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CLINICAL AND MOLECULAR EFFECTS OF AZACITIDINE IN THE MYELODYSPLASTIC SYNDROMES

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“For every complex problem there is an answer that is clear, simple, and wrong.”

H.L. Mencken, 1917

ABSTRACT

The myelodysplastic syndromes (MDS) constitute a heterogeneous group of malignant bone marrow disorders characterized by peripheral cytopenia and increased risk of leukemic progression. In higher-risk MDS, Azacitidine has been shown to prolong survival and modulate the epigenome, although the precise mechanisms by which the drug exerts its effect are unknown.

Paper I reports the result from a Nordic study enrolling 30 transfusion-dependent Epo-refractory lower-risk MDS patients. Patients were treated with 6 cycles of Azacitidine and terminated the study if they reached transfusion independence, while non-responding patients received another 3 cycles combined with Epo. Five (21%) and one patient responded after Azacitidine and the combined treatment, respectively, and only 2 patients (10%) responded for more than 6 months. Toxicity was substantial, mainly consisting of infections. We conclude that Azacitidine can be effective in this cohort of patients but that the low response rate and relatively high toxicity precludes its recommendation as standard treatment. Targeted sequencing revealed a high frequency of recurrent MDS mutations without clear relation to response.

In paper II we cultured CD34+ progenitors from higher-risk MDS and normal bone marrow (NBM) with or without Azacitidine and studied the effects on DNA methylation and histone acetylation. We showed that the MDS genome at the global level is hypermethylated compared to NBM and that Azacitidine induced profound demethylation. Histone acetylation was decreased by treatment, which theoretically would counteract the transcriptional activation resulting from reduced DNA methylation. To further explore these effects, we repeated the same culture experiment in paper IV to study the effects of Azacitidine on both DNA methylation and gene expression. We confirmed the marked demethylating effect of Azacitidine, and by using RNA seq we could show that Azacitidine significantly increases gene expression but without association with demethylated regions. Interestingly, the repressive histone mark H3K9me3 increased in three demethylated genes without increased expression, providing a potential explanation for the lack of association between demethylation and increased expression.

In paper III we searched for factors associated with response to Azacitidine by studying clinical parameters (n=134); mutations (n=90); and DNA methylation (n=42) in patients treated with Azacitidine. Among the clinical variables, only disease duration before treatment predicted for poor response and survival. The group of mutations involved in histone modulation (*ASXL1, EZH2, MLL*) was associated with prolonged survival, contrasting previous reports on mixed MDS cohorts. Furthermore, DNA methylation profiles differed significantly between responding and non-responding patients. Analysis of 200 differentially methylated regions showed enrichment in pathways involved in differentiation and development. Methylation level of the most significant DMR, the *HOXA5/A6*-locus, was associated with survival.

To summarize, these studies show that epigenetic modifications play a significant role in the pathogenesis and response to treatment in MDS and that further understanding of chromatin modifications will be important in order to develop therapeutic strategies in MDS.

LIST OF PUBLICATIONS

I. LIMITED CLINICAL EFFICACY OF AZACITIDINE IN TRANSFUSION-DEPENDENT, GROWTH FACTOR RESISTANT, LOW- AND INT-1 RISK MDS. RESULTS FROM THE NORDIC NMDSG08A PHASE II STUDY
M Tobiasson, I Dybedahl, M Skov Holm, M Karimi, L Brandefors, H Garelius, M Grövdal, I Högh-Dufva, K Grønbæk, M Jansson, C Marcher, L Nilsson, A Olsnes Kittang, A Porwit, L Saft, L Möllgård, E Hellström-Lindberg. *Blood Cancer J. Mar 2014; 4(3): e189.*

II. AZACITIDINE INDUCES PROFOUND GENOME-WIDE HYPOMETHYLATION IN PRIMARY MYELODYSPLASTIC BONE MARROW CULTURES BUT MAY ALSO REDUCE HISTONE ACETYLATION.
M Grövdal, M Karimi, M Tobiasson, L Reinius, M Jansson, K Ekwall, J Ungerstedt, J Kere, D Greco, E Hellström-Lindberg. *Leukemia (2014) 28, 411–413*

III. MUTATIONS IN HISTONE MODULATORS AND HOXA5 METHYLATION LEVELS PREDICT SURVIVAL IN AZACITIDINE TREATED MDS PATIENTS
M Tobiasson, M Karimi, M Dimitriou, Y Qu, M Jansson, A Ben Azenkoud, M Jädersten, H Abdulkadir, J Ungerstedt, S Lehmann, A Lennartsson , K Ekwall, E Hellström-Lindberg. *Submitted*

IV. IN VITRO EXPOSURE TO AZACITIDINE INDUCES DEMETHYLATION AND INCREASED GENE EXPRESSION IN PRIMARY MDS PROGENITOR CELLS.
M Tobiasson, H Abdulkadir, A Lennartsson, S Katayama, F Marabita, M Karimi, Y Qu, K Krjutshkov , E Einarsdottir, M Grövdal, M Jansson, A Ben Azenkoud, S Lehmann, K Ekwall, J Kere, E Hellström-Lindberg* J Ungerstedt*
(* shared senior author). *Manuscript*

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

I. EARLY DETECTION OF RELAPSE IN PATIENTS WITH MYELODYSPLASTIC SYNDROME AFTER ALLO-SCT.
M Tobiasson, R Olsson, E Hellström-Lindberg, J Mattsson. *Bone Marrow Transplantation 2011 May;46(5):719-26*

II. MYELODYSPLASTIC SYNDROMES ARE PROPAGATED BY RARE AND DISTINCT HUMAN CANCER STEM CELLS IN VIVO.
Woll PS, Kjällquist U, Chowdhury O, Doolittle H, Wedge DC, Thongjuea S, Erlandsson R, Ngara M, Anderson K, Deng Q, Mead AJ, Stenson L, Giustacchini A, Duarte S, Giannoulatou E, Taylor S, Karimi M, Scharenberg C, Mortera-Blanco T, Macaulay IC, Clark SA, Dybedal I, Josefsen D, Fenaux P, Hokland P, Holm MS, Cazzola M, Malcovati L, Tauro S, Bowen D, Boultwood J, Pellagatti A, Pimanda JE, Unnikrishnan A, Vyas P, Göhring G, Schlegelberger B, Tobiasson M, Kvalheim G, Constantinescu SN, Nerlov C, Nilsson L, Campbell PJ, Sandberg R, Papaemmanuil E, Hellström-Lindberg E, Linnarsson S, Jacobsen SE. *Cancer Cell. 2014 Jun 16;25(6):794-808*

CONTENTS

LIST OF ABBREVIATIONS

ANC	Absolute neutrophil count
AML	Acute myeloid leukemia
BM	Bone marrow
CR	Complete remission
CMML	Chronic myelomonocytic leukemia
DAC	Decitabine
G-CSF	Granulocyte colony-stimulating factor
Hb	Hemoglobin
HI	Hematological improvement
HSC	Hematopoietic stem cell
IPSS	International Prognostic Scoring System
IPSS-R	Revised IPSS
RA	Refractory anemia
mCR	Marrow complete remission
MDS	Myelodysplastic syndrome
MPN	Myeloproliferative neoplasm
PB	Peripheral blood
PD	Progressive disease
PR	Partial remission
RAEB	Refractory anemia with excess of blasts
RARS	Refractory anemia with ringsideroblasts
RCMD	Refractory cytopenia with multilineage dysplasia
RCMD-RS	Refractory cytopenia with multilineage dysplasia and ringsideroblasts
SCT	Stem cell transplantation
SD	Stable disease
WBC	White blood count
WHO	World Health Organization
WPSS	WHO-based Prognostic Scoring System

1 THE MYELODYSPLASTIC SYNDROMES

1.1 BACKGROUND AND EPIDEMIOLOGY

The myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal hematopoietic stem cell (HSC) disorders characterized by cytopenia(s), dysplasia in one or more cell lineages, ineffective hematopoiesis, and increased risk of progression to acute myeloid leukemia (AML). MDS can arise *de novo* or as a consequence of previous chemotherapy or radiation. Although the majority of MDS are characterized by progressive bone marrow (BM) failure, the clinical course varies substantially in the different subtypes with survival ranging from a few months to decades[1].

