the unclassifiable category.

Table 2. The WHO 2001 classification of MDS.[26]

Disease	Blood findings	Bone marrow findings
Refractory anemia (RA)	Anemia No or rare blasts	Erythroid dysplasia only <5% blasts <15% ringed sideroblasts
Refractory anemia with ringed sideroblasts (RARS)	Anemia No blasts	Erythroid dysplasia only <5% blasts ≥15% ringed sideroblasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenias (bicytopenia or pancytopenia) No or rare blasts No Auer rods <1x10^9/L monocytes	Dysplasia in ≥10% of cells of 2 or more myeloid cell lineages <5% blasts No Auer rods <15% ringed sideroblasts
RCMD and ringed sideroblasts (RCMD-RS)	Cytopenias (bicytopenia or pancytopenia) No or rare blasts No Auer rods <1x10^9/L monocytes	Dysplasia in ≥10% of cells in 2 or more myeloid cell lineages <5% blasts No Auer rods ≥15% ringed sideroblasts
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenias <5% blasts No Auer rods <1x10^9/L monocytes.	Unilineage or multilineage dysplasia 5-9% blasts No Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenias 5-19% blasts +/- Auer rods <1x10^9/L monocytes	Unilineage or multilineage dysplasia 10-19% blasts +/- Auer rods*
Myelodysplastic syndrome, unclassified (MDS-U)	Cytopenias No or rare blasts No Auer rods	Unilineage dysplasia in one myeloid cell lineage <5% blasts No Auer rods
MDS associated with isolated del(5q)	Anemia <5% blasts Platelet count usually normal to increased	Normal to increased megakaryocytes with hypolobated nuclei <5% blasts No Auer rods Isolated del(5q) cytogenetic abnormality

* If the diagnostic criteria for MDS are fulfilled and Auer rods are present, the patient should always be categorized as RAEB-2

2.1.5 Cytogenetic features

Chromosomal aberrations are present in half of all *de novo* MDS patients, and a number of recurrent abnormalities have been described (Table 3).[27-30] Several cytogenetic abnormalities observed in MDS are also seen in AML, thus supporting a common origin of a fraction of these two disease categories.[28] Certain karyotypes are more frequently associated with MDS than AML, in particular del(5q) and del(20q).[28] Interestingly, balanced translocations are much less frequently seen in MDS compared to AML.[28] In AML, several fusion-genes as results of balanced translocations have been identified, such as *PML-RARA* (t[14;17]), *AML-ETO* (t[8;21]), and *CBFB-MYH11* (inv[16]), each constituting a separate subgroup in the WHO classification.[26] Currently, the only subgroup of MDS defined by cytogenetics according in the WHO classification is the 5q- syndrome, with a sole deletion involving 5q31-32.[26]

Table 3. Chromosomal abnormalities in *de novo* MDS. Based on cytogenetic data from MDS patients reported to the Mitelman Database of Chromosome Aberrations in Cancer by May 2001.[28,30]

Cytogenetic features	n (total=1377)	%
3p−	**16**	**1.2**
−5	**92**	**6.7**
5q−	**349**	**25.0**
5q− (sole)	**184**	**13.0**
−7	**171**	**12.0**
−7 (sole)	**78**	**5.7**
7q−	**69**	**5.0**
Der(1;7)	**25**	**1.8**
+8	**287**	**21.0**
+8 (sole)	**177**	**13.0**
11q−	**35**	**2.5**
Der(12p)	**82**	**6.0**
13q−	**23**	**1.7**
−17	**59**	**4.3**
Der(17p)	**48**	**3.5**
−18	**54**	**3.9**
20q−	**82**	**6.0**
20q− (sole)	**51**	**3.7**
−21	**35**	**2.5**
−Y (sole)	**64**	**7.6**

2.1.6 Pathogenesis

2.1.6.1 Clonal stem cell disorder

MDS is a heterogeneous disease, ranging from chronic states of cytopenia to pre-leukemic disorders generally progressing rapidly to AML.[22] Most types of MDS are considered to be clonal disorders of an early hematopoietic progenitor or stem cell. Clonality has been demonstrated using fluorescence in situ hybridization (FISH) analysis in patients with known cytogenetic aberrations, generally demonstrating clonal involvement of the hematopoietic stem cells and all myeloid lineages, and less often also B and NK cells.[31-34] In addition, several studies have shown a nonrandom X-inactivation pattern in all MDS categories, including patients with RARS.[35-38] A proportion of the patients with MDS have an autoimmune attack on the hematopoiesis, although the most likely the initiating event is a malignant transformation of a hematopoietic stem cell, as further discussed in section 2.1.6.5.

2.1.6.2 Genetic alterations

MDS is considered to require multiple hits, and to date, no single genetic lesions has been shown to be sufficient for the development of the disease. The first identified molecular lesion in MDS was an activating mutation of the *NRAS* oncogene.[39] The reported average frequency of *NRAS* mutations is 12.5% (range 6 - 48%) with higher frequency in patients with increased blast counts[40,41] Mutated *NRAS* correlates to an increased risk of AML evolution, however, larger studies are needed to confirm whether it is an independent risk factor.[40,41]

The tumor suppressor gene *TP53* is the most frequently mutated gene in cancer and is of major importance for the genomic integrity and stability. However, *TP53* is only mutated in 8-14% of patients with *de novo* MDS, and it is often associated with loss del(17p13), complex karyotype, resistance to chemotherapy, and an exceedingly poor outcome.[40,42,43]

Several genetic aberrations have prognostic implications in AML, however, these are less often seen in MDS. Mutations of *AML1* occur in around 2% MDS patients without blast increase and in 19% in patients with excess blasts[44] Recently, *AML1* mutations have been associated with adverse outcome in MDS.[45] *FLT-3* mutations are found in less than 1% of MDS patients, *KIT* mutations in 1.2%, and *MLL* partial tandem duplications in 2.7%.[41]

A particular type of CMML carries a t(5;12)(q33;p13) translocation resulting in a fusion gene of the tyrosine kinase domain of platelet-derived growth factor β (*PDGFR beta*) and the gene *tel*, resulting in a constitutive activation.[46] This rare subgroup responds well to the tyrosine kinase inhibitor imatinib.[47]

2.1.6.3 Mutations of the nucleophosmin gene

The nucleophosmin gene (*NPM1*) is the most commonly muted gene in AML with normal karyotype,[48-50] however, its role in MDS is less well studied.[51-54] Mutations can be detected through gene sequencing or by immunohistochemistry showing aberrant cytoplasmic nucleophosmin (NPMc).[55] *NPM1* mutations are rarely found in MDS or AML with abnormalities of chromosome 5, and has never been reported in 5q- syndrome.[51,52,54,56]

NPM1 encodes a nuclear phosphoprotein shuttling between the nucleus and the cytoplasm playing an important role in ribosome biogenesis, chromosome duplication, and genomic instability by regulating p53 levels and activity.[55] Furthermore, mice heterozygous for *NPM1* develop MDS like features and are susceptible to tumor development, in particular myeloid malignancies.[57]

2.1.6.4 Epigenetic alterations

The most studied epigenetic alterations in cancer are promoter hypermethylation and histone deacetylation, although several other ways of epigenetic modulation of gene expression exist.[58,59] Hypermethylation most often occurs in the CpG islands of gene promoter regions, reducing the gene expression. The genes silenced in this way are most often tumor suppressors. In contrast, there is generally a low global methylation of CpG dinucleotides occurring at other locations within the genome, indicating an active overall gene transcription and conceivably leading to a greater genomic instability and loss of imprinting.[58,59]

In MDS, hypermethylation of any of the tumor suppressor genes $p15^{INK4b}$, *HIC1*, *ER*, *CDH1* is associated with adverse survival and increased risk of leukemic evolution.[60] Despite the promising clinical effects of hypomethylating agents in MDS, the methylation status of specific genes poorly predicts the probability of response to this treatment. However, a recent study suggests that patients with hypermethylation of $p15^{INK4b}$, *HIC1*, and *CDH1* have a low probability of response to intensive chemotherapy.[61]

Histone deacetylation is associated with reduced transcriptional activity.[58,59] Clinically, histone deacetylase (HDAC) inhibitiors have had moderate efficacy in MDS,

and currently more potent drugs as well as combinations with hypomethylating agents are under investigation.[62]

2.1.6.5 Immune mediated attack on the hematopoietic progenitors

A minority of MDS patients reside on the diagnostic border between MDS, aplastic anemia, and paroxysmal nocturn hemoglobinuria (PNH). These MDS patients most often have a hypocellular bone marrow and there is a clear link to the HLA haplotype DR15.[63,64] Immunosuppressive therapy can induce long lasting responses in this subcategory, and it is conceivable that some of these patients do not have a clonal disease.[38,65,66]

It is clear that there is an oligoclonal expansion of T-cells in MDS.[67,68] Furthermore, autologous CD8+ T-cells can suppress the growth of erythroid and granulocytic progenitors both of MDS and normal origin, suggesting the presence of "collateral damage".[68-70] It remains to be determined whether the lymphocyte attack on the bone marrow progenitors is the primary disease mediating event or the result of inherent changes in the malignant cells leading to recognition by the immune system.[66]

2.1.6.6 Increased apoptosis in the bone marrow

Most patients with MDS have a normo- or hypercellular bone marrow, with an expansion of the progenitor compartment. Increased apoptosis in the progenitors, which is a hallmark of MDS, results in peripheral cytopenias.[71] Both the extrinsic and the intrinsic pathways of apoptosis have been shown to be involved.

The death receptors (Fas, TNF-α, and TRAIL), and the Fas-associated death domain (FADD) can be over-expressed in MDS.[72-75] However, blocking the extrinsic pathways have generated conflicting results *in vitro*,[74,76-78] and clinical studies utilizing tumor necrosis factor α (TNF-α) inhibitors failed to demonstrated any significant activity.[79]

The intrinsic pathway also plays a major role in the apoptosis observed in MDS. The pro-apoptotic members of the Bcl-2 family are up-regulated in low-risk disease, leading to increased apoptotic signaling.[80,81] Defects in the mitochondrial function may also be present. The ringed sideroblasts observed in RARS are in fact iron-overloaded mitochondria, with the iron bound to aberrant mitochondrial ferritin.[82,83] In patients with RARS, there is a constitutive leakage of cytochrome *c* from the mitochondria, leading to subsequent caspase activation and increased apoptosis.[84] Furthermore, mutations of the mitochondrial DNA are present in as much as half of the patients with MDS.[85]

2.1.6.7 Therapy related MDS

Therapy related MDS (t-MDS) together with therapy related AML (t-AML) constitute a unique entity in the WHO 2001 classification.[26] The strongest associated exposures are alkylating agents, topoisomerase II inhibitors, and radiation.[86] Alkylating agents are typically associated with delayed development of t-MDS with the cytogenetic abnormalities del(5q)/-5 or del(7q)/-7, while topoisomerase II inhibitors more frequently induce t-AML early after exposure, with balanced translocations involving 3q26, 11q23, and 21q22.[28,54] The most commonly mutated genes are *TP53* (24-46%) and *AML1* (13-38%).[54] *TP53* mutations are associated with chromosome 5 abnormalities, whereas *AML1* mutations occur more frequently in patients with del(7q) or -7.[54] In addition, abnormalities leading to a deletion of 17p (including the *TP53* locus) are more frequently observed in t-MDS than in *de novo* MDS, and are strongly associated with *TP53* mutation.[28,54,87] *NPM1* mutations and *FLT3* mutations or internal tandem duplications are less often seen in t-AML compared to *de novo* AML, and are rarely seen in t-MDS.[54,88] Complex karyotypes are almost twice as common in therapy related vs. *de novo* MDS, occurring in around 20% of patients.[28,54] The clinical outcome in t-MDS and t-AML is equally poor.[89]

2.1.6.8 The 5q- syndrome

2.1.6.8.1 History

The 5q- syndrome was first described by Van den Berghe *et al* in 1974 in three patients with refractory anemia characterized by erythroid hypoplasia, hypolobulated megakaryocytes, normal to elevated platelet counts, and an interstitial deletion of the long arm of chromosome 5.[90] The sole deletion at 5q was the second chromosomal abnormality, after the Philadelphia chromosome (t[9;21]) recognized to be linked to a certain malignancy. The 5q- syndrome was acknowledged as a separate disease entity in the WHO classification of 2001.[26]

2.1.6.8.2 Origin at the hematopoietic stem cell level

Lars Nilsson *et al* showed that 5q- syndrome originates at the HSC level by demonstrating that 99% of the $CD34^{+}CD38^{-}Thy\text{-}1^{+}$ HSC carry the deletion.[31,32] Furthermore, del(5) is not limited to the myeloid progenitors, it can in some cases be detected also in B- and NK-cells, suggesting an origin at a multipotent stem cell level.[31-34] However, the 5q- HSC are in several ways abnormal. Functional studies show that 5q- HSC do not repopulate lethally

irradiated mice, and they fail to grow in long-term culture-initiating assays *in vitro*.[31] Gene expression profiling shows that del(5q) and normal HSCs have a highly similar expression profile, with few but intriguing exceptions, including up-regulation of the HSC renewal factor *BMI1*.[91]

2.1.6.8.3 Commonly deleted region

Jacqueline Boultwood *et al* first described a small commonly deleted segment (CDS) at 5q31-32 of only 1.5 megabases.[92,93] The CDS includes around 44 genes, and several of them have been implicated in other forms of cancer (Figure 2). Interestingly, research groups studying high-risk MDS or AML with del(5q), have indentified commonly deleted regions located more centromeric on 5q.[94-96] This suggests another disease mediating mechanism in high-risk myeloid disorders as compared to the classical 5q- syndrome, although most patients have a deletion spanning over all the described segments.

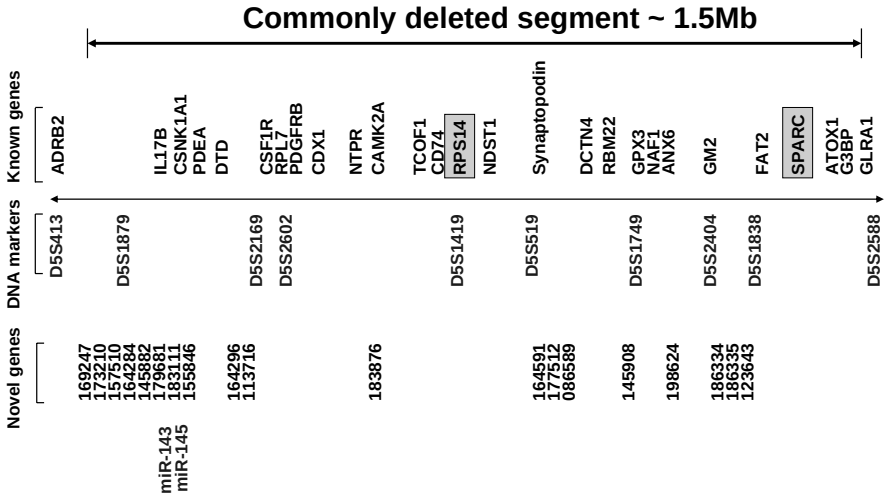

Figure 2. The commonly deleted segment in 5q- syndrome includes 44 genes, including the ribosomal gene *RPS14* and the tumor suppressor gene *SPARC*. Adapted from Boultwood *et al* Blood 2002; 99:4638-41.