The incidence of MDS has been reported to between 4-10 new cases / 100 000 inhabitants / year.[2-7] In the Swedish quality registry covering >90% of newly diagnosed patients, 1247 new cases of MDS and the mixed group of myelodysplastic and myeloproliferative disorders (MDS/MPN) were reported between 2009 and 2012, corresponding to a yearly incidence of 4 / 100 000 inhabitants. It is a disease mainly of the elderly and the incidence is one of the most common hematologic malignancies in patients over the age of 70 years, among which the annual incidence exceeds 20 per 100,000 persons. Median age at diagnosis is around 70-75 years, with a slight dominance of men, see Figure 1.[2-7] Ninety percent of MDS cases are idiopathic (*de novo* MDS) while around 10% are secondary to previous treatment with cytostatic drugs, in particular alkylating agents, or radiotherapy.[7] Other risk factors identified are tobacco use, occupational exposure to solvents or agricultural chemicals and having a relative with hematological disease although these factors show only a week association with the disease.[8, 9] The risk of MDS is markedly increased in certain rare genetic syndromes with bone marrow failure syndromes, often as a result of telomere dysregulation (i.e. Fanconi anemia, Shwachman-Diamond syndrome, severe congenital neutropenia, Dyskeratosis congenita, Diamond-Blackfan anemia).[10]

1.2 CLINICAL PRESENTATION

The clinical presentation and the natural course of MDS varies largely among diagnostic subgroups and between individuals. An increasing number of patients are diagnosed through routine blood samples obtained by the family doctor for non-MDS related problems. Symptoms from MDS are often related to the cytopenias where a majority of the patients have anemia, most often macrocytic, and some also have

leukopenia and / or thrombocytopenia. Around half of the patients have a severe anemia (<100 g / L) at diagnosis.[11] Anemia-related symptoms (e.g. fatigue, dyspnea and head-ache) constitute the most common clinical presentation of MDS. Other symptoms include infections and bleedings as a result of leukopenia and thrombocytopenia. These symptoms are also the major cause of MDS-related mortality.[12, 13] A minority of the patients suffer from autoimmune manifestations such as arthritis, pulmonary effusions and vasculitis. [14-16] Around one third of the patients transform to a secondary acute myeloid leukemia (AML).[11]

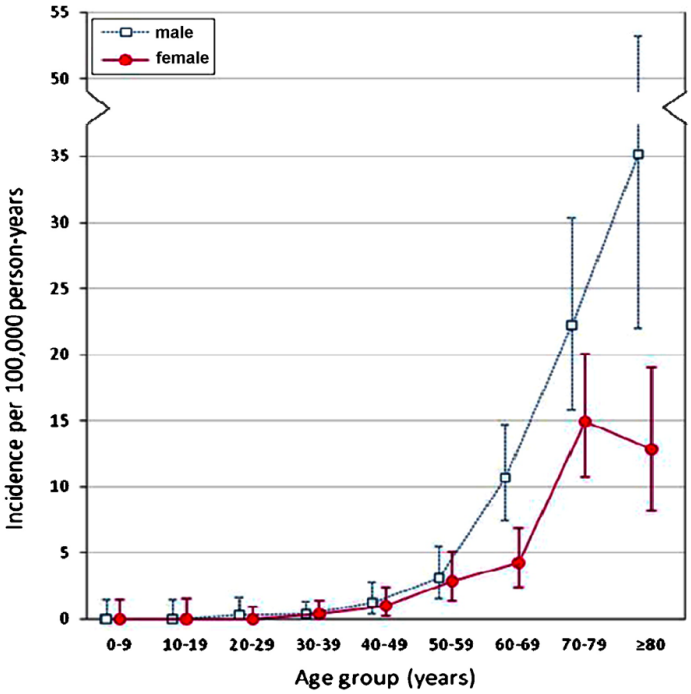

Figure 1: Incidence for different age groups. Neukirchen et al, Leuk Res 2011

1.3 BONE MARROW MORPHOLOGY AND CYTOGENETICS

The bone marrow of MDS is typically hypercellular although it can be normo- or hypocellular. As defined by the WHO criteria (see below), at least 10% of the progenitors of one cell line (erythroid, myeloid or megakaryocytic) must show significant dysplasia for a diagnosis of MDS. Examples of dysplasia include nuclear abnormalities, poor granulation of the cytoplasm of granulocytes or presence of ringed sideroblasts. Number of cell lines involved, presence of ringed sideroblasts and percentage of blasts, which might be normal (<5%) or elevated defines together with the cytogenetic analysis the subgroup classification.[17, 18]

Cytogenetic abnormalities are important determinants in the pathogenesis, diagnosis, and prognosis of MDS and have major impact on therapeutic decision-making in

individual patients.[19-22] Chromosomal abnormalities are detected in approximately 50% of patients with de novo MDS and in up to 80% in therapy-related MDS, see illustration in Figure 2.[19, 21] Aberrations are most common in the RAEB-subtypes.

In MDS, unbalanced chromosomal abnormalities reflecting gain or loss of chromosomal material are more prevalent in comparison to AML, while balanced translocation are rare.[20] The most frequently observed chromosomal abnormality in de novo MDS is the interstitial deletion of the long arm of chromosome 5, with or without additional karyotypic abnormalities.[20, 21]

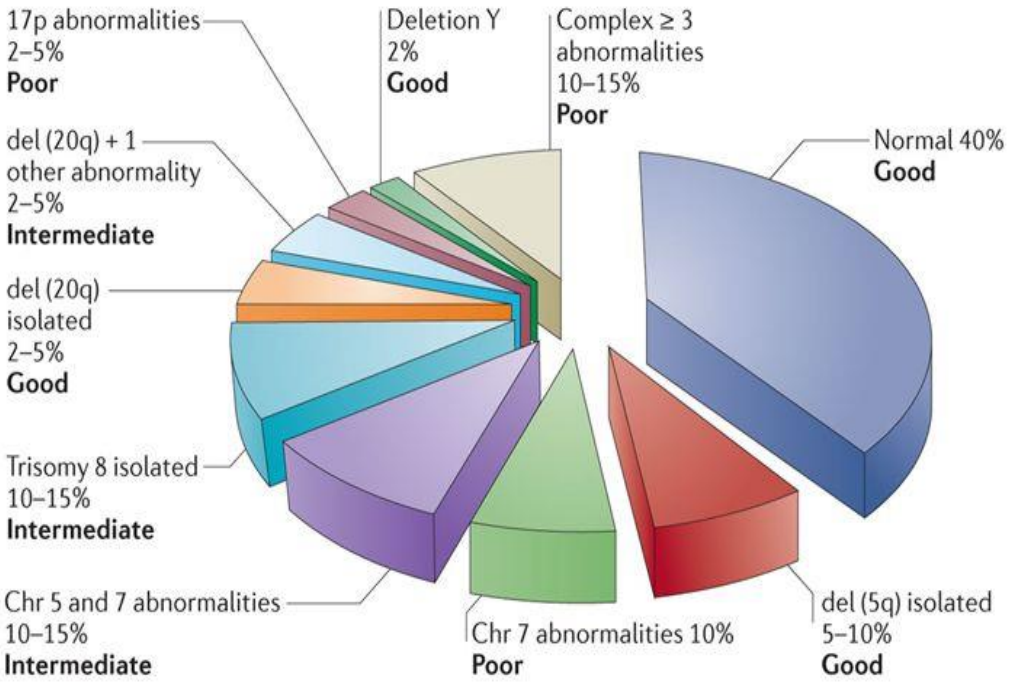

Figure 2: Common chromosomal abnormalities in MDS including International Prognostic Scoring System predictions. Raza et al, Nature Reviews Cancer 2012

1.4 CLASSIFICATION

The World Health Organization (WHO) classification of MDS was presented in 2001 and updated in 2008, see Table 1. It provides at date the best diagnostic approach to MDS. The WHO classification has considerable prognostic relevance, and its implementation into clinical practice is mandatory for optimal management of MDS.[13] The WHO classification replaced the older French-American-British (FAB) classification. The basis for the classification is determination of number of dysplastic cell lines, presence of ringed sideroblasts, percentage of blasts, myeloproliferative features, number of monocytes in blood and deletion of chromosome 5. In addition to the MDS WHO group, there is a group defining the mixed myelodysplastic /

myeloproliferative conditions including chronic myelomonocytic leukemia (CMML) and atypical chronical myeloid leukemia (aCML), see Table 2.

WHO subtype	Peripheral blood	Bone marrow findings
Refractory cytopenias with unilineage dysplasia (RCUD) Refractory anemia (RA) Refractory neutropenia (RN) Refractory thrombocytopenia RT)	Unicytopenia or bicytopenia* No or rare blasts (<1%) **	Unilineage dysplasia: ≥10% of the cells in one linage <5% blasts <15% of erythroid precursors are ring sideroblasts
Refractory anemia with ring sideroblasts (RARS)	Anemia, no blasts	≥15% of erythroid precursors are ring sideroblasts. Erythroid dysplasia only, <5% blasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s) No ore rare blasts (<1%) No Auer rods <1x10/L monocytes	Dysplasia in ≥10% of the cells in ≥ 2 myeloid lineages <5% blasts in BM No Auer rods, +/- 15% ring sideroblasts
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenia(s), <5% blasts, no Auer rods <1x10/L monocytes	Unilineage or multilineage dysplasia 5-9% blasts** No Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenia(s) <5% blasts Auer rods +/-*** <1x10/L monocytes	Unilineage or multilineage dysplasia 10-19% blasts Auer rods +/-
Myelodysplastic syndrome-unclassified (MDS-U)	Cytopenia(s) ≤1% blasts**	Unequivocal dysplasia in <10% of cells in ≥1 cell lines accompanied by a cyto-genetic abnormality; considered as presumptive evidence for MDS, <5% blasts
MDS associated with isolated del(5q)	Anemia; usually normal or increased platelet count No or rare blasts (<1%)	Normal to increased mega-karyocytes with hypolobated nuclei; <5% blasts Isolated del(5q) abnormality No Auer rods
**Bicytopenia may occassionally be observed. Cases with pancytopenia should be classified as MDS-U; **If the marrow blast percentage is <5% but there are 2-4% myeloblasts in the blood, the diagnostic classification is RAEB 1. Cases of RCUD and RCMD with 1% myeloblasts in the blood should be classified as MDS-U; ***Cases with Auer rods and <5% myeloblasts in the blood and <10% in the bone marrow should be classified as RAEB-2*		