2.1.6.8.4 The search for disease mediating genes

During the last two decades there has been an intense research regarding the key disease mediating genes within the CDS. Boultwood *et al* has sequenced all genes within the CDS without identifying any point mutations.[97,98] This led to the hypothesis that

haploinsufficiency of one or more genes within the CDS may mediate the expansion of 5q- progenitors in the bone marrow. In 2008, Ebert *et al* assessed the effect of down regulation of each gene within the CDS, using RNA interference, and demonstrated that decreased expression of *RPS14* causes a block specifically in the erythroid maturation, as is typically seen clinically in the 5q- syndrome.[99] *RPS14* is a component of the ribosomal 40S subunit, and interestingly another other ribosomal gene *RPS19* was recently found to cause the congenital disease Diamond-Blackfan anemia – which is characterized by an erythroid hypoplasia in the bone marrow and chronic anemia, thus resembling the clinical picture of the 5q- syndrome.[100,101]

2.1.7 Prognosis

2.1.7.1 The International Prognostic Scoring System

The first generally accepted and widely used prognostic score for MDS was developed by Peter Greenberg and the International MDS Risk Analysis Workshop (IMRAW) in 1997.[102] The score was named "International Prognostic Scoring System" (IPSS) and was based on percentage of bone marrow blasts, number of cytopenias, and karyotype (Table 4). The study cohort consisted of 816 primary MDS patients from Europe, the United States, and Japan. All patient data were reassessed in detail by the workshop. Four risk categories were proposed based on a multivariate analysis: Low, Intermediate-1 (Int-1), Intermediate-2 (Int-2), and High risk, all of which distinctly predicted overall survival and risk of AML evolution (Figure 3). Patients were also stratified according to age, and within the Low and Int-1 risk categories there was a significantly longer overall survival in patients ≤70 years of age. The association of age and survival was not evident in the higher risk categories, conceivably due to the high disease related mortality. There was no association of age with the rate of AML evolution.

2.1.7.2 Prognostic impact of rare cytogenetic abnormalities

The IMRAW categorization of karyotypic abnormalities in MDS has been a valuable tool when assessing patients for prognosis and optimal treatment. However, due to the limitation of patient number, rare abnormalities (non-complex) were placed in the intermediate risk karyotype group.[102] Haase *et al* recently published data on the prognostic impact of karyotypic abnormalities in 2124 MDS patients.[103] The size of the study cohort enabled recognition of infrequent aberrations with good (+1/+1q, t[1q], del[5q], t[7q], del[9q], t[11q], del[12p], del[15q], t[15q], -21, -X, -Y), intermediate (Rea 3q, -5, del[7q], -7,

Table 4. The International Prognostic Scoring System (IPSS) for MDS.[102]

Prognostic variable	0 points	0.5 points	1.0 point	1.5 points
Number of cytopenias*	0 – 1	1 – 2	-	-
Karyotype†	Good	Intermediate	Poor	-
Bone marrow blasts (%)	<5	5-10	-	11-20

Risk group	Total score
Low	0
Intermediate-1	0.5-1.0
Intermediate-2	1.5-2.0
High	≥2.5

* Cytopenias defined as a hemoglobin-level below 100 g/L, platelet counts below 100 x 10^9/L, and absolute neutrophil counts below 1.8 x 10^9/L.
† Good: normal, -Y, del(5q), del(20q); Poor: complex (≥3 abnormalities) or chromosome 7 anomalies; Intermediate: all other abnormalities.

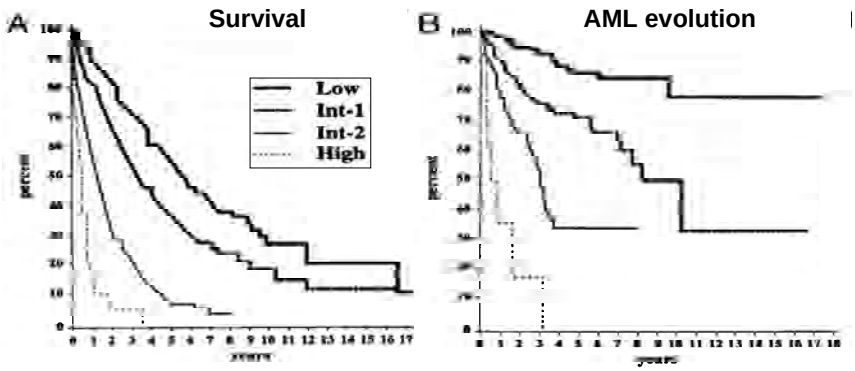

Figure 3. International Prognostic Scoring System (IPSS).[102]

Kaplan-Meier curves describing the estimated probability of (A) survival and (B) freedom of AML-evolution in the four risk groups.

Adapted from Greenberg *et al* Blood 1997; 89:2079-88.

+8, del[11q], t[11q23], +19, complex karyotype of 3 abnormalities), and poor (t[5q], complex karyotypes of ≥4 abnormalities) prognostic implications. Of particular interest was the observation that patients with translocations involving the long arm of chromosome 7 (t[7q]) were placed in the good prognostic category, whereas the IMRAW categorized them as poor risk. Also, the heterogeneous IPSS intermediate risk group was hereby risk-stratified in greater detail, and the novel poor risk group only incorporates the extremely high-risk karyotypes, including complex karyotypes with ≥4 abnormalities, and perhaps less expected t(5q).

2.1.7.3 Prognostic impact of multilineage dysplasia, transfusion dependency, and iron overload

Several studies have demonstrated that multilineage dysplasia is associated both with shorter survival and increased risk of AML evolution.[104-107] The stratification of RA and RARS based on the presence of multilineage dysplasia therefore constitutes one of the most important additions of the WHO 2001 classification.

The IMRAW workshop in 1997 clearly identified anemia (defined as a hemoglobin [Hb] level <100 g/L) at time of diagnosis to be associated with shorter survival.[102] Luca Malcovati *et al* dissected this issue further; in a time-dependent multivariate Cox model they demonstrated that the development of a RBC transfusion need during the course of the disease was significantly associated with shorter survival and increased risk of leukemic evolution.[107,108] Furthermore, the total number of RBC units received as well as the number of RBC units per month were also associated with survival and risk of transformation to AML.

Malcovati *et al* also demonstrated a significant correlation between transfusion need and the risk of leukemic evolution. Therefore, the presence of RBC transfusion requirement most likely reflects an adverse biology of the disease.[107,109] However, chronic RBC transfusions also increased the risk of iron overload, which also may affect survival mainly due to a higher rate of congestive heart failure.[107] Interestingly, progressive iron overload was associated with a 40% increased risk of death for every 500 ng/mL increase in serum (S) ferritin above 1000 ng/mL.[107] The negative effect of iron overload was most evident in the low-risk WHO-categories RA and RARS, with longer expected survival.

2.1.7.4 WHO-classification based prognostic scoring system

The IPSS scoring system has proved to be highly useful in clinical decision making, and is currently the standard risk score in prospective studies. However, the IPSS is based on data from diagnosis and is therefore not designed to be used during the course of the disease or at time of progression.[102] Furthermore, it does not account for the recently acknowledged prognostic implications of transfusion-dependency or multilineage dysplasia. In order to encompass all of the above, Luca Malcovati *et al* developed a time-dependent prognostic scoring system in 2007 called the WHO-classification based prognostic scoring system (WPSS)[109]. The study cohort consisted of 426 primary MDS patients (1992-2004) from Pavia, Italy. All patients were reclassified according to the WHO criteria and data on RBC transfusion need were recorded. In a previous study by Malcovati *et al*, WHO-category, karyotype risk group, and RBC transfusion requirement had been identified as the main disease related prognostic factors, and thus, these three variables were used in the WPSS model. The WPSS recognizes five prognostic groups, ranging from very low to very high risk. Interestingly, in patients above 70 years of age and with very low risk MDS, the overall survival was not significantly shorter than that of the general population. The scoring system was also validated in a cohort of 739 untreated MDS patients (1982-2003) from Düsseldorf in Germany.

The main advantage of the WPSS is its ability to identify patients within the FAB RA and RARS subgroups with adverse prognosis, namely those with multilineage dysplasia or RBC transfusion need. In addition, 271 and 193 Italian and German patients, respectively, were assessed with repeated measures and were used to generate a time-dependent model, with the advantage of allowing repeated assessment of the prognosis of a patient during the course of the disease and at time of progression.

2.1.8 Treatment

2.1.8.1 Transfusion therapy

The vast majority of all MDS patients develop a red blood cell (RBC) transfusion need during the course of their disease.[22] Anemia *per se* is associated with severe impairment of quality of life and constitutes a risk factor for heart failure related death, as discussed in section 2.1.7.3. Furthermore, several studies have shown that the quality of life increases linearly when elevating the Hb level up to the normal range in patients with anemia.[110,111] Hence, a modern chronic transfusion therapy aims at maintaining a Hb level of

90 - 100 g/L or higher, depending on age, co-morbidities, and the patient's preference.[112,113]

Around 20% of patients with MDS present with a significant thrombocytopenia, and a proportion of these require platelet transfusions to prevent bleeding symptoms.[114] Unfortunately, platelet transfusions are costly and often give rise to allo-immunization, leading to poor response.[114] Matched donors is an option, but is limited by high expenses and poor availability. Treatment with fibrinolysis inhibitors such as tranexamic acid can ameliorate in particular bleeding symptoms from mucous membranes, and danazol has been shown to have some efficacy.[115] AMG531, a new thrombopoiesis stimulating peptibody, is currently under investigation in MDS.[116,117]

2.1.8.2 Iron chelation

Transfusion related hemochromatosis affecting the heart and liver, is a reality in patients receiving chronic RBC transfusions.[118] Iron chelation therapy is life saving in patients with beta thalassemia major,[119] however, the benefit of chelation in MDS is insufficiently studied.[120,121] The risk of cardiac events in thalassemia increases significantly beyond a S-ferritin level of 2500 ng/mL. Even with full chelation therapy it can take months or even years to adequately reduce a greatly elevated S-ferritin, and thus, it is important to view chelation as a preventive measure rather than a therapeutic. Most patients reach a S-ferritin level of above 1500 ng/mL after having received 20-25 RBC units, although some patients present with an elevated S-ferritin already at diagnosis as the result of an ineffective erythropoiesis. International guidelines recommend iron chelation to transfusion dependent MDS patients with a reasonable expected survival and a S-ferritin exceeding 1500 ng/mL.[24,113,122,123] Recent data supports this strategy since significant iron-overload and excessive heart failure related deaths are mainly observed in MDS categories with favorable prognosis,[107] conceivably due to the fact that it takes several years to develop a symptomatic iron-overload, and this exceeds the expected survival in high-risk patients.

The first line therapy is deferoxamine given as subcutaneous or intravenous infusion.[24,113,122,123] Unfortunately the parenteral administration negatively affects compliance. Two oral chelators are currently available, deferiprone and deferasirox, and both have efficacy in MDS.[120,123] Deferiprone carries approximately 1% risk of agranulocytosis and 5% risk of less severe neutropenia, which can occur at any time during treatment. Deferasirox can impair the renal function, and the S-creatinine level has to be monitored closely.[123]

2.1.8.3 Erythropoietic growth factors

Treatment with erythropoietic growth factors is an effective treatment of anemia in MDS, and is recommended by all major international guidelines.[24,113,122,124,125] Response rates in low-risk MDS varies between 32 and 82%, depending on patient selection.[126] Recombinant human erythropoietin (EPO) is given as subcutaneous injections one to three times per week, in a weekly dose of 30 000 to 60 000 units (U). Two randomized studies in MDS have demonstrated superiority of EPO over placebo, however, in the largest one (n=87) the RA subtype was the only one for which the difference reached significance.[127,128] The median response duration to erythroid growth factors is around two years.[129-131]

Darbepoetin-α (DAR) is a long-acting form of EPO, and a series of phase II studies in MDS have shown response rates similar to those observed with EPO treatment.[132-135] Interestingly, there are reports of MDS patients responding to DAR after being primary refractory to standard treatment with EPO.[132,134] DAR is administered subcutaneously with intervals of 7 to 21 days, in doses of 150 to 300 μg per week.

During the last decades there have been several response criteria used in parallel, some stricter than others. An international working group (IWG) proposed uniformed response criteria for MDS in 2000,[136] and these were subsequently revised in 2006 in order to be more clinically relevant.[137] Due to the great difference in expected response rates depending on patient selection, as well as different dosing regimens and varying response criteria, it is a difficult task to determine whether any specific type of erythroid growth factor or treatment regime is superior of another. A recent meta-analysis compared treatment with EPO-α (n=589; 9 studies) vs. DAR (n=389; 8 studies) and concluded that the response rates for both drugs were highly similar (58 and 59 %, respectively, P=0.82), according to the IWG 2000 criteria.[126]

The addition of granulocyte colony-stimulating factor (G-CSF) to EPO significantly enhances the response rate compared to using EPO alone. The evidence of a synergistic effect rests on data from two randomized trials[138,139] as well as several studies describing patients not responding to EPO but responding to the combination, and moreover, that such patients may loose their response when G-CSF is with drawn and regain it when G-CSF is reintroduced.[129,140]

It has been consistently shown that the response rate correlates strongly to S-EPO level and degree of transfusion need,[131,141-144] and based on these factors Hellström-Lindberg *et al* developed a predictive model for response.[130,142] The model identifies patients with low, intermediate, and high probability of erythroid response (Table 5).

Treatment with subcutaneous EPO at the high dosing recommended in MDS gives a maximum S-EPO concentration of around 800 U/L,[145] making the cut-off level of endogenous S-EPO of 500 U/L associated with poor predicted response to growth factor treatment intuitive. In addition, patients with unilineage dysplasia respond better than patients with multilineage dysplasia, supporting the use of the WHO classification,[106,131] and low-risk categories according to the IPSS respond better than high-risk categories.[131] The probability of response also decreases with longer interval from the time of diagnosis to start of treatment.[131] A recent large multivariate analysis, adjusting for all major prognostic variables, identified the following four independent predictors for higher response rate: S-EPO <200 U/L, absence of RBC transfusion need, IPSS risk groups Low/Int-1, and shorter interval between diagnosis and start of treatment.[131] Finally, patients with refractory anemia and ringed sideroblasts respond better to EPO and G-CSF than to EPO alone, and should be given the combination up-front.[113,129-131,146]

Several studies have shown that EPO with or without G-CSF improves quality of life in MDS, in particular decreasing the experience of fatigue.[130,133,139,147] Most likely this is attributable to maintaining a higher mean Hb level compared to being chronically transfused, but other effects, such as lower degree of iron-accumulation may also have a positive impact.