Table 1: The WHO classification 2008 of the myelodysplastic syndromes

Disease	Peripheral blood	Bone marrow findings
Chronic myelomonocytic leukaemia (CMML)	Peripheral blood monocytosis > $1x10^9$/l No BCR/ABL-1 fusion gene <20% blasts	Dysplasia in one or more myeloid lineage[1] <20% blasts. Blasts include myeloblasts, monoblasts and promonocytes. No rearrangement of PDGFRA or PDGFRB
Atypical chronic myeloid leukaemia, BCR-ABL1 negative (aCML)	Leukocytosis, neutrophilia Neutrophilic dysplasia Neutrophil precursors ≥10% of leukocytes Blasts <20% No BCR-ABL1 fusion gene No rearrangement of PDGFRA or PDGFRB Minimal basofilia Monocytes < 10% of leukocytes	Neutrophil dysplasia with or without dysplastic lineages <20% blasts
Juvenile myelomonocytic leukaemia (JMML)	Peripheral blood monocytosis >$1x10^9$/l <20% blasts Usually WBC > $10x10^9$/l	<20% blasts. Evidence of clonality
Myelodysplastic/myeloproliferative neoplasm, unclassifiable (MDS/MPN)	Mixed MDS and MPN features No prior diagnosis of MDS or MPN No history of recent growth factor or cytotoxic therapy to explain MDS or MPN features No BCR-ABL1 fusion gene of rearrangements of PDGFRA or PDGFRB	Mixed MDS and MPN features <20% blasts
[1]Refractory anaemia with ring sideroblasts associated with marked thrombocytosis (RARS-T) (provisional entity)[2]	Persistent thrombocytosis >$450x10^9$/l Anaemia BCR-ABL1 negative Cases with t(3;3)(q21;q26), inv(#)(q21q26) and isolated del(5q) are excluded	Morphologic features of RARS; ≥ 15% of erythroid precursors are ring sideroblast Abnormal megakaryocytes similar to those observed in BCR-ABL1 negative MPN

[1] If myelodysplasia minimal or absent, CML can still be diagnosed if the other requirements are met and there is an acquired clonal cytogenetic or molecular genetic abnormality. Bicytopenia may occasionally be observed. Cases with pancytopenia should be classified as MDS-U

[2] If the marrow myeloblast percentage is <5% but there are 2-4% myeloblasts in the blood, the diagnostic classification is RAEB-1. If the marrow myeloblast percentage is <5% and there are 1% myeloblasts in the blood, the case should be classified as MDS-U.

[3]Cases with Auer rods and <5% myeloblasts in the blood and <10% in the marrow should be classified as RAEB

Table 2: The WHO classification 2008 of myelodysplastic/myeloproliferative neoplasms

1.5 PROGNOSIS, RISK ASSESSMENT AND FOLLOW-UP

The natural history of MDS varies considerably; while some patients experience an indolent course, others show short overall survival and rapid transformation to AML. Therefore, risk stratification is critical for both prognostic assessment and formulating treatment goals. Several prognostic scoring systems have been developed to identify MDS subtypes with different outcomes and to stratify patients into lower and higher risk subgroups. These schemes are based on morphology, peripheral blood counts and cytogenetics. The most commonly used prognostic tool is the International Prognostic Scoring System (IPSS) from 1997.[11] The original IPSS was recently revised (IPSS-R) and now includes five instead of three cytogenetic prognostic subgroups; it splits marrow blast percentage <5% into two groups and introduces more levels of cytopenia (Table 3).[21] A third scoring system, the WHO classification based prognostic score system (WPSS) uses the WHO classification subgroups, karyotype and in addition transfusion dependency which is an independent negative prognostic factor.[13] In addition to the factors included in the scoring systems above, presence of fibrosis and co-morbidity has been identified as independent negative factors.[23, 24]

Prognostic variable	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**	**3.0**	**4.0**
Cytogenetics	Very good		Good		INT	Poor	Very poor
BM blasts, %	≤2%		>2-<5%		5-10%	>10%	
Hemoglobin (g/dl)	≥10		8-<10	<8			
Platelets (x10/L)	≥100	50-<100	<50				
ANC (x10/L)	≥0.8	<0.8					
Risk category	**Risk score**						
Very low	≤1.5	**Prognostic subgroups:** **Very good**: del(11q), -Y **Good**: normal,del(5q), del(12p), del(20q), double including del(5q) **Poor:** Inv(3)/t(3q)/del(3q), double including -7/del(7q), Complex karyotype: 3 abnormalities, -7 **Very poor**: complex karyotype (>3 abnormalities)					
Low	>1.5-3						
Intermediate	>3-4.5						
High	>4.5-6						
Very high	>6						

Table 3. Revised IPSS, adapted from Greenberg *et al*., Blood, 2012

Real-life survival data, as documented in the Swedish quality registry between 2009 and 2012 demonstrate a median overall survival of the lower risk groups (IPSS low or intermediate-1) of around 4 years, while median overall survival for the higher risk groups (IPSS intermediate-2 or high) is only around one year, see Figure 3.

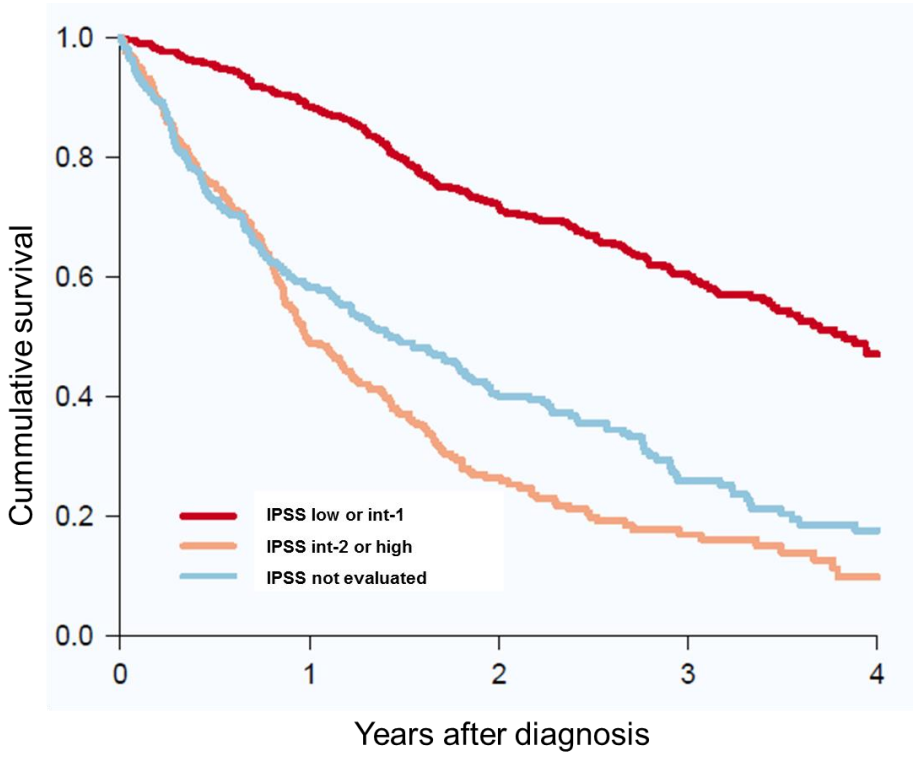

Figure 3: Cumulative survival stratified for IPSS risk group. Data from the Swedish quality registry 2009-2012.

1.6 PATHOGENESIS

1.6.1 Stem cell disease

The MDS clone has its origin in the hematopoietic stem cell compartment as demonstrated by clonal genetic markers in sorted HSCs and by repopulation and development of the MDS phenotype in mice transplantations. [25-29] Studies of fractionated compartments of the MDS clone have demonstrated that both genetic and epigenetic alterations can be traced back to the stem cells.[30, 31] Furthermore, it has been shown that transplantation of MDS stem cells into immunodeficient mice is sufficient to develop the disease.[29] Evidence of a prevailing MDS-clone, as demonstrated by FISH for del5q- in the stem cell compartment has been shown after lenalidomide treatment despite morphological and cytogenetic remission.[27] Similarly, the phenotypical MDS clone within the stem cell compartment failed to be eradicated by Azacitidine, despite morphological remission.[32] The non-curative potential of these drugs could thus be explained by a remaining MDS clone within the stem cell compartment which will eventually proliferate, resulting in treatment failure.