There has been a concern that the anti-apoptotic and pro-proliferative effects of erythroid growth factors may increase the risk of AML evolution in MDS. No randomized trial has yet been performed with survival or risk of leukemic evolution as endpoints. However, a preliminary reported randomized and placebo controlled study (n=102), originally designed to evaluate the effect on neutropenia and risk of infectious complications in MDS, showed no association between chronic treatment with G-CSF and the risk of leukemic evolution .[148]

The American Society of Hematology and American Society of Clinical Oncology guideline from 2007 states that the Hb target value should be 120 g/L, based on increased risk of thrombo-embolic events and concern of adverse effects on outcome in patients with cancer, as further discussed in section 2.2.7.[125]

Table 5. Predictive model for erythroid response to EPO and G-CSF treatment of anemia in MDS.[130,142]

Variable	Value	Score
Transfusion-need	<2 U	0
(RBC/month)	≥2 U	1
Serum-EPO	<500 U/L	0
	≥500 U/L	1

Predictive group	Total score	Response rate
Good	0	74%
Int	1	23%
Poor	2	7%

2.1.8.4 Immunosuppressive treatment

Immunosuppressive treatment in the form of anti-thymocyte globulin (ATG) or cyclosporine-A (CyA) has been evaluated in a number of studies in low-risk MDS, demonstrating highly variable response rates and durations.[63,64,67,149-151] Several studies have identified bone marrow hypocellularity, lower age, and presence of HLA DR15 as positive predictive factors.[63,64,150] Evidence also suggests that RA and RCMD patients respond better than patients with ringed sideroblasts, and that shorter duration of transfusion dependency increases the probability of response. ATG depletes the T-cells which greatly increases the risk of infections in particular during the first months post-treatment. ATG also carries a significant risk of serum-sickness and is poorly tolerated in patients above 70 years due to considerable toxicity, including cardiac events.[151]

Limited evidence supports the use of CyA maintenance after ATG therapy, in analogy to the current standard of care in aplastic anemia.[64] CyA alone has been shown to give tri-lineage and long-standing responses, although the efficacy is considerably lower than for ATG.[66] CyA can cause nephropathy, and the renal function needs to be monitored regularly.

2.1.8.5 Lenalidomide

The immunomodulatory drug lenalidomide has a US label for MDS with del(5q) since December 2005 based on the dramatic effects demonstrated in this subgroup; 67% major

erythroid responses and 45% complete cytogenetic remissions.[152,153] The major side effects in MDS are neutropenia and thrombocytopenia, which occur in 55 and 44% of patients, respectively, and supportive G-CSF treatment is frequently required. The median response duration is around two years, and is longer for patients reaching a complete cytogenetic remission.[154] Interestingly, several complete remissions have been observed also in patients with complex karyotypes that include del(5q), which may translate into a positive effect on outcome in these high-risk patients.[152,153,155] However, due to reports of an unexpectedly high rate of leukemic transformation, the European Medicines Agency (EMEA) decided against approval of the drug in Europe in January 2008 and requested more detailed data on safety.

Lenalidomide also has a clinically significant activity in non-del(5q) MDS, where 26% of low-risk patients become transfusion independent, with a median response duration of 41 weeks.[156] Interestingly, less neutropenia and thrombocytopenia is seen in non-del(5q) patients in comparison to patients carrying the deletion.

2.1.8.6 Hypomethylating agents

The most widely studied hypomethylating agent 5-AZA cytidine (5-AZA) has a potent effect in patients with MDS and is recommended by recent guidelines as first-line therapy in high-risk disease.[62,113,122] In a randomized phase III trial in 2002, Silverman *et al* demonstrated an overall response rate of 60%, and 7% complete remissions.[157] Moreover, treatment with 5-AZA was significantly associated with prolonged time to AML evolution or death. A recently reported phase III trial in 358 patients with an IPSS Int-2 or High, also including RAEB-t according to the FAB classification, demonstrated 9 month longer median survival compared to conventional care regimens (24 vs. 15 months, respectively, P=0.0001).[158] Conventional care in this study was to be decided *a priori*, and consisted of AML-like induction therapy, low-dose ara-C, or supportive care only. The study was not powered to assess each subgroup separately, however, treatment with 5-AZA was associated with better survival in all three groups, although not reaching significance in patients receiving induction chemotherapy. In addition, there was a trend of better over all survival in patients receiving 5-AZA in all strata when stratifying for age, WHO category, cytogenetics, and proportion of bone marrow blasts. The hazard ratio (HR) for survival in a multivariate analysis was 0.58 for treatment with 5-AZA vs. conventional care.

Decitabine has also been evaluated in MDS, and the response characteristics in a randomized phase III study were comparable to those of 5-AZA.[159] Results from a

large phase III EORTC (European Organisation for Research and Treatment of Cancer) trial designed to assess survival and time to AML evolution is pending.

The Nordic MDS Group recently performed a study of 5-AZA maintenance treatment after achieving a marrow complete remission (CR) following a conventional daunorubicine and ara-C induction regimen.[61] For patients that reached CR and started 5-AZA maintenance therapy, the median progression free survival was 13 months and the overall survival 17 months.[160] Interestingly, none of the patients with promoter hypermethylation of all three tumor suppressor genes studied (*p15^{INK4b}*, *HIC1*, and *CDH1*) reached CR upon induction treatment, and therefore they did not receive 5-AZA due to the study design.[61] Future studies will clarify the role of 5-AZA in pre-induction and pre-conditioning regimens, as well as in maintenance therapy.

2.1.8.7 Low-dose chemotherapy

Low-dose ara-C is the most studied type of low-dose chemotherapy, yielding response rates of around 30%.[161,162] There are also reports of good efficacy in patients with 5q- syndrome.[163,164] The treatment carries significant bone marrow toxicity, and a randomized phase III trial demonstrated no effect on long-term outcome.[161]

Hydroxyurea has some efficacy in CMML, where it can be considered as a palliative treatment.[165] Hydroxyurea or thioguanine are clinically also used in the palliative setting in MDS patients with proliferative disease and an increase of blasts.

Oral low-dose melphalan may be an attractive treatment for selected RAEB patients with a normal karyotype and hypoplastic bone marrow.[166-168] The treatment can be given with few side effects, and the reported response rate is around 30%.

2.1.8.8 Intensive chemotherapy

The CR rate of AML-like intensive chemotherapy in high-risk MDS is 40 to 50% and the median survival for responders is less than two years.[169,170] No survival benefit over supportive care has been demonstrated, unless CR is followed by allogeneic stem cell transplantation. In light of the recent data for 5-AZA the role of intensive chemotherapy in MDS is not well defined. It is still indicated in young patients with a blast increase of more than 10% that are eligible for a subsequent allogeneic stem cell transplantation (allo-SCT), and non-transplant candidates with a highly proliferative disease in transformation, provided they are medically fit.[113] There is no convincing evidence that consolidation courses are of any benefit in MDS.

2.1.8.9 Stem cell transplantation

Allo-SCT is currently the only curative approach to MDS, and all patients should be considered for a potential transplantation at the initial assessment. The most favorable outcome, with the lowest transplant-related mortality (TRM) and the lowest risk of relapse is seen in low-risk disease.[171] However, many low-risk MDS patients can live a decent life for a number of years only with supportive care, and this makes the timing of the transplant a delicate matter since it carries a considerable morbidity and mortality. Cutler *et al* developed a Markov model in order to investigate the influence of delayed transplantation in the four IPSS risk categories.[172] In patients with IPSS Int-2 or High, immediate transplant correlated with the greatest over all survival benefit, while patients with IPSS Low benefited from a delayed transplant – carried out at the time of clinical progression. The optimal timing of transplant for patients with IPSS Int-1 could not be determined, and factors such as young age, high-risk karyotype, severe clinical symptoms, as well as patient's choice should be considered.[113,122,124]

2.2 ERYTHROPOIETIC GROWTH FACTORS

2.2.1 History - a winding road from the lab to the clinics

In 1906 Carnot and DeFlandre demonstrated that plasma from a bled rabbit could induce reticulocytosis in control rabbits, and postulated the presence of a humoral factor that induced erythropoiesis that they named "hemopoietin".[173] In 1977, Miyake *et al* were the first to purify human EPO.[174] This was heroically done by collecting 2550 liters of urine from patients with aplastic anemia, in order to get sufficient amount of EPO. Miyake's group was also the first to clone the EPO gene in 1985, although Lin *et al* published similar results the same year.[175,176] Both groups also managed to produce functional hormone in transfected cell lines *in vitro*, which paved the way for the first clinical trial with EPO by Eschbach *et al* in anemic patients with end-stage renal disease in 1987.[177]

2.2.2 Molecular features

EPO is a glycoprotein of 30.4 kDa, consisting of four α-helical bundles, in total 165 amino acids, and four carbohydrates chains including three complex N-linked oligosaccharides important for stabilization in the circulation and one small O-linked oligosaccharide of uncertain significance.[178] The clinically most studied types of recombinant human EPO are epoetin alfa, epoetin beta, and darbepoetin alfa, all produced in Chinese hamster ovary cell

lines. Darbepoetin alfa is a hyperglycosylated variant of epoetin alfa, containing two extra N-linked oligosaccharides, prolonging the plasma half-life.[178,179]

2.2.3 Production and metabolism

EPO is produced by peritubular cells in the renal cortex in response to decreased O_2 capacity, determined by the Hb level, pO_2, and the O_2 affinity of Hb.[178] Cellular hypoxia momentarily leads to decreased degradation of the hypoxia inducible factor-1α (HIF-1α), resulting in a rapidly increasing expression level. HIF-1α subsequently enters the nucleus and heterodimerizes with HIF-1β, forming a transcription complex increasing the EPO gene transcription.[178] Negative regulation of EPO gene transcription can be mediated by inflammatory factors (including TNF-α, nuclear factor κB [NFκB], interleukin-1 [IL-1], and GATA-2) that may contribute to the anemia of chronic disease.[178]

The metabolism of EPO is incompletely understood. The main route of elimination of EPO is known to be internalization of the EPO/EPO-receptor (EPO-R) complex.[178,180,181] In patients with normal hematopoiesis, this is primarily dependent on the rate of erythroid cell production, following non-linear kinetics, while also extra-medullary sites cells expressing EPO-Rs contribute to a minor part of the elimination, via first order kinetics.[181,182] A minimal proportion of EPO is cleared via the kidneys and liver.[180]

The plasma half-life is 6-8 hours for recombinant EPO and around 24 hours for darbopoietin, following intravenous injection.[178-180] Subcutaneously administered preparations are slowly absorbed and the bioavailability is only around 30%. Despite the low bioavailability, subcutaneous administration requires around 30% lower dosing than the intravenous route, thanks to the extended terminal half-life of 24 hours (EPO) and at least 48 hours (Darbepoetin), respectively.[180]

2.2.4 Signaling pathways

The EPO-R is primarily expressed on erythroid progenitors from the CFU-E (colony-forming unit erythroid) to the pronormoblast stage of differentiation.[178,179] Cells from several other organs have been shown to harbor EPO-R, including the heart, kidney, pancreatic islets, placenta, and brain.[178] The EPO-R undergoes homo-dimerization upon the binding of EPO, resulting in an activation of the Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), phosphatidyl-inositol-3-kinase (PI-3K), and mitogen activated protein kinase (MAPK) signaling cascades leading to cell survival, proliferation, and erythroid differentiation.[178,179] The signaling cascade is

terminated by hemopoietic cell phosphatase (HCP) catalyzing JAK2 dephosphorylation, whereby the EPO-R is internalized and degraded.[178]

2.2.5 Resistance mechanisms

There are several reasons why some anemic patients are primary refractory to EPO or eventually loose their response. A common reason for treatment failure is functional iron deficiency, defined as failure to provide enough iron to the erythroid progenitors despite sufficient iron stores. The main cause of functional iron deficiency is considered to be chronic inflammation although other factors such as vitamin-C deficiency may play a role.[183] In patients with cancer, several studies have shown a significant benefit of combining parenteral iron with erythroid growth factors.[184-186]

Progressive disease or leukemic evolution should always be ruled out in patients with MDS who lose their response to EPO, although this can be demonstrated only in 18-28% of patients, leaving most relapses essentially unexplained.[131,187]

Development of pure red cell aplasia (PRCA) attributable to the development of antibodies towards EPO is exceedingly rare, but should also be considered. Between 1998 and 2003 the incidence of PRCA peaked at 0.03% per year, due to a certain type of pre-filled syringes with epoetin alfa.[188,189] The increased immunogenicity of EPO was caused by an interaction with organic compounds leaking from the rubber stopper due to a reaction with the solvent polysorbate 80 (Tween 80).[190] Since the implantation of silicone coating on the rubber stoppers, the incidence has dropped 10-fold.[188]

Experiments in rats suggest that there is no down-regulation of EPO-Rs as a consequence of high doses of administered EPO. The main cause of anemia evolving under the EPO treatment in rats was identified as an exhaustion of the erythroid progenitor pool.[191] Whether this occurs in MDS patients, with limited numbers of normal progenitors *a priori* due to the expansion of the malignant clone, remains to be determined.

2.2.6 Adverse effects

Erythroid growth factors carries an increased risk for thrombo-embolic events, including deep vein thromboses, pulmonary emboli, strokes, and myocardial infarctions, with a relative risk of around 1.7.[192] The baseline risk of thrombosis should therefore always be considered. In patients with chronic renal failure, it has been demonstrated the cardiac mortality and the risk of thrombo-embolic events is higher if the Hb levels are driven into the normal range (above 135 g/L) as compared with a target of 105-115 g/L.[193]

Flu-like symptoms, arthralgia, and cutaneous reactions can be observed especially at the initiation of treatment. Hypertension may also occur, and should be monitored.[194]

2.2.7 Growth factor treatment in cancer

Several trials have demonstrated an increased quality of life in cancer patients treated with EPO.[111,195-198] In addition, both experimental and clinical studies have suggested an increased chemo- and radio-sensitizing effect of EPO.[199] This led to the hypothesis that treatment with EPO may improve the survival of patients receiving cancer therapy and early studies were promising. However, recent data from several randomized studies have failed to confirm these results. In fact, concerns have been raised that growth factors may increase the risk of venous thrombosis, increase the risk of relapse, and negatively affect overall survival in patients with cancer.[193,199] The effects on mortality and relapse have up until now only been seen in trials recruiting patients with borderline or even no anemia, and the study designs have been suboptimal. In addition, the results are not consistent; several large randomized studies found no negative association with survival.[199] Although this issue has been highly debated, the American Society of Hematology and American Society of Clinical Oncology updated their guideline in 2007 and state that evidence only supports treatment with erythropoietic growth factors in cancer patients with chemotherapy induced as supposed to disease related anemia; MDS constitutes the only exception to this rule.[125] Furthermore, they state that the Hb target value should be 120 g/L.

Expression of EPO-Rs on tumor cells has been proposed as the main mechanism why treatment with EPO could affect the risk of relapse, since signaling via the EPO-R has proliferative and anti-apoptotic effects. However, it is currently unclear whether the EPO-Rs on cancer cells are functional; there is currently no good method to determine this, and *in vitro* results are conflicting.[199]

2.3 LENALIDOMIDE

2.3.1 A tragic history but a promising future

Thalidomide, the parent compound to lenalidomide, was first marketed by the German company Chemie Grünenthal in 1957 as a non-addictive sedative and an anti-emetic drug in particular effective in the treatment of morning sickness in pregnant women (history reviewed by Rajkumar[200] and Melchert & List [201]). Pre-clinical studies in rodents found the drug remarkably non-toxic, and LD50 doses could not even be established. Furthermore, intentional or accidental overdosing in humans up to 140 times the normal dose did not have lethal outcomes. Thalidomide gained tremendous popularity in part due to the lack of suitable alternatives; sedatives such as barbiturates were highly addictive were dangerous to overdose. Thalidomide was marketed under different names in more than 40 countries world wide, however, not in the United States thanks to Dr. Frances Kelsey at the Food and Drug Administration (FDA) who disapproved the application due to lack of sufficient safety data. In November and December 1961, respectively, Widukind Lenz, a German pediatrician, and William McBride, an Australian obstetrician, independently of each other presented convincing evidence that thalidomide, if taken during pregnancy, was associated with a high risk of severe birth defects.[202,203] The drug was immediately withdrawn from the German market, although it took as long as one year until the drug was completely off the market world wide. It is estimated that 10 000 children (5 000 in Germany, 131 in Sweden) were born with birth defects attributed to thalidomide, although the true prevalence including less severe birth defects most likely is much higher. Animal studies addressing the teratogenic potential were performed soon after the withdrawal of thalidomide from the market, and interestingly not all animals were susceptible; similar changes as in humans were initially only demonstrated in New Zealand white rabbits.[204]

A true serendipitous finding was made by Dr Jacob Sheskin in Jerusalem in 1964.[205] He cared for a patient with erythema nodosum leprosum (ENL) – a feared complication of lepra - with fever, joint pain, skin lesions, and difficulties to sleep. Having access to a stock of thalidomide, he prescribed the drug as a sedative, and interestingly, within a few days the ENL completely resolved. After further studies confirming this dramatic activity and following intense discussions, the FDA approved thalidomide for treatment of ENL in the United States. The anti-inflammatory properties of thalidomide were investigated further, and in 1991 it was shown to potently inhibit TNF-α.[206] The drug

has been shown to have activity in a number of inflammatory diseases, including Behçet's syndrome, systemic lupus erythematosus, and graft-vs.-host disease.[207]

Already in the 1960's, thalidomide had been tried in patients with cancer, based on the hypothesis that if it harms the rapidly growing fetus, it may also harm the cancer cells. However, no activity was demonstrated. In 1994 came the first reports of thalidomide's antiangiogenic properties, and this led to an increased interest in the drug since the importance of neo-angiogenesis in cancers was well known.[207] In 1997, came the first report of the successful treatment of a patient with relapsed and refractory patient myeloma, where thalidomide was given as compassionate use. Thalidomide and its derivatives are now widely used and highly active drugs in myeloma.[208,209]

Following the increased interest in thalidomide after discovering its anti-TNF-α activity and antiangiogenic properties, several structural analogues to thalidomide have been synthesized and named immunomodulatory drugs (IMiDs; Figure 4).[207] Studies have shown that several of these compounds including CC-5013 (lenalidomide) have greater activity and less toxicity thalidomide, although the spectrum of effects varies between the different IMiDs.[207] The IMiDs are currently undergoing investigations in various clinical settings, and the results are promising.

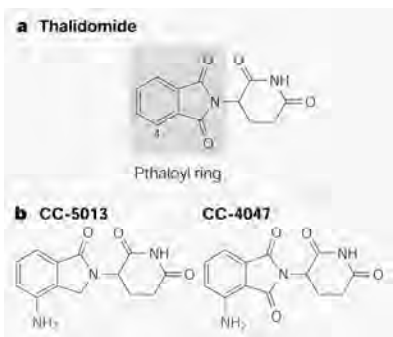

Figure 4. Chemical structure of thalidomide and its analogues.

CC-5013, lenalidomide; CC-4047, pomalidomide

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2.3.2 Mechanisms of action

Lenalidomide and the other IMiDs exert multiple functions (Figure 5) although each drug has its unique activity profile.[207] Lenalidomide is antiangiogenic, as shown by inhibition of the formation of new vessels (rat aorta assay) and attenuation of the effects of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF).[210,211] Thalidomide has an anti-adhesive effect, demonstrated by reduction of the expression of the adhesion molecules ICAM-1 (CD54), VCAM-1 (CD106) and E-selectin on human umbilical vein endothelial cells (HUVEC), and also functionally in decreasing the cell-cell

contact between human leukemic T-cells and HUVEC cells.[212,213] Lenalidomide potently co-stimulate $CD4^+$ and $CD8^+$ T-cells that already are partially activated via the T-cell receptor.[214] This is in part mediated by phosphorylation of CD28, increasing the co-stimulation of the T-cells via the CD28/B7 pathway.[215] Another important immune mediated mechanism of lenalidomide is enhancement of NK cell mediated lysis, demonstrated in multiple myeloma cell lines.[216] The IMiDs also affect several important cytokine circuits, including a highly potent inhibition of TNF-α production.[217,218] Finally, there is a direct effect of lenalidomide on several types of cancer cells, including cell lines with del(5q), inducing growth arrest and apoptosis.[219-221]

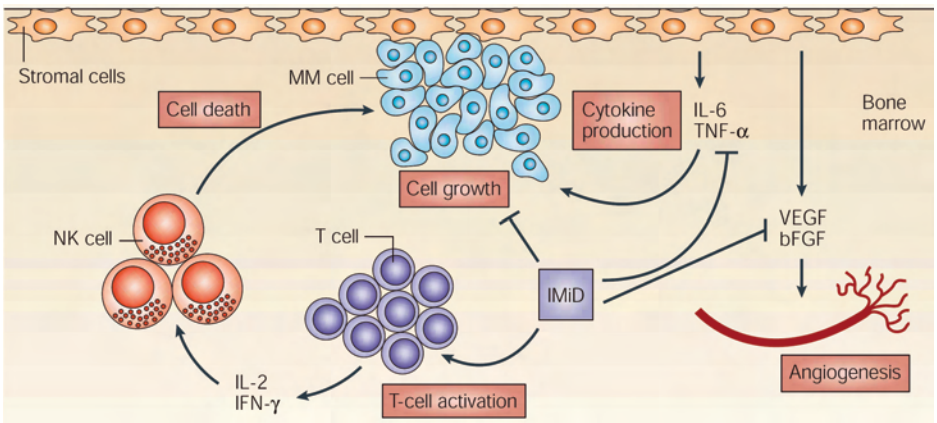

Figure 5. Biological functions of immunomodulatory drugs.

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2.3.3 Pharmacokinetics

Lenalidomide is a lipophilic compound rapidly absorbed upon oral ingestion. It crosses the blood-brain and blood-placenta barriers, and is distributed throughout the body. The C_{max} is reached after 0.6-1.5 hours, and a dose of lenalidomide 25 mg gives an estimated C_{max} of around 2.2 μM.[221,222]

The metabolism of lenalidomide is insufficiently studied. More than 80% of the drug is eliminated unchanged via the kidneys, and the plasma half-life is 3-4 hours, if the kidney function is normal.[222] In patients with end stage renal disease the half-life is

prolonged to around 15 hours, suggesting alternative routes of elimination.[222] Also, lenalidomide is thought to undergo spontaneous hydrolyzation in aqueous solutions, as has been shown for thalidomide, where after the metabolites are eliminated via the kidneys.[201] Liver metabolism is not considered to play a major role.

2.4 SPARC

2.4.1 Physical properties

SPARC (secreted protein acidic and rich in cysteine; also known as osteonectin and BM-40) is a 32 kDa glycoprotein containing three modules: a C-terminal extracellular module with two Ca^{2+} binding EF hands, a follistatin-like module, and an N-terminal acidic module.[223,224] SPARC has a high degree of evolutionary conservation, and is located at chromosome 5q31.3 in humans.[223]

2.4.2 Functions

SPARC belongs to a group of matricellular proteins defined as molecules that directly interact with the extracellular matrix or indirectly via growth factor/proteases, but are not in themselves part of the matrix.[224] Other members of the group include SPP1 (secreted phosphoprotein 1; also known as osteopontin), THBS1 and THBS2 (thrombospondin 1 and 2), TNC (tenascin C), CTGF (connective tissue growth factor), and Sparcl1 (SPARC-like 1; also known as hevin, Ecm2, and mast9).[224]

SPARC exerts diverse functions which may differ depending on type of cell or tissue. The major effects are deadhesion, inhibition of angiogenesis, anti-proliferation, and regulation of extracellular matrix.[224-226] SPARC knock-out mice have several phenotypic abnormalities, including osteopenia and reduced number of osteoblasts,[227] accelerated wound closure,[228] increase in number and size of adipocytes,[229] reduction of the tensile strength of the dermis and reduced collagen formation.[230] Interestingly, SPARC null mice have significantly lower platelet counts and impaired ability to form erythroid burst-forming units (BFU-E) compared to wild-type animals.[231]

2.4.3 Associations with cancer

SPARC has been associated with numerous types of malignancies, however, its role is diverse. SPARC can be up or down regulated in different types of cancer (Table 6). Down-regulation of SPARC can be achieved by promoter hypermethylation or deletion of

the *SPARC* locus at 5q31, as regularly is the case in AML and MDS, although it also occurs in other hematological malignancies and solid tumors .[232-234]

SPARCs role in cancer is complex; it functions as a tumor suppressor gene in several types of cancer, but is associated with invasive growth and metastasis in others (Table 6) SPARC generally inhibits proliferation of malignant cells, even in tumors where SPARC is associated with more aggressive disease. However, results in knock-out mice makes the picture less clear cut; mammary carcinoma cells injected into *SPARC* null mice showed decreased proliferation but a massive parenchymal infiltration,[235]and lung cancer and T-cell lymphoma cell lines generated larger tumors in the *SPARC* null mice in comparison to the wild type counterparts.[236] SPARC is produced both in the malignant cells in the surrounding stroma. In melanoma, the regulation of cancer cell growth is regulated by SPARC produced by the malignant cells themselves, rather than by exogenous SPARC derived from the stromal cells.[237] The situation is less clear in other types of tumors. In conclusion, it is clear that SPARC has diverse effects depending on the type tissue of the cancer originates in, and that SPARC plays an important role in modulating the interaction of the malignant cell with its surrounding stroma.

2.5 THE BONE MORROW STROMA AND THE STEM CELL NICHE

2.5.1 Hematopoietic stem cells

Hematopoietic stem cells (HSC) are functionally described by their ability to mediate long-term repopulation of all hematopoietic lineages after lethal irradiation. Animal studies have shown multilineage repopulation even after transplantation with a single HSC. Furthermore, bone marrow from a transplanted animal can be retransplanted to secondary and even tertiary recipients, without loss of HSC self-renewal and multilineage differentiation capacity.[238,239] Phenotypically, the HSC are considered to reside within the $CD34^+CD38^-CD90^+lineage^-$ compartment, constituting around 0.1% of the bone marrow mononuclear cells.[240] In mice, it has been shown that even more primitive HSC are in fact $CD34^-$, and evidence suggests that this might be the case also in humans.[240] However, mouse studies have shown that $CD34^+$ HSC can revert to $CD34^-$ and also display full self-renewal and repopulating potential.[240] Also, clinical studies have shown that CD34 enriched cells used for autologous or allogeneic SCT do not lead to significantly increased risk of graft failure.[240]

Table 6. The role of SPARC in cancer.

Tumor type	Expression in cancer vs. non-malignant cells	Expression in adjacent stroma	Promoter hypermethylation	Effect of SPARC *in vitro* or *in vivo*
Ovarian cancer	Down[241]			Growth inhibition[241,242]
Pancreatic adenocarcinoma	Down[243]	Up[243]	Yes[243]	Growth inhibition[243]
AML with *MLL* rearrangements	Down, in adult and pediatric patients[244]		Yes, in cell lines; not in primary cells[244]	Growth inhibition[244]
AML without *MLL* rearrangements				No effect on growth[244]
Non-small cell lung cancer	Down[245]	Up[245,246]	Yes, correlates to poor prognosis[246]	
Multiple myeloma	Up in 2 of 6 samples;[247] low expression if promoter hypermethylation[248]		Yes, in 8%, correlates to poor prognosis[248]	
Esophageal carcinoma	Up-regulation correlates to lymphnode metastasis[249]	Up[249]		
Bladder cancer	Up[250]			
Prostate cancer	Up-regulation in metastatic cells[251]		Yes, in cell lines[252]	
Invasive meningioma	Up[253]			
Malignant melanoma	Up-regulation correlates to poor prognosis[254]			Endogenous SPARC inhibits growth[237]
Gastric cancer	Up-regulation correlates to lymph node metastasis and invasive growth[255]			
Colo-rectal cancer	Down[256]		Yes[257]	Enhances apoptosis via interaction with pro-caspase 8[258]
Breast cancer		Up[259]		Growth inhibition[260]
Hepatocellular carcinoma		Up[261]		
Cervical cancer			Yes[262]	
Neuroblastoma				Inhibits growth and angiogenesis[263]
Glioma				Inhibits growth but promotes invasion[264]

2.5.2 MDS originates at the hematopoietic stem cell level

MDS originates at the hematopoietic stem cell level, and multilineage dysplasia can often be demonstrated. All myeloid lineages are frequently involved, although clonality of B and NK cells also have been described in patients with the chromosomal aberrations del(5q), -7, and trisomy 8, suggesting malignant transformation in an early HSC.[31-34]

2.5.3 The stem cell niche

The HSCs reside close to the endosteum, in proximity of the fenestrated endothelium of the bone marrow sinusoids, where several types of cells provide supportive signals.[239,265] The endosteal and perivascular areas may constitute separate niches, although recent evidence suggests that they in fact are part of a common niche.[265] In addition, vascular cells in the liver and spleen are able to support HSCs for long periods of time, suggesting the presence of extramedullary HSC niches.[265]