1.6.2 Genetic aberrations

Through next generation sequencing, the landscape of the MDS genome has been unraveled and recurrent mutations in more than 40 genes have been reported to be associated with the MDS disease. [33-37] These genes can be divided into functional classes including spliceosome factors e.g. *SF3B1* and *SRSF2*; epigenetic regulators e.g. *TET2* and *ASXL1*; DNA methylation regulators e.g. *TET2* and *DNMT3A*; histone regulators e.g. *ASXL1* and *EZH2*; transcription factors e.g. *RUNX1* and *ETV6*; signaling factors e.g. *JAK2* and *CBL*; and cohesion factors e.g. *STAG2* and *RAD21*. Some of the mutations are present in all subgroups of MDS e.g. *ASXL1* while some are enriched in specific subgroups e.g. *SF3B1* in RARS.[35].Some of the mutations appear mutually exclusive to each other e.g. all spliceosome mutations while others show mutual association e.g. *IDH2* and *SRSF2*.[35] Around 70-90% of the patients have been reported to carry one or several mutations and the number of mutations correlates with survival.[35, 36] Several of the mutations are associated with shorter survival e.g. *ASXL1, EZH2, RUNX1* and *TP53*.[34-36, 38] Our research group has demonstrated that mutations of *TP53* in patients with del(5q) patients are common, and are associated with significantly shorter survival.[39] Only *SF3B1* mutation have been reported to be a positive prognostic marker.[40] The impact of mutations on response to Azacitidine is reviewed in detail in chapter 2.10.3. One study reporting the effect of mutations after transplantation demonstrated a negative impact on survival of *TET2, DNMT3A* and *TP53*.[41]

The high sensitivity of next generation sequencing has enabled a more detailed characterization of the MDS clone architecture, revealing a dynamic evolution of different subclones over time where Darwinian mechanisms subsequently lead to dominance of the subclones with the greatest survival advantage, see illustration in Figure 4. [31, 42, 43]

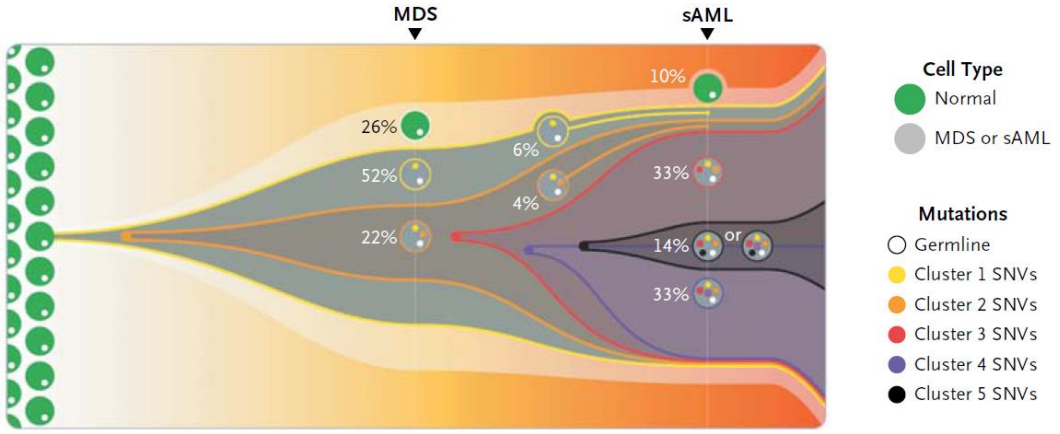

Figure 4: Clonal evolution during progression from MDS to AML. Walter et al, New England Journal of Medicine, 2012. Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society

1.6.3 Epigenetics in normal and cancerous cells

The term epigenetics refers to changes in phenotype which are inherited over cell division, without alterations in the DNA sequence. The epigenetic regulation are important for cellular differentiation.[44] There are two main mechanisms for epigenetic regulation. The most studied epigenetic mechanism is DNA methylation, in which cytosine residues of the DNA are methylated. Methylated cytosine is often located within or close to CpG islands, which are rich in cytosine followed by a guanine residue. These are often located in gene promoters and methylation in these sites results in inactivated transcription. The role of methylation in non-promoter regions is less known. A second important epigenetic mechanism is regulation of chromatin structure which can be formed as heterochromatin, associated with down-regulated transcription or euchromatin, associated with active transcription. Chromatin structure is regulated by enzymes which modulate the chromatin e.g. by adding histone modifications or by replacing the histone proteins with histone variants. Both DNA methylation and histone modifications are dynamically regulated during hematopoietic differentiation.[45, 46]

Epigenetic dysregulation is a hallmark of cancer, where general hypomethylation as well as promotor hypermethylation are typical features.[47] Another important feature of the cancer epigenome is increased epigenetic variation, as a result of stochastic drift due to loss of epigenetic regulation.[48]

1.6.4 Epigenetics aberrations in MDS

Several of the genes recurrently mutated in MDS are in fact epigenetic regulators and mutations in these genes are likely to affect the epigenome.[35, 36] The first group of mutated genes is involved in the regulation of DNA methylation. *TET2*, which is

mutated in 15-25% of MDS patients, is involved in production of hydroxymethylation, an intermediate chemical group during DNA demethylation.[49] It has been demonstrated that mutations in *TET2* lead to reduced hydroxymethylation although the functional consequences are not clear.[50] *IDH1/2* is involved in the metabolism of hydroxymethylation and mutations in these enzymes result in a hypermethylated phenotype.[51] Mutations in *IDH1/2* have also been shown to block histone demethylation.[52] *DNMT3A* is involved in de novo methylation and *DNMT3A*-mutated patients cluster together in genome-wide methylation analyses although the functional effects are yet to be described.[53] The DNA methylation pattern has been characterized in a few studies, demonstrating an aberrant methylation pattern with common promoter-hypermethylation in line with the general pattern in cancer epigenetics.[54-56] Average methylation increases over time and is thought to contribute to progression of the disease.[55] Several single genes, with p15 being the most studied, have been shown to be hypermethylated in MDS and several of them are reported to be associated with worse prognosis.[57-63] Our research group showed in a previous study that promoter hypermethylation is associated with higher resistance to chemotherapy. [63]

The second group of genes is involved in regulation of chromatin structure. *EZH2* together with the rare mutations in *EED, JARID2* and *SUZ12* are part of the polycomb repressing complex 2 (PRC2), involved in trimethylation of histone 3, lysine 27 (H3K27me3), important for stem cell function and differentiation.[64-68] It is known that the *EZH2* mutations in myeloid disorders result in loss of function and increased transcription of polycomb-target genes, e.g. *HOX*-genes. *ASXL1* also affects PRC2 although the exact mechanisms are still unclear.[69, 70] Histone modifications and chromatin structure are much less studied than DNA methylation, partly due to technically more complicated assays, hence reports on histone patterns in MDS are sparse. One study on the activating histone mark H3K4me3 showed an increase in 36 genes enriched for innate immunity signaling.[71]

Several transcription factors important for hematopoietic differentiation, including PU.1 and GATA1 have been shown to be epigenetically dysregulated. [72, 73] Furthermore, aberrant methylation of ribosomal RNA has been demonstrated in MDS.[74]

There are principally two different hypotheses why epigenetic aberrations occur. The first is mutations in epigenetic regulators, resulting in dysregulation due to altered function of these regulators. This hypothesis is supported by clustering of epigenetic data based on mutations in specific regulators e.g. *DNMT3A*.[51, 53, 54, 69, 75] Indirect

impact on epigenetic regulators has in a similar way been shown, for instance *JAK2* mutation downregulates a histone methyltransferase.[76] According to the second hypothesis, epigenetic aberrations are a consequence of stochastic epigenetic drift with increased variation, supported by the fact that epigenetic variation is increasing with age and is considered a hallmark of cancer.[45, 48, 77, 78]

Intriguingly, it has been postulated that age-related epigenetic drift, results in a decreased ability of stem cells to respond to stress and to self-renew. In this context, mutations in epigenetic regulators e.g. *TET2* and *ASXL1*, gives these cells a survival advantage and an escape from the negative pressure of epigenetic aberrations.[79]

1.6.5 Immunological mechanisms

Patients with MDS sometimes present with inflammatory manifestations such as arthritis or vasculitis and immunosuppressant therapy is efficient in a subset of MDS patients.[14-16, 80] The composition of the immune system has thus been of great interest in MDS research and several immunological imbalances have been identified, in particular within the T-cell lineage. Up-regulation of cytotoxic T-cells have been demonstrated in lower-risk MDS, while regulatory T-cells are up-regulated in higher risk MDS.[81-87] The role of the immune system seems thus rather contrary between lower and higher risk MDS, where lower risk MDS is characterized by a pro-inflammatory environment while higher risk MDS is characterized by immune escape.[88] Studies on NK cells have revealed a decreased NK-cell reactivity in MDS patients[89-91]. Dendritic cells show reduced number and functionality in MDS with unknown consequences.[92, 93]

1.6.6 Disturbances in the microenvironment

Disruption of the BM microarchitecture is a common finding in MDS encompassing altered localization of hematopoietic cells within the bone marrow and alterations in components that comprise the microenvironment including edema, fibrosis, vascular proliferation, lymphoid aggregates and inflammatory changes.[94] These findings indicate that MDS is not only a disease of hematopoietic cells but also of the tissue. However, whether these BM stromal changes are an epiphenomenon or a pathogenetically important element of the disease itself remains unclear. In human MDS, xenotransplant models using immunodeficient mice have consistently shown poor engraftment of myelodysplastic cells and failure to confer the clinical

hematopoietic phenotype of human MDS suggesting a crucial role of the microenvironment to support the clone. Several groups have reported that abnormalities in stromal cells can induce MDS (or AML) in otherwise normal bone marrow cells.[95, 96] On the basis of these studies, a "niche-based" model of leukemogenesis in MDS has been proposed.[97]

1.7 TREATMENT

The choice of treatment in MDS is based on clinical symptoms, risk groups, cytogenetic and morphological features, and age. The arsenal of therapeutic possibilities ranges from basic treatments such as transfusions to advanced treatments including intensive chemotherapy and allogeneic transplantation.