CXCL12 (stromal derived factor-1 [SDF-1)] is a chemokine that is secreted by several types of stromal cells in the bone marrow including osteoblasts and perivascular reticular cells.[265] The interaction between CXCL12 and its receptor CXCR4 on the HSCs is crucial for retaining the HSC in the bone marrow and for establishing normal hematopoiesis.[239,265] HSC maintenance also requires thrombopoietin and angiopoietin. Both factors are secreted in part by the osteoblasts, although thrombopoietin is mainly produced in the liver and kidney, while angiopoietin is secreted by perivascular cells in the bone marrow, including magakaryocytes.[265] The Rho GTPases RAC1 and RAC2 regulate the actin cytoskeleton and its interaction with cell surface adhesions molecules, which is necessary for the homing of the HSCs to the bone marrow stem cell niche.[266] Crosstalk between the HSCs and the cells of the niche is also mediated via other molecules including KIT and membrane-bound stem cell factor (SCF), VLA4 and VCAM1, Notch and Jagged, and homotypic interaction between N-cadhering, although the relative importance of these interactions are not fully understood. (Figure 6).[239,265]

2.5.4 Stromal defects in MDS

The level of CXCR4 on $CD34^+$ progenitors from MDS patients is similar to that of normal progenitors, however, the migration toward CXCL12 is abrogated, which may contribute to the ineffective hematopoiesis.[267] Stromal cells from MDS patients show decreased ability to support normal hematopoietic cells, and evidence suggests disruption of the Notch signaling pathway.[268]

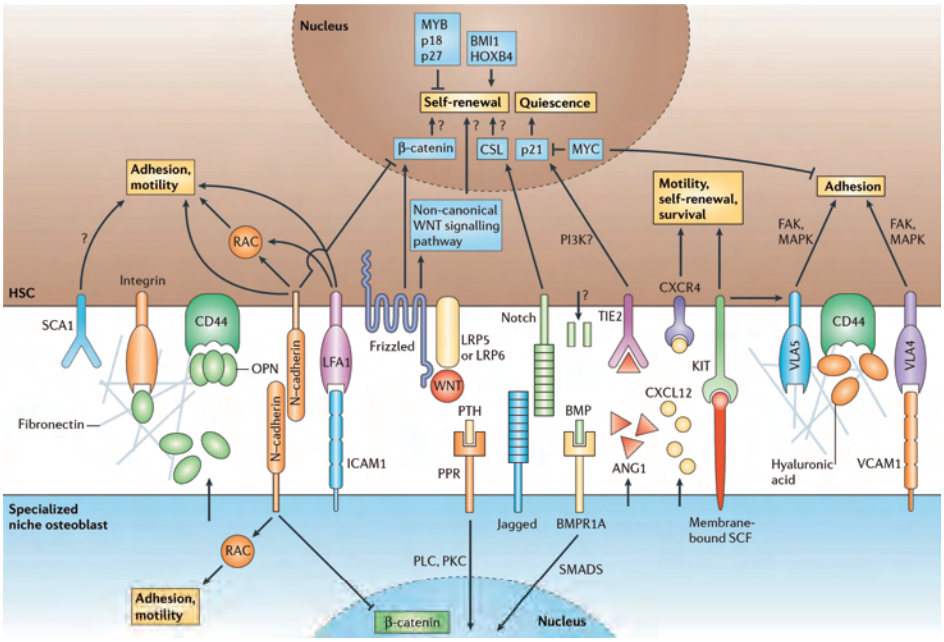

Figure 6. The interaction of the hematopoietic stem cell with its niche.

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Wilson & Trumpp, Nat Rev Immunol 2006; 6:93-106 ©

There is conflicting evidence if the stromal cells in MDS can be part of the malignant clone, which could be one explanation for an altered function of the stroma. However, long-term MDS marrow cultures demonstrated a low percentage of remnant macrophages, and FISH assessment of chromosomal abnormalities never reached above the proportion of macrophages plus the detection threshold of the probes, suggesting that the stromal cells were not part of the clone.[269] Recently, del Cañizo *et al* reported that mesenchymal stem cells of patients with 5q- syndrome may be of clonal origin (3rd Symposium on Recent Advances on Myelodysplastic Syndromes, Salamanca, Spain, May 2008). In summary, it is clear that the interaction between the HSCs and the stroma may be disturbed in several ways in MDS, providing a strong rational for targeting molecules involved in this interaction.

2.6 SURVIVAL ANALYSIS IN CANCER

2.6.1 Non-parametric procedures

Survival analysis constitutes a statistical test were the associations with a terminal event are estimated taking the duration of follow-up into account. The terminal event may be the outcome (such as death or development of AML) or censoring, the end of follow-up.[270,271] The most widely used type of survival analysis in clinical studies is the Kaplan-Meier estimate of the survivor function (describing the estimated probability of survival at time *t*), where the projected median survival is determined.[272] The Kaplan-Meier estimate requires no assumption of the underlying hazard function and is therefore considered to be non-parametric. The Kaplan-Meier procedure can also estimate the hazard function, describing the estimated probability of having experienced a terminal event by a specific time point *t*.

To determine whether the survival is significantly different between two strata, the most commonly used method is the Mantel-Haenszel procedure (log-rank test; Mantel-Cox analysis).[273] The Mantel-Haenzel procedure assumes proportional hazards in the two groups and is therefore in fact a semi-parametric test. The test statistic is derived from the rank of the survival times of the subjects within each stratum. To determine if there is a significant difference across more than two strata, the log-rank test for trend can be used.

2.6.2 Modeling survival data

When comparing the survival of two or more groups of patients they may differ substantially in a variety of characteristics, including age and other factors relevant to disease risk. One way to address this is to use a multivariate model, adjusting for important features that may be related both to the exposure of interest and the outcome (confounding). The proportional hazards model (Cox-regression model) is the most commonly used multivariate survival analysis in medical science.[274] It requires an assumption of proportional hazards, which means that the hazard of death at any given time for an individual in one group is proportional to the hazard at that time for a similar individual in another group. However, it allows variations in the baseline hazard with time, and is therefore considered to be a semi-parametric model. Using the proportional hazards model, it is possible to determine the HR for each covariate adjusted for in the model (such as age and various risk factors). A HR of 1.0 indicates similar hazard as the reference group, for example that treated patients have similar hazard as untreated. A HR of 2.0

indicates a doubled probability of death at each time point, whereas a HR of 0.5 indicates that the probability of death is only half of that of the reference group. If the baseline hazard function is known, as is conceivable in special cases, it is possible to utilize parametric tests, which have the advantage of estimating the absolute risk of death each time point, not only the difference in hazard.[271]

Another challenge is to properly account for the effects of delayed entry into a study. If a patient was diagnosed three years prior to study entry, he or she may not have the same risk of death as a patient diagnosed more recently, even if the measured risk factors would be equivalent. In such situations it is possible to use a variant of the proportional hazards model, accounting for the delayed entry (Cox regression with delayed entry; left truncation).[275] In such a model, we compare outcomes in subject A with the those in other subjects after a period of *x* days since diagnosis, for all subjects who were under observation in the study *x* days after being diagnosed. In this way, patients with a long period of delay until study entry are compared only with patients surviving at least as long from the time of diagnosis. This analysis requires the assumption that patients entering the study at a certain period of time after diagnosis are representative of patients in the entire study base after the same time period.

3 AIMS OF THE THESIS

The overall aims of this thesis were to investigate the long-term clinical effects and molecular mechanisms of the currently most widely used drugs in the treatment of anemia in MDS.

Specific aims were as follows:

I. To assess the long-term efficacy of treatment of anemia in MDS with EPO and G-CSF.

II. To investigate if treatment with EPO and G-CSF affects survival or risk of leukemic evolution in patients with MDS.

III. To study how lenalidomide affects growth, differentiation, and gene expression of bone marrow cells from patients with low-risk MDS and del(5q).

IV. To investigate the presence of pre-treatment molecular lesions in low-risk MDS patients with del(5q) treated with lenalidomide, who subsequently underwent disease progression.

4 MATERIALS AND METHODS

4.1 CLINICAL STUDIES OF TREATMENT WITH EPO AND G-CSF

4.1.1 Patients

The EPO + G-CSF treated cohort consisted of all 129 evaluable patients from three previous Nordic MDS Group studies (1990-99) on treatment of anemia in MDS with EPO + G-CSF (EPO-G). The inclusion criteria were RA, RARS, or RAEB, according to the FAB classification, in combination with a Hb level below 100 g/L or a regular RBC transfusion need. Exclusion criteria were ongoing bleeding, transfusion dependent thrombocytopenia, or eligibility for allo-SCT.

In paper I, the untreated control group consisted of 334 MDS patients selected from the cohort used by the International MDS Risk Analysis Workshop (IMRAW) to develop the IPSS in 1997 (Table 1, paper I, page 804).[102] The selection criteria were identical to the inclusion criteria in the EPO-G studies. The patients included in the original IMRAW cohort was a merger of seven previous studies (performed in Europe, America, and Japan), spanning from the early 1980's to early 90's.

To address several deficits in the IMRAW dataset (such as lack of recording of transfusion requirements, S-EPO level, and presence of multilineage-dysplasia) we used a more suitable control cohort in paper II, consisting of 272 untreated MDS patients from Pavia, Italy. All patients were reclassified according to the WHO classification by two independent cytologists.[107] Except for supportive care, the patients remained untreated during the follow-up period in line with the current practice in Italy at the time.

Both the Nordic and the Pavia cohorts were enrolled during the same time-period in Western Europe, with detailed recording of important prognostic factors, and only a few patients received iron-chelation therapy. Eight of the 129 EPO-G patients and 35 of the 272 untreated patients lacked information about ≥1 variables included in the multivariate analysis, and therefore 121 EPO-G treated and 237 untreated patients were included in the final analysis (Table 7).

Table 7. Characteristics of the EPO and G-CSF (EPO-G) treated and untreated cohorts of MDS patients in paper II.

Variable	EPO-G (n=121)	Untreated (n=237)	Cohort differences (*P*-values§)
Median age, years (interquartile range)	71 (65-79)	66 (58-73)	<0.0001
Sex, n (%)			0.17
Male	66 (54.6)	147 (62.0)	
Female	55 (45.4)	90 (38.0)	
WHO-group*, n (%)			0.007
RA/RARS/5q-	33 (27.3)	87 (36.7)	
RCMD/RCMD-RS	42 (34.7)	67 (28.3)	
RAEB-1	30 (24.8)	32 (13.5)	
RAEB-2	16 (13.2)	51 (21.5)	
IPSS group†, n (%)			0.003
Low	31 (25.6)	54 (22.8)	
Intermediate-1	57 (47.1)	86 (36.3)	
Intermediate-2	22 (18.2)	33 (13.9)	
High	4 (3.3)	22 (9.3)	
Missing karyotype	7 (5.8)	42 (17.7)	
Transfusion-dependent, (%)			<0.0001
No	38 (31.4)	148 (62.5)	
Yes	83 (68.6)	89 (37.6)	
Predictive group for response‡, n (%)			<0.0001
Good	59 (48.8)	58 (24.5)	
Intermediate	43 (35.5)	28 (11.8)	
Poor	14 (11.6)	4 (1.7)	
Unknown	5 (4.1)	147 (62.0)	

*WHO-group RA: refractory anemia, RARS: RA with ringed sideroblasts, 5q-: 5q- syndrome, RCMD: refractory cytopenia with multilineage dysplasia, RCMD-RS: RCMD with ringed sideroblasts, RAEB-1: refractory anemia with excess blasts (5-9% bone marrow blasts), RAEB-2: refractory anemia with excess blasts (10-19% bone marrow blasts)
†IPSS = International Prognostic Scoring System[102]
‡Predictive group for erythroid response to EPO-G according to a validated predictive model based on level of transfusion-need and S-EPO level.[130,142]
§*P*-values were calculated using the Pearson's chi^2 test, except for age where the Wilcoxon Rank-sum (Mann-Whitney) test was used

4.1.2 Treatment

Induction treatment with EPO and G-CSF was given for 12 to 18 weeks, and followed by maintenance treatment at the lowest effective dose in case of a response. [129,130,187,276]

The definition of complete erythroid response (CER) was an increase in hemoglobin level to at least 115 g/L without transfusion need, while a partial erythroid response (PER) required an increase in hemoglobin level of 15 g/L for patients with non-transfused anemia, or an abolished transfusion need. Both response-criteria fulfilled the revised International Working Group criteria for erythroid response.[137] The date of relapse was defined as the date of first RBC transfusion.

4.1.3 Statistical analyses

4.1.3.1 Paper I

The outcome measures were death and time of leukemic evolution, and all patients were followed-up per December 1, 2002. The Kaplan-Meier procedure[272] was used to estimate survival, evolution of AML, and response duration from start of study in the long-term follow-up of the EPO-G cohort. The log-rank test (Mantel-Haenszel procedure[273]) was used to test significance.

In order to assess the effects of treatment on outcome, overall survival and time to AML evolution was measured in months from of start of study in the EPO-G cohort and from time of diagnosis in the IMRAW cohort. We used an intention-to-treat approach, including also EPO-G treated patients having discontinued treatment prematurely (n=6). Multivariate Cox regression was used to compare the outcome of treated and untreated patients,[274] adjusting for important prognostic variables (age, number of cytopenias, karyotype according to the IPSS, % bone marrow blasts, and sex).

4.1.3.2 Paper II

Similarly to paper I, we compared the outcome of the Nordic EPO-G cohort in an intention-to-treat approach with untreated patients from Pavia. Overall survival, in months, was here measured from time of diagnosis to death, end of follow-up, or time of allogeneic bone marrow transplantation (n=7; in the untreated cohort only).

To adjust for the variable time between diagnosis and start of EPO-G treatment (in median 6 months [interquartile range 2.0-16.3]), a multivariate Cox model with delayed entry, or left truncation, was used.[275] This allowed measurement of the

survival from time of MDS diagnosis also in the treated patients. Adjustment was made for all major prognostic variables, and they were modeled as continuous (age, number of RBC units per month, absolute neutrophil count, S-EPO, S-LDH, and platelet count) or as indicator (EPO-G treatment, WHO-group, karyotype risk-group, and sex) covariates (fixed, not time-dependent).

We investigated the proportional hazard assumption by testing for a non-zero slope in a generalized linear regression of the scaled Schoenfeld residuals on functions of time. For both survival and AML the test was non-significant, thus indicating no major deviations from this assumption.

In order to address possible differences in age-specific mortality between the countries, the directly standardized mortality rates (SMRs) of Italy and Sweden were calculated by applying the calendar-year, age, and sex specific mortality rates of each country (as provided by the respective national institutes of statistics) to a reference population with uniform age and sex distribution.[277]

4.2 IN VITRO STUDIES

4.2.1 Study subjects

In paper III, bone marrow samples were taken with informed consent from 15 MDS patients with a karyotype involving del(5)(q31) and three MDS patients with a karyotype not involving del(5)(q31) (Table 1, paper III, page 11407). Ten healthy voluntary donors were also sampled.

In paper IV, the patients with 5q- syndrome were part of a clinical study (Celgene MDS004) assessing the effects of lenalidomide in MDS with del(5q).

4.2.2 Study drug

Lenalidomide (Celgene, Warren, NJ) was solved in 10% dimethyl sulfoxide (DMSO). Based on data on multiple myeloma[219] as well as unpublished data on AML cell lines and MDS,[278] the concentration of lenalidomide to be used in our experiments was set to 10 μM. This concentration is similar to those used in subsequent studies.[221,279]

4.2.3 Cells and cultures

Bone marrow (BM) mononuclear cells were separated on a density gradient, and $CD34^+$ progenitor cells were separated using a magnetic labeling system. The $CD34^+$ cells were

cultured according to a method developed to study the generation of erythroblasts.[84] Briefly, $CD34^+$ cells were cultured for 14 days in medium supplemented with recombinant human (rh)IL-3, rhIL-6, and rh-stem cell factor (SCF), and during the second week with the addition of 2 units/ml EPO. The cells were cultured at a concentration of 0.1 x 10^6 cells per ml in two positions with or without 10 μM lenalidomide. The separation procedures and culture methods are further described in paper III, page 11410.

In paper III, $CD34^-$ mononuclear cells isolated from two MDS del(5q) patients were cultured for 7 days at 0.5 x 10^6 cells/ml in the presence or absence of 10 μM lenalidomide.

4.2.4 Chromosome banding analysis

After 20-48 hours of culture, metaphases of BM were prepared and fluorescence R-banding was performed, as described earlier.[280] Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[281]

4.2.5 Fluorescence in situ hybridization (FISH)

Interphase FISH was performed using a probe for the locus 5q31, as described in detail in paper III, page 11410, as well as earlier.[282] Depending on the cytogenetic aberrations detected, probes for the *MLL*-locus in 11q23, for the *RB1*-locus in 13q14, for the *TP53*-locus in 17p13, for the *BCL2*-locus in 18q21, and for the *AML1*-locus in 21q22 were applied.

4.2.6 M-FISH

In paper IV, M-FISH analysis was carried out using an M-FISH kit (MetaSystems, Altlussheim, Germany) as described previously.[282] Fluorochromes were sequentially captured using specific single-band pass filters in a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany). M-FISH ISIS software (MetaSystems) was used for image analyses.

4.2.7 Flow cytometry (FACS)

In paper III, FACS phenotyping was performed at day 14 of culture and, if cell counts allowed, also at day 7. Analyses were performed using a FACSCalibur (BD) operating with the CellQuest Pro software (BD).

4.2.8 Statistical analysis of cell culture data

Mann-Whitney *U* test was used for comparison of different groups regarding fold increase of cell counts or proportion of cells positive for specific antigens as determined by FACS analysis. $P<0.05$ was considered statistically significant.

4.2.9 Bone marrow assessment and immunohistochemistry

In paper IV, consecutive BM samples were routinely stained, and morphological assessments were made of biopsies/clot preparations and of smears/imprints (described in detail in paper IV, supplement 1).

Immunohistochemistry for p53 and NPM was performed on paraffin-embedded sections using mouse monoclonal antibodies.

4.2.10 Gene expression profiling

$CD34^+$ progenitors were cultured for one week and then resuspended in TRIZOL where after total RNA was extracted. After a two-cycle amplification, gene expression profiling was performed using the GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix) platform and data analysis, as described in detail in paper III and elsewhere.[98]

Genes significantly differentially expressed ($P<0.001$) between lenalidomide-treated and untreated MDS samples were mapped to the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database (http://www.genome.jp/kegg/pathway.html) via DAVID Bioinformatic Resources (http://niaid.abcc.ncifcrf.gov).

4.2.11 Real-time quantitative PCR

Real-time quantitative PCR was used to validate microarray expression data for selected genes. The expression level of the *ABL1* gene was used to normalize for differences in input cDNA. Pre-developed TaqMan Assays were used and reactions were run on a LightCycler 480 Real-Time PCR System. Each sample was performed in triplicate and a reverse-transcriptase negative control was also tested to exclude any contaminating DNA amplification. The expression ratio between each lenalidomide-treated sample and the corresponding untreated sample was calculated using the $\Delta\Delta C_T$ method.[283]

Immunofluorescent staining of SPARC

Cytocentrifuged cells were fixed in paraformaldehyde 4% and permeabilized with saponin 0.1% containing 0.5% bovine serum albumin, the cells were incubated for 60 minutes with an anti-SPARC antibody (clone ON1-1, Zymed laboratories, South San Francisco, CA) at a concentration of 10 μg/ml, and subsequently for 30 minutes with a FITC-conjugated anti-IgG1 antibody. Antibody incubation was performed in the presence of saponin and BSA in the concentrations above. Nuclei were stained with DAPI (4',6 diamidino-2-phenylindole).

4.2.12 TP53 sequencing

In paper IV, DNA-sequences spanning exons 5-8 of *TP53* were amplified by polymerase chain reaction (PCR), using published primer sequences.[284] PCR products were purified and directly sequenced using the BigDye Terminator v1.1 kit (Applied Biosystems).

5 RESULTS

5.1 ERYTHROPOIETIN AND G-CSF IN MDS

5.1.1 Erythroid response rate (Paper I)

We evaluated the erythroid response rate of treatment with EPO and G-CSF in a pooled cohort of MDS patients from three previous Nordic MDS Group studies; 30 RA, 41 RARS, and 58 RAEB according to the FAB classification.[129,130,276] The overall erythroid response rate was 39% (48 of 123 evaluable patients), with 22% and 17% complete and partial erythroid responses, respectively. Patients in the IPSS Low/Int-1 risk-categories had a higher response rate compared to patients with IPSS Int-2/High, 46 and 27% respectively. Twenty-five of 85 (29%) transfusion-dependent patients became transfusion-independent as a result of treatment.

5.1.2 Response duration (Paper I)

The response duration was more durable in patients with IPSS Low or Int-1 risk compared with IPSS Int-2 or High (in median 25 vs. 7 months, P=0.002; Figure 1, paper I, page 806). Complete erythroid responders had significantly longer median response duration compared to partial responders (29 vs. 12 months, P=0.006; Figure 1, paper I, page 806). Patients in the good and intermediate predictive groups of response had comparable response durations of around two years, while the single responding patient in the poor predictive group (consisting of 16 patients) only had a partial response of three months.

5.1.3 Reasons for loss of response (Paper I)

The reasons for relapse of anemia or discontinuation of treatment were due leukemic evolution or significant increase of marrow blasts only in 7 of 39 (18%) responders, leaving most relapses essentially unexplained.

5.1.4 Maintenance doses of EPO and G-CSF (Paper I)

The median nadir dose of EPO was 30 000 U/week and of G-CSF 225 μg/week.

5.1.5 EPO and G-CSF and long-term outcome (Papers I and II)

5.1.5.1 Descriptive long-term survival

The median overall survival from the initiation of EPO-G treatment was 31 months (range 2-142+). Patients in the good predictive group for erythroid response had significantly longer survival compared to the intermediate and poor predictive groups (*P*=0.01; Figure 2, paper I, page 807).

5.1.5.2 Descriptive long-term evolution of AML

The cumulative incidence of AML evolution at four years from start of EPO-G treatment was 30%. Only 2 out of 40 RARS (according to the FAB classification) patients developed AML. The time until 25% of patients developed AML in the good and intermediate predictive groups for erythroid response was significantly longer than in the poor predictive group (52 vs. 13 months, *P*=0.008; Figure 2, paper I, page 807). No more than 1 of 20 patients responding longer than two years developed AML.

5.1.5.3 No association with survival or risk of AML evolution in comparison with untreated patients from the IMRAW database

In paper I, we developed a multivariate Cox-regression model adjusted for karyotype, bone marrow blast count, number of cytopenias, age, and sex. There was no significant difference in survival between patients treated with EPO and G-CSF compared with untreated patients from the IMRAW cohort (HR 0.9; 95% 95% confidence interval [CI] 0.7-1.2; *P*=0.56), and no significant difference in the risk of AML evolution (HR 1.3; 95% CI 0.7-2.2; *P*=0.40).

5.1.5.4 Optimized statistical comparison using a more suitable comparison group

In paper II, we used another comparison group consisting of untreated MDS patients from the Pavia cohort in order to sharpen our analysis by enabling adjustment for all major prognostic factors, out of which some were unknown in the IMRAW cohort. We also applied another statistical model, Cox regression with delayed entry, in order to more correctly adjust for the time interval between diagnosis and study entry.

In order to address differences in mortality in Sweden vs. Italy, the SMR rate ratio between the two countries was calculated and found to be similar (1.03 to 1.13) for the enrollment period 1990-2000.

5.1.5.5 Treatment associated with better survival in comparison to untreated patients from the Pavia database

In a multivariate analysis (adjusted for WHO-group, karyotype risk-group, number of transfused RBC units per month, age, sex, and platelet and absolute neutrophil counts) treatment with EPO and G-CSF was associated with better overall survival (HR 0.61; 95% CI; 0.44-0.83, *P*=0.002; Table 2, paper II, page 3610), and also decreased risk of non-leukemic death (HR 0.66; 95% CI 0.44-0.99; *P*=0.042). There was no association between treatment and the risk of AML evolution (HR 0.89; 95% CI 0.52-1.52; *P*=0.66; Table 2, paper II, page 3610).

In order to investigate the association between disease risk and effect of treatment, we defined low and high risk based on BM blasts below or above 10%, and found a positive association of treatment on survival in both groups (HR_{low} 0.68; 95% CI 0.47-0.99; *P*=0.046; HR_{high} 0.29; 95% CI 0.12-0.69; *P*=0.006). However, there was no significant association with AML evolution in any risk group (data not shown).

5.1.5.6 Positive association with survival limited to patients with low pre-treatment transfusion requirement

Patients requiring <2 units of RBC per month have a higher probability of response to EPO-G according to the predictive model.[130,142] We found a significant interaction between treatment and transfusion need in a multivariate analysis (*P*=0.039). We therefore stratified the patients based on transfusion requirement of <2 ($n_{treated}$=75, $n_{untreated}$=196) and ≥2 ($n_{treated}$=46 $n_{untreated}$=41) units of RBC per month. Treatment with EPO-G was associated with enhanced survival only in patients receiving <2 units per month ($HR_{<2\ U/month}$ 0.44; 95% CI 0.29-0.66; *P*<0.001, $HR_{\geq 2\ units/month}$ 1.04; 95% CI 0.57-1.89; *P*=0.91; Figure 7). Furthermore, there was no association between treatment and risk of leukemic transformation in patients with low or high transfusion need (HR 0.87; 95% CI 0.45-1.66; *P*=0.67 and HR 0.92; 95% CI 0.28-3.03; *P*=0.89, respectively; Figure 7).

The response rate was higher for the less compared to the more heavily transfused patients, 56% vs. 18%, respectively (*P*<0.001). As expected due to the higher response rate, the less transfused patients received growth factors for a longer time period than the more heavily transfused patients (47% and 11%, respectively, were on therapy ≥6 months, *P*<0.001).

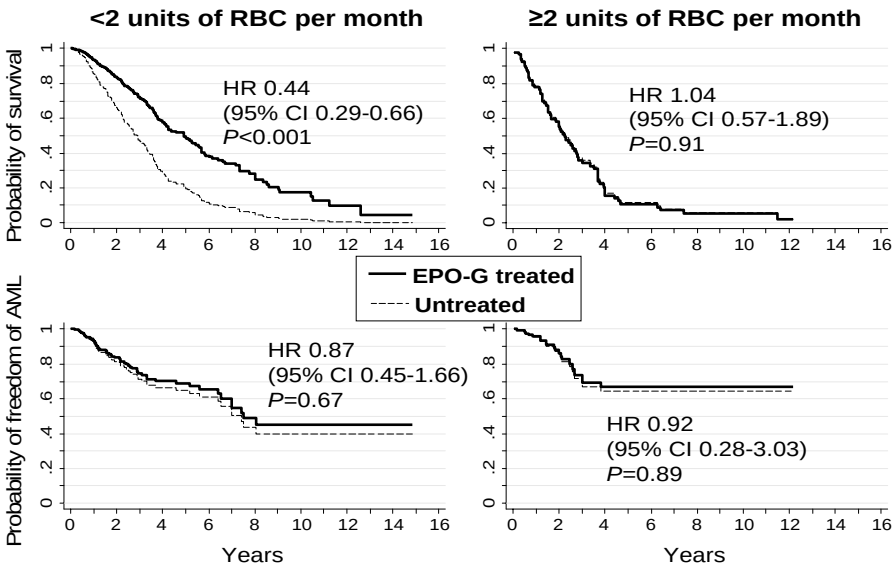

Figure 7. EPO and G-CSF treatment associated with better survival in patients with low transfusion need.

5.1.5.7 Treatment response associated with favorable outcome

Next, we modified the overall Cox analysis by modeling the treatment covariate as binary dummy-variables: untreated, responder, non-responder. The positive association with survival was only seen in responders (HR 0.40; 95% CI 0.26-0.62; *P*<0.001). Non-responders showed no such association (HR 0.80, 95% CI 0.56-1.14, *P*=0.21). Neither response nor non-response was significantly associated with the rate of AML evolution (HR 0.60; 95% CI 0.29-1.24; *P*=0.17, and HR 1.13; 95% CI 0.61-2.10; *P*=0.69, respectively). Finally, there was a close association between treatment response and improved non-leukemic survival ($HR_{responders}$ 0.39, 95% CI 0.22-0.67, *P*=0.001, $HR_{non\text{-}responders}$ 0.97, 95% CI 0.62-1.52, *P*=0.91).

5.2 LENALIDOMIDE IN DEL(5Q) MDS

5.2.1 Effects of lenalidomide on cell growth (Paper III)

5.2.1.1 Lenalidomide inhibits expansion of MDS del(5q) cells but not cells from healthy donors

Lenalidomide did not inhibit proliferation (measured by [^{3}H]thymidine incorporation) of bone marrow mononuclear cells from healthy donors in doses titrated up to 500 μM.

$CD34^+$ cells isolated from the bone marrow of 13 MDS del(5q) patients and 10 healthy controls were cultured according an erythroblast protocol.[84,285] During the first week of culture the proportion of del(5q) cells remained high, however, during the second week it decreased due to an outgrowth of cytogenetically normal cells (Figure 8 B).

By correlating the proliferation index to the proportion of del(5q) cells as determined by FISH, we could estimate the proliferation of the normal and malignant cells independently, as previously described.[285] Lenalidomide significantly inhibited the expansion of cells carrying the del(5q) at day 14 (P=0.04; Figure 8 A), but had no inhibitory effects on cells from healthy controls or on the cytogenetically normal cells in the MDS cultures (Figure 8 C and D).

Finally, we assessed the effect of lenalidomide on $CD34^-$ mononuclear cells from two MDS del(5q) patients. At day 0, around 1% of the cells were erythroblasts, as determined by morphology, thus, any effects observed would be on non-erythroid progenitors. There was a clear inhibition of cell growth of the del(5q) clone by day 7 (34% and 65%), compared with the cytogenetically normal cells in the same culture.