1.7.1 Transfusion therapy

A majority of the patients will develop transfusion-dependent anemia at some time and more than 50% have a severe anemia already at diagnosis.[11] Anemia is associated with increased morbidity / mortality and with decreased quality of life which is improved by transfusions.[13, 98, 99] The hemoglobin level should be adapted on an individual basis by the patient and the physician, taking into account co-morbidities and symptoms.

1.7.2 Growth factors

Around 50% of anemic patients respond to growth factor treatment, i.e. erythropoiesis stimulating agents (ESA) with or without the addition of granulocyte colony stimulation factors (G-CSF), and the median duration of response is 2 years.[100-104] A predictive model, based on serum levels of erythropoietin (S-Epo) and the red blood cell (RBC) transfusion rate was developed by our group to select patients with good probabilities for response to treatment, where S-epo and transfusion rate were negative prognostic factors for response.[105] A randomized phase III study has showen that responders to ESA have prolonged survival compared to non-responders and two large retrospective studies indicate an improved survival for patients treated with ESA compared to untreated patients.[101, 104, 106]

1.7.3 Iron chelation therapy

All patients with a chronic need of red blood cell transfusions will eventually develop iron overload. In thalassemia major patients with chronic transfusion dependency from

early childhood, it is well known that iron overload will cause organ damage (liver, heart and pancreas) and eventually organ failure and death. Iron chelation decreases both morbidity and mortality in these patients.[107-109] Excess iron is seen also in organs of heavily transfused MDS patients. Several retrospective studies have shown a survival benefit for patients receiving iron chelation therapy; whether this reflects a chelation-effect or is confounded by a more severe disease or comorbidities is however unclear.[110-113] No prospective, randomized studies have addressed this question but extrapolated from the experience of thalassemia patients, iron chelation is generally recommended for patients with lower risk disease with a chronic transfusion need and an expected survival counted in years.[114] Reducing the iron overload seems to improve bone marrow function in some cases and patients achieving transfusion independency when treated with iron chelation have been reported.[115, 116]

1.7.4 Immunosupression

A small group of patients with low-risk MDS can benefit from immunosuppressive treatment with anti-thymoglobulin (ATG) + cyclosporine-A, and response rates of around 30% have been reported. Hypocellular bone marrow, age below 60 years, and HLA DR15 positivity have been reported as associated with response.[80, 117-119]

1.7.5 Immunmodulatory drugs

Lenalidomide represents the first targeted therapy for MDS and is approved for transfusion dependent patients with lower risk MDS and deletion of chromosome 5 del(5q). The specific activity in del(5q) MDS was first observed in the MDS-001 study in which 12 of 43 lower risk patients had del(5q) and 83% of these responded.[120] The unique activity in lower risk del(5q) MDS was confirmed in a subsequent MDS-003 study which led to approval in the United States.[121] By contrast, the European drug authorities did not approve the drug as they could not exclude an association between treatment and leukemic transformation. The randomized double-blind phase III MDS-004 trial aimed to validate the finding from MDS-001 and MDS-003 studies in which 205 patients were randomized to either 10 mg lenalidomide day 1-21 every 28 days, 5 mg daily, or placebo. The RBC-transfusion independence rate (≥26 weeks) was 56%, 42% and 6% for lenalidomide 10 mg, 5 mg and placebo, respectively, with corresponding cytogenetic response rates of 29%, 15% and 0%.[122] Median duration of transfusion independency in the 004 study was approximately two years, and the three

year overall survival and AML transformation risk were 56% and 25%, respectively in the lenalidomide cohorts combined. This study resulted in the European approval.

The use of lenalidomide in non-del(5q) lower-risk, transfusion dependent MDS was examined in the MDS-002 clinical trial.[123] This study had similar inclusion criteria and treatment schedule as the MDS-003 trial apart from the exclusion of patients with a del(5q) cytogenetic abnormality. The study enrolled 214 patients; 40% had RARS, and the majority of patients were low or INT-1 IPSS risk. The overall response rate was 43% with 26% of patients achieving transfusion-independency with a median duration of response of 41 weeks.

The observed difference in clinical responses between patients with del(5q) and non-del(5q) MDS led to the understanding of a karyotype-specific mechanism of action. The ability to induce apoptosis of progenitors harboring the del(5q) abnormality is thought to be linked to the haploinsufficiency of one or several genes on the long arm of chromosome 5. The fact that patients usually achieve TI within 4-5 weeks, before achieving cytogenetic remission, supports the concept of activation of residual normal erythropoiesis. Lenalidomide has also anti-angiogenic and anti-inflammatory properties.[124] In non-del(5q) disease, lenalidomide seems to enhance response of eryhroid precursors to different stimuli, including erythropoietin which is supported by the fact that lenalidomide promotes erythroid progenitor formation and expansion in CD34+ cells from healthy donors *in vitro*.[125]

The long-term effect of lenalidomide on del(5q) patients with regard to AML transfusion is yet to be determined since a prospective randomized study with this endpoint has not been conducted.

1.7.6 Chemotherapy

Before treatment with hypomethylating agents were available, intensive chemotherapy was the only disease-modulating option for patients not eligible for allogeneic stem cell transplantation. The rate of complete remission achieved with this treatment was around 50%, but the relapse rates were very high and long-term survival short. [126-128] Patients with a more proliferative disease, as reflected in higher cellularity, higher S-LDH and WBC have worse response to intensive chemotherapy.[129] After the advent of hypomethylating drugs, intensive chemotherapy is more rarely used but can be considered after failure to hypomethylating drugs in particular as a disease-controlling treatment prior to allogeneic stem cell transplantation.

1.7.7 Allogeneic stem cell transplantation

Since allogeneic stem cell transplantation (SCT) is the only potentially curative treatment in MDS and all patients should be evaluated for this option. However, due to the potentially severe complications, transplantation can only be performed in patients up to around 70 years. In younger patients (<50-60 years), a myeloablative conditioning is normally chosen, while older patients receive a reduced intensity conditioning which reduces transplantation-related mortality but increases the risk of relapse. Long-term survival rates of between 25% and 45% have been reported after transplantation.[130-135] Transplantation-related mortality (TRM) after myeloablative conditioning and reduced intensity conditioning has been reported to be 32% and 22% and relapse rate 22% vs. 45%, respectively.[130] Due to the high risk of TRM, timing of transplantation is of great importance where higher-risk patients is recommended to proceed to SCT upfront while lower-risk patients should follow a strict surveillance program and be transplanted in case of signs of progression.[136] All three prognostic scoring systems (IPSS, IPSS-R and WPSS) have been validated to also predict survival after allogeneic stem cell transplantation.[134, 137] We have previously demonstrated that surveillance by using a chimerism analysis which determines the proportions of hematopoietic donor / recipient cells can be used to predict an impending relapse after SCT. However, this analysis has low sensitivity / specificity and will most likely in the future, be replaced by molecular markers based on gene mutations.

1.7.8 Hypomethylating therapy

There are two hypomethylating drugs available: Azacitidine, which is reviewed in detail in Chapter 2, and Decitabine (DAC). Both drugs result in reduced DNA methylation which is thought to be the principal mechanism of action although other mechanisms are involved, see Chapter 2. The efficacy of DAC on patients with MDS have been evaluated in two randomized studies, both showing responses in around 30% of the patients, which was significantly better than the control arm consisting of supportive care, although none of the studies could observe any survival benefit for DAC-treated patients.[138, 139] DAC is registered for treatment of MDS in the United States but not in Europe.

1.7.9 Other epigenetic therapies

Histone deacetylase inhibitors (HDACi) target another epigenetic mechanism resulting in reduced histone acetylation and increased gene expression. One of the HDACi, vorinostat, is approved for treatment of cutaneous T-cell lymphoma. The efficacy of these drugs in MDS and AML has also been evaluated in several phase I / II studies showing clinical activity although limited to usually between 10-20% of the patients.[140-143] The toxicity profile includes both hematological toxicity and side effects on the central nervous system such as fatigue.