5.2.1.2 Effects of lenalidomide on expansion of cells from MDS patients without del(5q)

We determined the effect of lenalidomide on $CD34^+$ cells from three MDS patients without del(5q). Only one of three samples (from a patient with RA and normal karyotype) showed inhibition of cell expansion by day 14. Interestingly, when culturing cells from a patient with trisomy 8 there was no difference in expansion of the malignant clone vs. the cytogenetically normal cells.

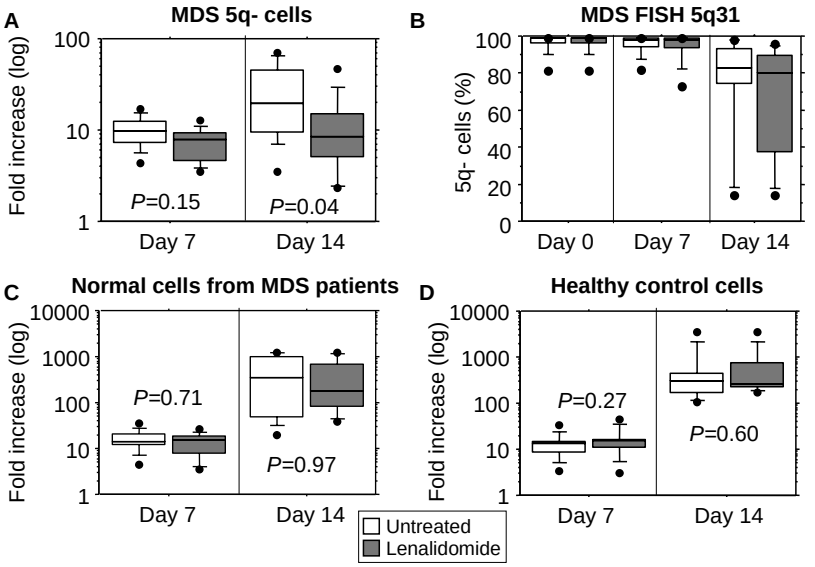

Figure 8. Lenalidomide specifically inhibits growth of MDS del(5q) cells.

5.2.2 Effects of lenalidomide on differentiation (Paper III)

At day 7 of culture, the $CD34^+$ progenitors had differentiated into intermediate erythroblasts and the lenalidomide-treated cells showed a phenotype similar to that of the untreated cells (Figure 1 D and F, paper III, page 11407). However, in the presence of EPO during the second week, the proportion of mature erythroid cells expressing the late erythroid marker glycophorin A (GPA) increased significantly more in cells derived from healthy controls than in cells from MDS del(5q) patients (P=0.001; Figure 1 E and G, paper III, page 11407). Lenalidomide treated MDS cells at day 14 showed less erythroid differentiation compared with untreated MDS cells (Figure 1 E, paper III, page 11407).

5.2.3 Effects of lenalidomide on gene expression (Paper III)

5.2.3.1 Gene expression profiles in cells from patients with MDS del(5q) and healthy controls

Gene expression profiling was performed on intermediate erythroblasts (at day 7 of culture) when a median of 98% of the MDS cells still carried the 5q deletion and thus represented the malignant clone.

Several genes were significantly down-regulated by lenalidomide, including genes involved in erythropoiesis. Four genes were up-regulated by lenalidomide at least 2-fold in all MDS del(5q) and all healthy control samples analyzed, *VSIG4*, *PPIC*, *TPBG*, and *SPARC*. Of the 44 genes mapping within the CDS of the 5q- syndrome, *SPARC* was the only one whose expression levels were significantly increased by lenalidomide (Figure 9 C). The average up-regulation of *SPARC* was 4.1-fold (range 2.4–8.1) in the MDS patients and 4.8-fold (range 3.2–9.5) in the healthy controls (Figure 9 A). In addition, the cancer related gene Activin A was one of the most significant differentially expressed genes in MDS vs. healthy controls.

Finally, lenalidomide significantly deregulated the following pathways: extracellular matrix (ECM) interactions (*P*=0.0007), hematopoietic cell lineages (*P*=0.0008), and focal adhesions (P=0.004).

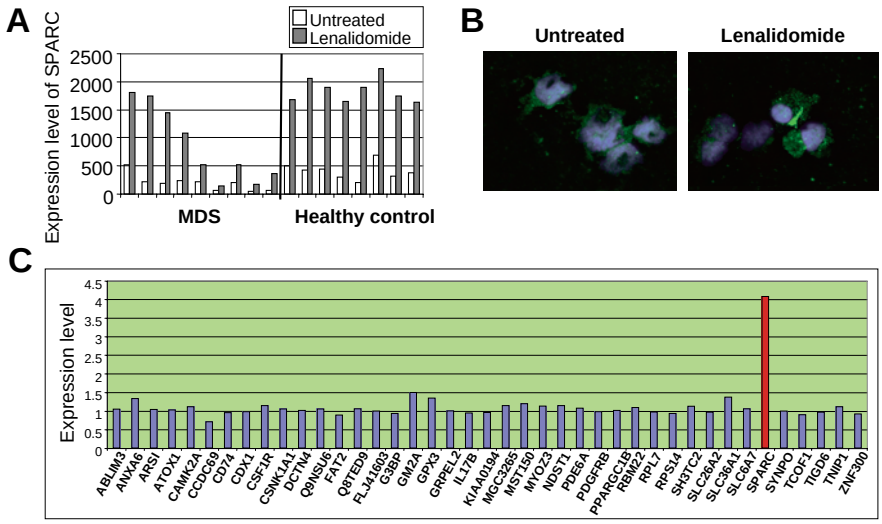

Figure 9. Increase of SPARC gene expression by treatment with lenalidomide. (A) Expression levels of the SPARC gene in erythroblasts from day 7 of culture. (B) SPARC immunofluorescent staining of cytocentrifuged MDS del(5q) cells from day 7 of culture, corresponding to the cells analyzed with gene expression profiling. (C) Effects of lenalidomide in the 5q- erythroblasts on the expression levels of the genes mapping to the commonly deleted segment of the 5q- syndrome.

5.2.3.2 SPARC and activin A expression in non-erythroid cells from MDS patients with del(5q)

$CD34^-$ mononuclear cells, morphologically consisting of around 99% non-erythroid cells, from two MDS patients with del(5) were cultured for 7 days. At least a two-fold increase of both *SPARC* and activin A expression by lenalidomide was observed at day 7.

5.2.3.3 Expression of SPARC and activin A in cells from MDS patients without del(5q)

In $CD34^+$ cells from MDS patients (n=2) without del(5q), lenalidomide treatment increased the expression of both *SPARC* (9.3-fold and 5.5-fold) and activin A (12.6-fold and 4.3-fold) in comparison with untreated cells.

5.2.3.4 Confirmation of gene expression data

Real-time quantitative PCR was used to validate the gene expression of selected genes (Figure 3, paper III, page 11409) and the concordance between the expression levels obtained with Affymetrix chips and with real-time quantitative PCR was high.

Immunofluorescent staining of SPARC was performed on cytocentrifuged cells from day 7 of culture, corresponding to the cells analyzed with gene expression profiling. We confirmed an increased expression of SPARC protein in lenalidomide treated samples (Figure 9 B).

5.2.4 Expansion of malignant subclones during treatment (Paper IV)

5.2.4.1 Case reports

We described two women with classical low-risk 5q- syndrome who after failure of erythroid growth factor treatment were treated with lenalidomide. Both became transfusion independent on lenalidomide, and reached partial cytogenetic responses. Interestingly, both patients lost their response and underwent disease progression to AML and RAEB-1, respectively, after 22 months of therapy, and both acquired a complex karyotype (Table 1, paper IV, page 14; Figure 10).

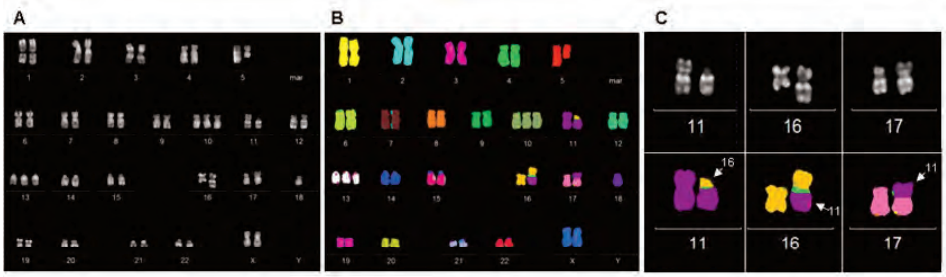

Figure 10. Karyogram of a metaphase from the bone marrow of patient 2.
(A) Fluorescence R-banding. (B) M-FISH. (C) Partial karyograms of the structural aberrant chromosomes 11, 16, and 17 shown in detail.

5.2.4.2 Identification molecular lesions

Due to emerging data on nucleophosmin (*NPM1*) mutations in patients with high-risk MDS, we investigated the presence of NPM aberrantly trapped in the cytoplasm (NPMc; a surrogate marker for *NPM1* mutations[51,52,55,286]) at the time of disease progression. Both patients were NPMc+ (5% and 20% of bone marrow cells, respectively; Table 1, paper IV, page 14; Figure 2, paper IV, page 16). Patient 2 had acquired a chromosomal deletion at 17p13 involving the locus of the tumor suppressor gene *TP53*, and using immunohistochemistry we could confirm aberrant overexpression of p53 (a surrogate marker of *TP53* mutation;[287] Table 1, paper IV, page 14; Figure 2, paper IV, page 16).

5.2.4.3 NPMc+ and p53 expressing cells demonstrated pre-treatment

We then assessed the presence of molecular lesions prior to disease progression, and demonstrated that both patients had around 5% NPMc+ cells before and during treatment. In patient 1 this subpopulation expanded to 20% in parallel to the blast count at the time of AML evolution (Table 1, paper IV, page 14; Figure 2, paper IV, page 16). Patient 2 demonstrated a small fraction of p53 overexpressing cells pre-treatment, and this population also increased in parallel to the blast count at the time of progression (Table 1, paper IV, page 14; Figure 2, paper IV, page 16).

In contrast, 10 normal bone marrows demonstrated the normal nuclear NPM staining only, and <0.01% of cells expressed p53. We also assessed three patients with 5q-syndrome with complete erythroid and cytogenetic responses to lenalidomide and no evidence of disease progression. In pre-treatment and follow-up marrow examinations

(after 4, 8 and 18 months of therapy, respectively) there were no p53 expressing cells, and NPMc was negative in two patients, while one demonstrated a minimal population of NPMc+ granulocytic precursors (<5%) before and during treatment.

5.2.4.4 Gene expression analysis

Pathway analysis demonstrated altered apoptosis and integrin signaling at treatment failure compared to pre-treatment. We also assessed the expression of individual genes, selected *a priori* based on the current data and paper III. *SPARC* and *Activin-A* expression were up-regulated *in vitro* by lenalidomide in both patients (3.6-7.1 and 2.5-5.1 fold, respectively).

5.2.4.5 Confirmation of TP53 mutations before and after treatment with lenalidomide

TP53 was sequenced in pre- and post-treatment samples of both patients. In patient 2, a heterozygous A>G mutation in exon 5 (Y163C) was found in the pre-treatment sample. In the post-treatment sample, this mutation was seen as homozygous.

6 DISCUSSION

6.1 TREATMENT WITH EPO AND G-CSF IN MDS

6.1.1 Long-term responses to EPO and G-CSF

EPO and G-CSF is an effective therapy of anemia in low-risk MDS provided that the patients are stratified pre-treatment according to the predictive model for response (based on S-EPO level and degree of transfusion need[130,142]). We demonstrate that patients in the poor predictive group are not eligible for treatment due to an exceedingly low probability response in addition to a high risk of leukemic evolution and a poor overall survival.

We report median response duration to EPO and G-CSF of two years, which is in line with recent data from a large retrospective study performed by the Groupe Francophone des Myélodysplasies.[131] Similar response durations but with significantly higher risk of serious adverse events have been reported for two second line treatments of low-risk MDS, namely ATG[63,64,150] and lenalidomide.[152-154]

The Nordic MDS Group as well as other investigators have demonstrated that response to growth factor therapy improves quality of life.[130,133,138,147,288] We demonstrate in our long-term follow-up that 20% of the responders remained transfusion-independent for more than four years during maintenance therapy, which no doubt had great implications for their quality of life.

We also analyzed the reasons for relapse of anemia. Most patients who loose their response appear to escape the effect of growth factor treatment without signs of disease progression, which corresponds well to recent French data.[131] For the majority the reasons for relapse of anemia are unknown. Whether some patients loose their response due to development of functional iron deficiency or exhaustion of the normal erythroid progenitor pool remains to be determined.

6.1.2 EPO and G-CSF associated with improved survival

In paper I, we compared patients treated with EPO and G-CSF with untreated from an international cohort that was used by the IMRAW group to develop the IPSS risk score. Using multivariate Cox regression, we were able to demonstrate for the first time that treatment with EPO and G-CSF in MDS is not associated with leukemic evolution.

In paper II, we increased the accuracy of the statistical comparison by reclassifying all Nordic patients according to the WHO 2001 criteria and by utilizing a more suitable cohort of untreated patients from Pavia, Italy. We also used another statistical model, multivariate Cox regression with delayed entry, where we more appropriately accounted for the delay between diagnosis and start of treatment with EPO and G-CSF. Despite that patients treated with EPO-G were significantly older and more frequently transfused than the untreated patients, which *per se* would imply a worse prognosis,[102,107,109] we demonstrated that treatment is associated with significantly enhanced overall and non-leukemic survival. These results are in line with a recent report by the French group, using a methodology similar to the one we used in paper I.[131]

Importantly, treatment with EPO and G-CSF is the first treatment of low-risk MDS where a survival benefit has been demonstrated, thus consolidating its place as first-line therapy in international guidelines.[24,113,122,124,125]

In a pre-determined subgroup analysis, we found that the association with improved survival is restricted to patients requiring <2 units of RBC per month, which was not unexpected since these patients responded better to EPO-G compared to the more heavily transfused.

The reason for the improved survival is most likely explained by the correction of the anemia *per se*. Anemia is associated with reduced physical performance in elderly patients,[289] and with poor outcome in patients with heart failure.[290] In MDS, anemia is associated with lower survival[102] and an increased incidence of heart failure.[291] In addition, Malcovati *et al* have demonstrated that the onset of RBC transfusion need in MDS worsens the survival in part due to a higher risk of heart failure-related death.[107,109]

Another positive effect of treatment response may be attributed to the prevention of progressive iron overload, by elimination of the transfusion requirement. It is known that progressive iron overload is inversely correlated to survival in transfusion-dependent MDS patients.[107]

Other potential effects of EPO such as modulation of the immune response against the tumor cells may also play a role,[292,293] although their relative contribution to the effect on the anemia *per se* remains to be determined.