2 AZACITIDINE

2.1 BACKGROUND AND HISTORICAL OVERVIEW

Almost 50 years ago, 5-azacitidine (Azacitidine) and 2'-deoxy-5-azacitidine (Decitabine) were developed as classical cytostatic agents given at high doses but were soon replaced by other drugs such as 5-ara-C for treatment of acute leukemias[144, 145] In the 1970s the differentiation-promoting effects of Azacitidine were discovered and could later be coupled to the reduction of DNA methylation.[146-149]. New clinical studies, using lower-dosing "non-cytostatic" schedules with the hypothesis that demethylation could have an anti-leukemic effect, started again in the 1990s primarily on patients with MDS.[150]

2.2 CLINICAL RESULTS IN HIGHER-RISK MDS

Early phase I / II studies in the 1990s indicated clinical activity in higher risk MDS.[150, 151] Later, two large randomized phase III studies have been conducted. In the first, CALGB9221, 191 patients of all subtypes of MDS were enrolled and randomized to receive either Azacitidine at a dose of 75 mg / m^2 subcutaneously for 7 of 28 days or to receive supportive care only.[152, 153] Crossover from the control arm to Azacitidine was allowed after 4 months. Responses were evaluated in both arms in accordance with the International Working Group criteria which groups patients into complete remission (CR), marrow complete remission (mCR), partial remission (PR), hematological improvement (HI), stable disease (SD) or progressive disease (PD).[154] Among patients randomized to receive supportive care, 5% met the criteria for improvement; no patients on this arm achieved a CR or PR. Of the 99 patients randomized to receive Azacitidine, 60% (n = 60) achieved a response ($p < 0.0001$). Responses were scored as CR in 7% (n=7), PR in 16% (n=16), and HI 37% (n=37). Forty-nine patients crossed over to receive Azacitidine, of these 47% (n=23) responded and 10% (n=5) achieved a CR. Patients treated with Azacitidine had a median progression free survival (PFS) of 21 months vs. 12 months in those patients treated with supportive care alone, and this was statistically significant ($p = 0.007$), median overall survival (OS) in an intention to treat analysis was 20 months in the Azacitidine-treated patients vs. 14 months for those randomized to supportive care ($p = 0.10$). Thus, no statistically significant survival could be demonstrated, partly due to the cross-over design of this study. A landmark analysis performed at 6 months could, however, demonstrate a survival benefit for

Azacitidine-treated patients (p=0.035). In addition, this study showed improvement in transfusion need, effect on white blood cell and platelet counts and an improved quality of life. This study resulted in approval of Azacitidine in the United States.

The second randomized study, AZA-001, was designed to demonstrate a difference in overall survival, which CALGB9221failed to do.[155] The control arm consisted of three possible treatments, chosen by the physician: intensive chemotherapy, low-dose cytarabine or supportive care. A total of 357 patients with MDS and IPSS int-2 or high, AML with 20-30% blasts and multilineage dysplasia or CMML with >10% blasts were enrolled. The primary OS endpoint of this study was met after a median follow-up of 21.1 months. The median OS for the Azacitidine-treated patients was 24.5 months vs. 15 months for patients assigned to the control arm ($p < 0.0001$), see Figure 5. Two-year OS also favored Azacitidine; 51% vs. 25% for the control arm ($p < 0.0001$). Subgroup analyses comparing Azacitidine and the three different therapies in the control arm demonstrated a survival benefit for Azacitidine compared with low-dose cytarabine (p=0.0006) and with supportive care (p=0.0045). There was also a survival benefit for Azacitidine compared with intensive chemotherapy (9.4 months) but it was not significant (p=0.51) probably due to the low number of patients in this group (n=42). Overall, 29% of those assigned to Azacitidine achieved either CR (17%) or PR (12%) compared with 12% (8% CR and 4% PR) assigned to the control arm ($p = 0.0001$). Any hematological improvement (HI) was observed in 49% of those treated with Azacitidine vs. 29% in the control arm ($p = 0.0001$). Furthermore, of the 111 patients with red cell transfusion dependence at the time of study enrollment, 50 (45%) became transfusion independent in the Azacitidine group vs. 13 (11.4%) in the control arm ($p = 0.0032$). In addition, treatment with Azacitidine was associated with delayed leukemic transformation (18 vs 12 months, p<0.0001). This study resulted in the approval of Azacitidine in Europe. Post-hoc analyses of the AZA001 cohort have demonstrated a survival benefit not only for patients achieving a response but also for patients with stable disease without progression during treatment.[156] Both randomized studies show that responses are normally not seen before the patient has received ≥3 cycles and best response is often seen several cycles after the initial response.[153, 155]

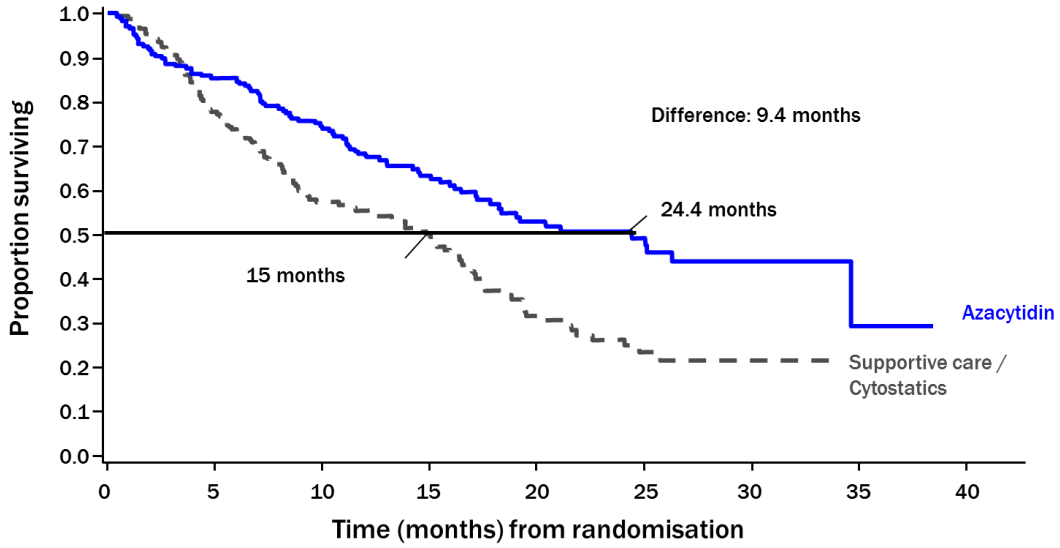

Figure 5: Survival in the AZ001 study. Feneaux et al, Lancet, 2010

Alternative dosing schedules have been evaluated in a study in which patients were randomized to receive 75 mg /m^2 daily for 5 days, off 2 days and then on 2 days (5-2-2), 50 mg/m 2 daily for 5 days, off 2 days and then on for 5 further days (5-2-5), and lastly 75 mg/m 2 daily for 5 days alone (5-0-0). These schedules seemed to result in similar hematological improvement rates (44%, 45%, 56%, respectively). This study was, however, not designed to produce statistically significant results, nor have these schedules been directly compared with the approved 7 day schedule.[157]

2.3 CLINICAL RESULTS IN LOWER-RISK MDS

In the US, the label encompasses all patients with MDS. A subgroup analysis of patients (n=22) with RA/RARS in the pivotal, prospective study CALGB9221 showed complete remission (CR), partial remission (PR) and hematological improvement (HI) in 9%, 18% and 32%, respectively.[152] A subgroup analysis from a second prospective study reported transfusion-independence (TI) after treatment with Azacitidine in 65% of previously transfusion-dependent (TD) patients (n=40) with lower-risk MDS[157]. Furthermore, two retrospective studies evaluated the effect of Azacitidine in lower-risk MDS populations. Musto et al reported CR, PR, HI of 16%, 10%, 20%, respectively and Prebet et al reported CR, PR, HI of 12%, 5% and 25%, respectively. None of these four studies employed ESA-resistance as an inclusion criterion. [158, 159] More recently, a prospective study on ESA-resistant patients with lower-risk MDS showed overall

response rate (ORR) of 47% and achievement of TI in previously TD patients in 33% of the patients.[160]

2.4 ALTERNATIVE ADMINISTRATION ROUTES

A few studies have evaluated Azacitidine given intravenously. The first compared pharmamacokinetic data between subcutaneous and intravenous administration.[161] Except peak concentration which was higher in intravenously treated patients, the pharmacokinetic data was almost identical. Both clinical trials using intravenous administration show similar response rates as previous studies on subcutaneous administration although they were not designed to detect survival benefit. [161, 162]. Use of oral Azacitidine has been limited by drug instability but development of a film-coated formulation has made oral administration possible and the initial phase-I studies are promising.[163-165] Larger studies on oral Azacitidine are ongoing.

2.5 COMBINATION OF AZACITIDINE AND OTHER DRUGS

The combination with HDACi has gained a lot of interest since it has been shown to have synergistic effects on gene expression.[166] A few smaller studies have evaluated the effect of Azacitidine in combination with different HDACis and show that the combination is feasible and effective, however, the response rates did not seem to differ remarkably from studies on Azacitidine as monotherapy.[167-169] The role of this combination is thus yet to be elucidated.

Lenalidomide is registered for treatment of MDS with isolated del 5q. Several phase I/II studies have evaluated the effect on the combination of Azacitidine and lenalidomide both in cohorts with a karyotype including del5q and mixed cytogenetic cohorts including patients without del5q.[170-174] The combination has shown to be feasible and with encouraging response rates exceeding the reported response rates on Azacitidine alone.