There was no association between treatment and AML evolution in the overall analysis, or in low- and high-risk patients in a stratified analysis. Hence, neither prolonged exposure in responding patients, nor short-term exposure in high-risk patients is associated with disease progression.

6.2 LENALIDOMIDE IN MDS

6.2.1 Lenalidomide specifically inhibits the malignant clone

In paper III, we demonstrate that lenalidomide specifically inhibits the growth of $CD34^+$ bone marrow progenitor cells of the malignant del(5q) clone, while not affecting normal cells. We found no or a less pronounced inhibition of lenalidomide on cells from three non-del(5q) MDS patients. Our data are in line with reports of enhanced sensitivity to lenalidomide in cell lines harboring a del(5q),[221,294] and of absence of growth inhibition in normal bone marrow progenitors.[279,294]

It is known that 99% of the hematopoietic stem cells in patients with 5q- syndrome are part of the malignant clone.[31] A potent inhibition of the del(5q) progenitors by lenalidomide in combination with a limited number of remaining normal hematopoietic stem cells could potentially cause delayed hematopoietic recovery in patients. This may in part explain the clinical experience that MDS patients with del(5q) often develop severe neutropenia and thrombocytopenia early during the treatment with lenalidomide.[152,153]

We observed a decrease in the proportion of erythroid progenitors in the MDS del(5q) cultures in the presence of lenalidomide, which is in line with previous experience in cells from healthy donors, where in addition a decrease in erythroid colonies and increase in myeloid has been observed.[279]

6.2.2 Lenalidomide up-regulates the tumor suppressor gene SPARC

We demonstrate that lenalidomide significantly alters the gene expression profiles of del(5q) bone marrow progenitors. Lenalidomide consistently down-regulated a number of erythroid genes and up-regulated four genes *VSIG4*, *PPIC*, *TPBG*, and *SPARC*. All four genes up-regulated have been implicated in cancer.[295-302]

The up-regulation of *SPARC* is of particular interest because of its location at 5q31, within the CDS of the 5q- syndrome,[93] and because of its functions as a tumor suppressor gene and a regulator of cell-cell / cell-matrix interactions.[224,303] Interestingly, we found that extra cellular matrix interaction was the pathway most significantly deregulated by lenalidomide. Morevover, SPARC is antiproliferative, antiadhesive, and antiangiogenic,[224-226,263,304] which are recognized as important effects of the immunomodulatory drugs.[207,213] Therefore up-regulation of SPARC may play a role in the mechanisms of action of lenalidomide in 5q- syndrome.

SPARC also functions as a tumor suppressor in several human malignancies, and decreased *SPARC* expression has been described in several types of cancers,[241-243,246] including multiple myeloma[248] and AML with rearrangements involving the mixed lineage leukemia (*MLL*) gene.[244] Reduction of *SPARC* expression is generally attributable to a deletion of 5q31 or promoter hypermethylation.

In addition, SPARC has been shown to stimulate the TGF-β signaling pathway,[305] and two genes in this pathway, activin A and activin A receptor, were significantly deregulated in response to treatment with lenalidomide. The activins are known to have effects on many physiological processes including cell proliferation, cell death, differentiation, and immune responses.[306,307]

6.2.3 The SPARC hypothesis of 5q- syndrome

No point mutation has yet been described in *SPARC* or any other of the 43 genes mapping within the CDS in 5q- syndrome, and therefore it seems probable that haploinsufficiency of one or more genes is the mechanism involved.[93,308,309] Ebert *et al* recently identified *RPS14* as the gene causing the erythroid maturation block characteristic for the 5q- syndrome.[310] *RPS14* is located within the CDS at 5q31 and encodes a protein that is a part of the ribosomal 40S subunit.[311] Mutations of another ribosomal gene, *RPS19*, have been found to cause the congenital disorder Diamond-Blackfan anemia.[100,101] It is unknown why ribosomal stress causes anemia, although up-regulation of the p53 pathway has been demonstrated.[312] The reason why maninly the erythroid lineage is affected is also unclear; differences in growth kinetics between erythroid and myeloid progenitors may be a part of the explanation.

It is unlikely that haploinsufficiency of *RPS14* is the sole genetic event underlying the 5q- syndrome, and it is conceivable that *SPARC* may also play a role in the pathogenesis. We therefore present the hypothesis that haploinsufficiency of *SPARC* leads to increased adhesion of the del(5q) hematopoietic stem cells to the supportive bone marrow stroma, allowing them to expand at the expense of the normal stem cells. Thus, the del(5q) cells may gradually overtake the stem cell compartment. In conjunction with an erythroid maturation block induced by the deficiency of *RPS14*, this may result in the observed clinical picture with an expanded del(5q) progenitor pool in combination with anemia (Figure 11). We are currently testing this hypothesis in our laboratory.

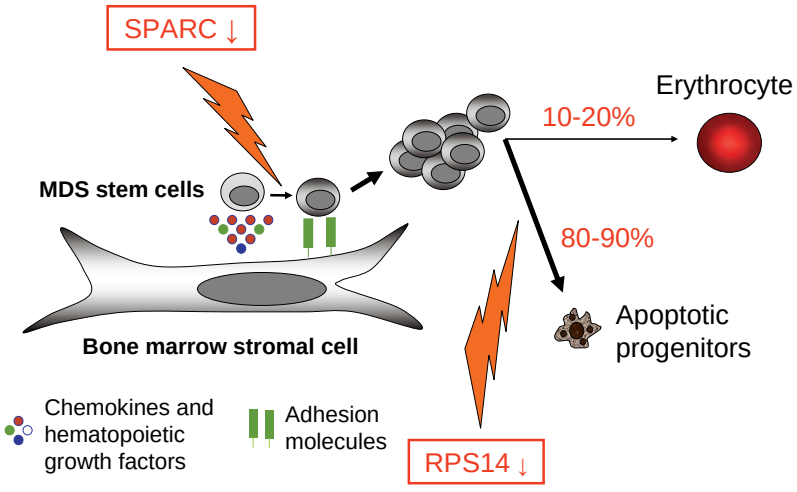

Figure 11. The SPARC hypothesis of 5q- syndrome.

6.2.4 Expansion of clones with molecular lesions during treatment

In paper IV, we describe two patients with 5q- syndrome and an excellent clinical and partial cytogenetic response to lenalidomide, who unexpectedly progressed to high-risk myeloid disease after 22 months of treatment. Interestingly, we detected abnormal NPMc+ BM progenitors in both patients, indicating a mutation of the *NPM1* gene, which represents a novel finding in 5q- syndrome and only rarely occur in high-risk MDS or AML with del(5q).[51,52,54,56] The NPMc+ subclones were detected already pre-treatment, and in one patient this abnormal clone expanded in conjunction with the blast counts at the time of AML transformation. In contrast, *NPM1* is the most frequently mutated gene in AML with normal karyotype, where it implies a favorable prognosis when it occurs in absence of *FLT3* internal tandem duplications.[49,286] *NPM1* encodes a nuclear phosphoprotein shuttling between the nucleus and the cytoplasm playing an important role in ribosome biogenesis, chromosome duplication, and genomic instability by regulating p53 levels and activity.[55]

Another intriguing finding in one of the patients was a small subclone of cells overexpressing p53 by immunohistochemistry, most likely due to mutation of the gene.[287] At time of disease progression the p53 overexpressing clone expanded in parallel to the blast counts, and sequencing confirmed a heterozygous *TP53*-mutation before treatment

and a homozygous mutation at progression. This was consistent with the acquisition of a complex karyotype including del(17p13), resulting in loss of the remaining non-mutated *TP53* allele. *TP53* mutations are exceedingly rare in MDS patients with an isolated del(5q).[56] However, they occur more often in conjunction with complex karyotypes that contain del(5q) and in therapy-related MDS, invariably implying a poor outcome.[40,42,43,54,313] *TP53* is the most frequently mutated tumor suppressor gene in cancer and plays a crucial role in genomic integrity and stability.

Pathway analysis based on the gene expression profiles of del(5q) progenitors post- vs. pre-lenalidomide showed significantly altered apoptosis and integrin signaling, which may reflect a more aggressive disease and an altered interaction between the MDS cells and the stroma. The tumor suppressor genes *SPARC* and *Activin-A* were up-regulated by lenalidomide *in vitro* at time of treatment failure, in line with our experience in samples from lenalidomide naïve patients in paper III, suggesting other mechanisms of resistance or potentially inactivation of down-stream targets.

6.2.5 Pre-treatment risk-stratification warranted

It cannot be excluded that the malignant transformations we observed reflect rare events during the natural course of the disease. In addition, lenalidomide may also affect immune surveillance or genomic stability, which potentially could increase the risk of clonal evolution. However, the NPMc+ and p53 mutated clones were detected before treatment and the clone-sizes were stable despite partial cytogenetic responses. Therefore, we argue that these clones consisted of cells with inherent genomic instability and pre-leukemic properties due to NPM and p53 abnormalities, which *per se* may have implications for the risk of disease progression. Moreover, the potent eradication of lenalidomide sensitive del(5q) cells during treatment potentially facilitated the expansion of the insensitive and genetically instable clones, potentially increasing their probability of acquiring additional cytogenetic abnormalities. Therefore, the development of a pre-treatment risk-stratification is warranted, in which screening for molecular lesions with immunohistochemistry is likely to be of value.

7 CONCLUSIONS

7.1 FAVORABLE LONG-TERM OUTCOME OF THERAPY WITH ERYTHROPOIETIC GROWTH FACTORS IN MDS

EPO and G-CSF is an effective treatment of anemia in low-risk MDS; the poor predictive group for response is not eligible for treatment.

Treatment with EPO and G-CSF is associated with improved survival in MDS patients requiring transfusion of less than two units of packed red blood cells per month, without any association with the risk of leukemic evolution.

7.2 MECHANISMS OF ACTION OF LENALIDOMIDE IN 5Q- SYNDROME AND THE POTENTIAL ROLE OF SPARC IN THE PATHOGENESIS OF THE DISEASE

Lenalidomide specifically inhibits the growth of bone marrow progenitor cells of the malignant clone, while not affecting normal cells.

Lenalidomide significantly alters the gene expression profile of bone marrow progenitor cells and up-regulates the tumor suppressor gene *SPARC*.

Up-regulation of *SPARC* may be of importance for the mechanisms of action of lenalidomide since its known effects overlap with those of lenalidomide.

Down-regulation of *SPARC* may be of importance for the pathogenesis of the 5q- syndrome since (a) *SPARC* is haploinsufficient due to its location at 5q31, (b) down-regulation of *SPARC* may increase the adhesion of the malignant hematopoietic stem cells to the supporting cells of the bone marrow, thereby facilitating expansion of malignant in expense of normal hematopoietic stem cells, and (c) lenalidomide restores the *SPARC* level to normal or supra-normal.

7.3 LENALIDOMIDE AND DISEASE PROGRESSION

In 5q- syndrome, molecular lesions affecting the genomic stability may be detected in subclones of bone marrow cells before lenalidomide treatment, conceivably increasing their probability of acquiring additional genetic abnormalities leading to drug resistance and disease progression.

There is a need risk-stratification before lenalidomide treatment; screening with immunohistochemistry for molecular lesions may be of value.

8 FUTURE PERSPECTIVES

8.1 ERYTHROPOIETIC GROWTH FACTORS IN MDS

EPO with or without G-CSF already plays a central role in the treatment of anemia in low-risk MDS. Despite this, no erythroid growth factor is currently approved for MDS by the FDA in the US or the EMEA in Europe. Therefore, patients in several countries are unable to receive growth factor therapy.

Here, we present long-term results of treatment with EPO and G-CSF in MDS, demonstrating the presence of long-term responders and an improved overall survival. We found no association with the risk of AML evolution. Due to the current standards of care and our long-term data, it is hard to ethically justify a large randomized trial comparing treatment with EPO vs. placebo. Moreover, it would require a long period of follow-up in order to detect any effect on survival or risk of AML evolution. Therefore, we hope that our results will be instrumental in the formal approval of EPO in MDS.

The probability of erythroid response to EPO and G-CSF can be determined by using the predictive model based on S-EPO level and degree of transfusion requirement.[142] However, the reason for relapse of anemia, most often without signs of disease progression, is insufficiently studied in MDS. Several factors may play roles, including development of functional iron deficiency, insufficiency of folic acid or vitamin-C, and exhaustion of the pool of normal erythroid progenitors. It is also conceivable that acquired promoter hypermethylation of certain genes during the natural course of the disease may lead to loss of response, and studies using the hypomethylating agent 5-AZA are currently ongoing. If the reasons for treatment failure can be better characterized in future studies, it may lead to enhanced responses.

8.2 THE 5Q- SYNDROME

After decades of intense research, a breakthrough in the understanding of the pathogenesis of the 5q- syndrome was made in 2007. Ebert *et al* first presented data on the important role of haploinsufficiency of the ribosomal gene *RPS14*, located within the commonly deleted segment at 5q31. Deficiency of *RPS14* most likely causes the observed block in erythroid differentiation. However, it is unlikely that this is the sole abnormality required for the development of the disease.

We present data supporting a role for a haploinsufficiency of the tumor suppressor gene *SPARC*, also located within the commonly deleted segment. Decreased expression of *SPARC* may increase the adhesion of the hematopoietic stem cells to the supporting stroma in the bone marrow, thus facilitating their gradual selective expansion. This may in part explain why the hematopoietic stem cell pool is completely overtaken by the malignant cells in patients with 5q- syndrome.

Functional studies and *in vivo* models testing the role down-regulation of *RPS14* and/or *SPARC* would clarify the relative importance of each genetic abnormality. We are currently running functional studies of the effect of up- and down-regulation of *SPARC* on cell growth characteristics and adhesion in 5q- syndrome, also in the context of lenalidomide.

8.3 LENALIDOMIDE IN MDS

The outstanding clinical efficacy of lenalidomide in 5q- syndrome constitutes a breakthrough in the treatment of the disease. However, we observed patients who initially responded well to therapy but after a period of time developed disease progression with unusual high-risk features. We were able to detect clones with molecular abnormalities already before treatment, which expanded upon disease transformation. Other groups have also observed patients unexpectedly transforming to AML during lenalidomide treatment, and this was why the EMEA decided against approval of the drug in January 2008, despite the earlier approval by the FDA in December 2005.

It would be of great value to perform a long-term follow-up of the initial studies on lenalidomide in MDS, and to retrieve study material in order to screen for molecular lesions before, during, and after lenalidomide treatment. Furthermore, it would be informative with a statistical comparison with an untreated cohort of patients with 5q- syndrome, retrospectively screened for the same molecular abnormalities, with the aim of identifying any associations of the molecular lesions with survival or risk of leukemic evolution. We have recently initiated these investigations.

We have proposed up-regulation of SPARC as one part of the mechanisms of action of lenalidomide in 5q- syndrome, however, further studies are warranted to unravel the complex effects of the drug in greater detail.

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