2.6 AZACITIDINE AND ALLOGENEIC STEM CELL TRANSPLANTATION

Two retrospective studies have evaluated the effect of Azacitidine as disease-controlling treatment prior to transplantation. The first study compared outcomes after SCT in 54 patients with MDS or CMML who either received or did not receive pre-transplantation Azacitidine and showed similar survival and relapse rates.[175] The second trial reviewed 68 patients who either received Azacitidine or intensive chemotherapy as pre-transplantation treatment. The estimated OS at 1 year was 57% in

those treated with Azacitidine and 36% in the chemotherapy group (p=0.24). The results from these retrospective studies indicate that Azacitidine is not inferior to intensive chemotherapy prior to transplantation although no prospective randomized studies have tested this conclusion.[176]

A few studies have reported the efficacy of Azacitidine used as salvage therapy after relapse and remission have been described for some patients.[177-181] Most of the patients in these studies received concomitant donor lymphocyte infusions (DLI). Azacitidine in combination with DLI have also been evaluated as preemptive treatment for impending relapse based on increasing levels of recipient cells in the chimerism analysis and prevention or delay of relapse have been reported with this combination.[182]

2.7 CLINICAL RESULTS IN ACUTE MYELOID LEUKEMIA

The efficacy of Azacitidine in AML has been assessed in several studies. Important in this setting is the fact that in the previous WHO classification, patients with a previous MDS diagnosis and between 20-30% blasts were classified as RAEB-t, i.e. a MDS-diagnosis, while WHO 2001 and 2008 classify these patients as AML. In the large randomized studies CALBG 9221 and AZA-001, patients with the former RAEB-t subgroup, now AML with 20-29% blasts and multilineage dysplasia, were included.[152, 155] Subgroup analysis of this cohort show superiority for Azacitidine-treated patients compared to the control arm and this subgroup is thus not different form the large cohort as a whole. Several studies have specifically evaluated the efficacy in AML-patients.[183-186] These studies show overall response rates of 32- 50%, with higher response rates for previously untreated patients. In summary, Azacitidine can thus be considered a highly active drug also in AML. Studies on the use of Azacitidine as consolidation therapy after remission achieved by intensive chemotherapy are ongoing.

2.8 PHARMOCOKINETICS

2.8.1 Basic pharmacokinetics

Azacitidine is a chemically relatively unstable drug with a half-life *in vitro* of around 7 hours[187-189]. Maximum concentration *in vivo* is reached within 30 minutes and half-life has been reported to be 0.3-0.7 and 0.1-0.4 hours after subcutaneous and intravenous injections respectively.[161, 190] The bioavailability has been reported to be > 90%.[161, 190] Systemic clearance exceeds glomerular filtration rate and additional clearance through deamination in liver and spleen has been proposed.[191] Concentration in patients treated

with standard dose (75 mg /m^2) has been measured to 3-11µM.[161, 190] Studies of the effect of factors such as age, gender, renal and hepatic impairments on pharmacokinetic data have not been conducted but based on the clinical studies, the safety profiles were not affected by these parameters.[152, 155] Clinical drug interaction studies have not been performed. Pharmacokinetic studies of oral Azacitidine show a bioavailability of 6-20%.[165] Maximum concentration was seen after 1 hour and mean elimination half-life was 0.6 hours.

2.8.2 Intracellular metabolism

Transportation of nucleosides across the cellular membrane are mediated through four different classes of transporters but it is still not known which transporters are important for the transportation of Azacitidine, see Figure 6.[192] However, one study shows correlation between expression of one of the transporters, ENT-1, and sensitivity to Azacitidine *in vitro*.[193] After transportation, Azacitidine is mono, di and tri phosphorylated. Exactly which enzymes that are involved in this processes are unknown, but uridin-cytidine kinase has been proposed to be of major importance.[192] A minor proportion (10-20%) of di-phosphorylated Azacitidine is being converted to 5-aza-deoxy-cytidine-triphosphate by the ribonucleotide reductase enzyme where the deoxy-converted analogue can be incorporated into DNA while the non-converted compound can be incorporated into RNA[194]. Interestingly, hydroxyurea has been shown to block the ribonucleotide reductase and thereby preventing Azacitidine from being incoportated to DNA.[195]

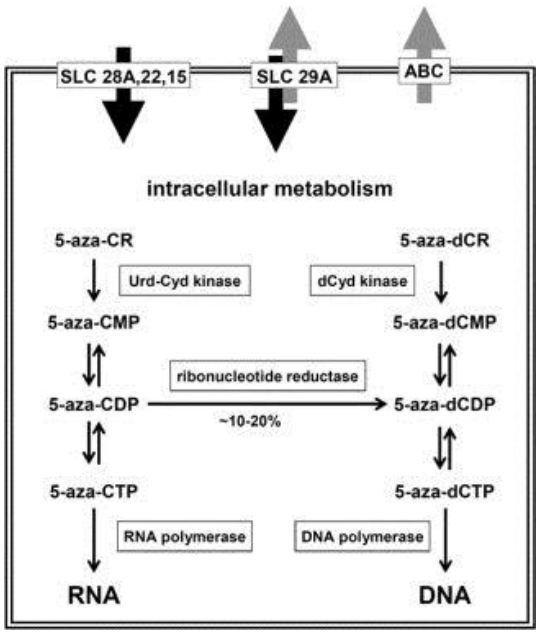

Figure 6: Intracellular metabolism of Azacitidine. Stresemann et al, Int J Cancer 2008

2.9 PHARMACODYNAMICS

2.9.1 Induction of apoptosis and differentiation

Several studies have demonstrated a direct cytotoxic effect on leukemic cells and the cytotoxic effect seems to be most pronounced in proliferating cells.[194, 196, 197] Apoptotic activity, involving several apoptotic pathways such as activation of the TRAIL receptor and the bcl-2 family proteins have been shown to increase early after exposure to Azacitidine *in vitro*[197-199]. Another apoptotic pathway described more in detail is the DNA damage response system which can be evoked by Azacitidine mediated through increased expression of the transcription factor *FOXO3A*. Decitabine has been shown to induce DNA double breaks followed by activation of the DNA damage response system and DNA is being repaired after drug removal.[200] Besides pro-apoptotic properties, it was early demonstrated that Azacitidine had the potential to induce cellular differentiation of leukemic cells.[146-149] A paradoxical effect of Azacitidine is observed in the clinical setting: where the kinetics of hematological toxicity observed during treatment, e.g. neutropenia and thrombocytopenia follows ordinary kinetics as compared to other cytostatic drugs with a cyclic decrease followed by recovery until next cycle, while the response effect arrives most often after several cycles of treatment, indicating a response effect other than the cytotoxicity seen in normal cytostatic treatment.

2.9.2 Effect on stem cells

Azacitidine can reduce clone size but repeated studies show that no complete eradication of MDS stem cells can be achieved.[27, 30, 32, 43]. However, long-term exposure *in vitro* has shown to inhibit self-renewal of malignant stem cells, which might contribute to the delay in leukemic transformation seen in Azacitidine-treated MDS-patients.[201] In contrast, both Decitabine and Azacitidine has been shown to rather increase the self-renewing capability of normal bone marrow.[202, 203]

2.9.3 Effects on DNA methylation

After Azacitidine has been metabolized to 5-aza-deoxy-cytidine-triphophate it becomes a substrate for the DNA replication machinery and will be incorporated into DNA as a substitute for cytosine. Methylation of DNA is exerted by the DNA methyl transferase (*DNMT*) family of enzymes where *DNMT1* is responsible for maintaining the methylation pattern during replication while *DNMT3A* and *DNMT3B* exert de novo methylation important during the differentiation process. The *DNMT* enzymes

recognize Azacitidine-guanine nucleotides which results in an irreversible binding and subsequently depletion of the enzyme which in turn lead to reduced methylation.[149, 204, 205] The affinity for the different types of *DNMT* are unknown, probably all three enzymes are affected although the cytotoxic effect of blocking *DNMT3A* and *DNMT3B* has been reported to be greater than for *DNMT1*.[206, 207] Besides the three *DNMT*s responsible for DNA methylation, there are two more *DNMT*s: *DNMT2* which methylates tRNA and *DNMT3L*, which function is unknown.

A numerous studies have shown a demethylating effect of Azacitidine, both *in vitro* and *in vivo*, demonstrating effect both using methods for global as well as for gene-specific methylation level.[58, 162, 167, 197, 208-215] Culture experiments on leukemic cell lines have showed increasing demethylation up to 48 h of incubation, starting already after 1 hour of incubation.[210] The hypomethylating effect is present at low to moderate doses of Azacitidine while the effect disappears with higher doses (>3uM) and demethylation is thus following a U-shaped curve.[211] *In vivo* treatment has demonstrated a transient reduction of methylation, which is being restored within 4 weeks, which is the time for the start of the next cycle.[212] The demethylating effect of Azacitidine seems to be non-random and a similar demethylation pattern appears after repeated experiments.[216] The demethylation effect is most prominent in regions with higher methylation levels and the patterns of which genes are targeted by Azacitidine are similar over several cell types.[215, 217]

2.9.4 Effects on chromatin structure

There is a complex cross-talk between DNA methylation and mechanisms regulating chromatin structure such as histone methylating and acetylating enzymes.[218] The effect of Azacitidine on chromatin structure e.g. histone modifications, histone variants and nucleosome positioning is sparsely investigated. A few studies have demonstrated gene-specific change in chromatin structure for cells treated *in vitro* with Azacitidine.[219-221] In a genome-wide study, chromatin accessibility was increased but only in a small minority of genes with reduced DNA methylation.[217] Another genome-wide study *in vitro* showed that the repressive marks H3K27me3 and H3K9me3 were reduced by Azacitidine.[222] Insertion of the histone variant H2A.Z has been shown to be an essential step in for Azacitidine-induced demethylation to result in increased gene expression.[223] Conversely, treatment with HDACi also results in DNA hypomethylation, another example of the cross-talk between the different epigenetic mechanisms.[224]

2.9.5 Effect on RNA and proteins

A small fraction (10%-20%) of Azacitidine is converted by ribonucleotide reductase to 5-aza-deoxy-cytidine which can be incorporated into DNA and block DNA methylation.[194] The reminding 80-90% is instead incorporated into RNA which has a destabilizing effect on the RNA resulting in reduced RNA levels and reduced total protein synthesis.[194, 211, 225-228] The effect of Azacitidine on RNA has mostly studied tRNA and rRNA although reduced stability of mRNA also has been reported. Azacitidine has also been shown to be a potent inhibitor of ribonucleotide reductase resulting in a reduced conversion of ribonucleotides to deoxyribonucleotides necessary for DNA synthesis[225]. This results probably in a destabilizing effect on DNA and anti-proliferative effect. Since ribonucleotide reductase is necessary for the conversion of Azacitidine to 5-aza-deoxy-cytidine, the blockage of ribonucleotide reductase by Azacitidine limits its own conversion resulting in a rapid decrease in the percentage Azacitidine being converted to DAC and thus a reduced inhibition of DNMTs over time. DNMT2 is methylating tRNA, however, it is not known if Azacitidine blocks DNMT2. Potentially, treatment with Azacitidine would result in reduced methylation and increased instability of tRNA.[229, 230]

2.9.6 Effects on gene expression

The ruling paradigm, explaining the mechanism of action of Azacitidine, has been as follows: demethylation of previously silenced tumor suppressor genes due to hypermethylated promotors result in re-expression of these genes. The evidence, at least *in vivo* is however sparse. There are studies supporting this hypothesis, which showed that genes with reduced methylation after treatment were accompanied by increased gene expression.[198, 201, 213, 222, 231] However, in the genome-wide studies only a small minority of the demethylated genes also show increased expression. First, in a study where cells from the celline HEK 293 were incubated with Azacitidine, only a small minority of the up-regulated genes could be explained by changes in methylation. [222] A second study, using a hypermethylated colon cancer cell line, showed that only 1.6% of the genes with Azacitidine-induced reduction of methylation also had gained chromatin accessibility. [217] However, of these genes, >90% were hypermethylated before start of treatment indicating that demethylation is a plausible mechanism for gained chromatin accessibility. Interestingly, treatment of the same cells with a HDACi, identified a non-overlapping set of genes, providing a rationale for the

combined treatment. In a third study where cells from the cell line HL60 were incubated with Decitabine, 160 genes were transcriptionally upregulated, corresponding to 3% of the total 4876 genes that were demethylated.[198] Interestingly, these 160 genes were enriched for polycomb target genes involved in differentiation and stem cell function. A fourth study on an AML-cell line show minor effect on gene expression despite clear demethylation.[215] A few additional studies confirm a decrease in methylation but modest effects on gene expression and lack of association between demethylation and gene expression.[201, 215, 232, 233] None of these studies have used primary MDS cells and none have used modern RNA sequencing for gene expression analysis.

Another study demonstrated that gene expression only occurs when demethylation is accompanied by an open chromatin structure.[234] Yet another possible mechanisms by which Azacitidine exerts its effect on gene expression could be the conformation change of *DNMT*1 induced by the binding of Azacitidine which possibly alters the interactions with transcription factors and chromatin regulators.[207]

2.9.7 Immunological effects

Several studies have demonstrated immunomodulatory properties of Azacitidine. Results from *in vitro* studies on regulatory T-cells (Tregs) are conflicting with reports of both increasing and decreasing number of Tregs as a result of Azacitidine treatment.[235-237] Several reports of *in vivo* effects show, however, a reduction of Tregs even though an initial increase was observed[235, 237-239] Interestingly, patients having a higher number of Tregs before start of treatment are less likely to respond. [235] Furthermore, Azacitidine can induce an expansion of cytototoxic T-cells partly as a result of immune sensitization due to activation of cancer testis antigens.[235, 238, 240, 241]

Studies on dendritic cells have shown that the cytokine production profile from these cells change during treatment although the functional consequences are unknown.[241] *In vitro* treatment of NK cells with Azacitidine results in an increase in inhibitory ligands and a reduced cytolytic activity.[242-244] The effects on NK cells *in vivo* remain to be explained.

2.10 RESPONSE FACTORS

2.10.1 Introduction

Response to treatment is evaluated according to the International working group criteria which scores patients into complete remission (CR), marrow CR (mCR), partial

remission (PR), hematological improvement (HI), stable disease (SD) and progressive disease (PD) , see Table 4.

Category	Response criteria (responses must last at least 4 weeks)
Complete remission	Bone marrow: ≤ 5% myeloblasts with normal maturation of all cell lines Persistent dysplasia will be noted Peripheral blood: Hgb ≥ 11 g/dL Platelets ≥ 100 × 109/L Neutrophils ≥ 1.0 × 109/L Blasts 0%
Partial remission	All CR criteria if abnormal before treatment except: Bone marrow blasts decreased by ≥ 50% over pretreatment but still > 5% Cellularity and morphology not relevant
Marrow CR†	Bone marrow: ≤ 5% myeloblasts and decrease by ≥ 50% over pretreatment Peripheral blood: if HI responses, they will be noted in addition to marrow CR
Stable disease	Failure to achieve at least PR, but no evidence of progression for > 8 weeks
Failure	Death during treatment or disease progression characterized by worsening of cytopenias, increase in percentage of bone marrow blasts, or progression to a more advanced MDS FAB subtype than pretreatment
Relapse after CR or PR	At least 1 of the following: Return to pretreatment bone marrow blast percentage Decrement of ≥ 50% from maximum remission/response levels in granulocytes or platelets Reduction in Hb concentration by ≥ 1.5 g/dL or transfusion dependence
Cytogenetic response	Complete: Disappearance of the chromosomal abnormality without appearance of new ones Partial: At least 50% reduction of the chromosomal abnormality
Disease progression	For patients with: Less than 5% blasts: ≥ 50% increase in blasts to > 5% blasts 5%-10% blasts: ≥ 50% increase to > 10% blasts 10%-20% blasts: ≥ 50% increase to > 20% blasts 20%-30% blasts: ≥ 50% increase to > 30% blasts Any of the following: At least 50% decrement from maximum remission/response in granulocytes or platelets Reduction in Hb by ≥ 2 g/dL Transfusion dependence

Table 4. International working group criteria for response. by Cheson et al, 2006

2.10.2 Clinical parameters

Basic clinical data such as morphology and cytogenetics give sparse predictive information although blast count > 15%, extensive transfusion need, abnormal karyotype and previous cytarabine treatment were reported as negative predictors by the French MDS group.[245]

2.10.3 Mutations and gene expression

Presence of *TET2*-mutations and/or *DNMT3A* has been reported as positively associated with response to treatment in several studies, although not statistically significant in more than 2 of the studies.[59, 246-248] High expression of *BCL2L10*, an anti-apoptotic member of the *Bcl-2* family, showed association with Azacitidine resistance in one study.[249]

2.10.4 DNA Methylation profiles

An initial reduction of methylation levels after the first treatment cycle in specific genes or on a global level was shown to predict a later clinical response.[58, 167, 213, 214, 231] A couple of studies reported correlation between methylation level of specific genes and responses. First, methylation levels of p15 were lower in responders compared to non-responders although not statistically significant.[250] Secondly, hypermethylaton of *BCL2L10* was linked to lower response rate which is intriguing since high expression of *BCL2L10* also has been linked to Azacitidine-resistance.[59, 249] In contrast, other studies report no correlation between baseline methylation levels and response.[58, 251] In a study on global levels of DNA methylation analyzed in peripheral blood of mononuclear cells, no association with response was observed.[209]

2.10.5 Other factors

Higher number of Tregs resulted in lower response rates possibly as a result of increased immune escape.[235] Furthermore, presence of aberrant clones in the FACS analysis of the MDS bone marrow was reported to be negatively associated with response.[252] Moreover, lower gene expression of UCK1, one of the enzymes involved in the intracellular metabolism was reported to be associated with Azacitidine resistance and shorter overall survival.[253]

3 AIMS OF THE THESIS

The purpose of this thesis was to study the clinical and molecular effects of Azacitidine in the myelodysplastic syndromes.

Specific aims were:

I. To evaluate the clinical effect of Azacitidine treatment in transfusion-dependent, Epo-refractory patients with lower-risk MDS

II. To investigate the cellular and epigenetic effects of Azacitidine in normal and higher-risk MDS progenitors *in vitro*

III. To identify clinical and molecular predictors for response in patients with MDS treated with Azacitidine

IV. To evaluate the differential effects on DNA methylation, gene expression and histone modifications in higher-risk MDS progenitors exposed to Azacitidine *in vitro*

4 MATERIAL AND METHODS

4.1 PATIENTS

4.1.1 Paper I

Thirty consecutive transfusion-dependent, Epo-refractory patients with MDS and IPSS low or intermediate-1 risk MDS were eligible for inclusion and enrolled from 11 centers across the Nordic region.

4.1.2 Paper II and IV

For these *in vitro* studies, we used bone marrow from patients with higher risk MDS (IPSS intermediate 2 or high), CMML-II or AML with multilinear dysplasia and 20-29% marrow blasts and bone marrow from healthy controls. Patients were previously untreated with regard to chemotherapy, Azacitidine or transplantation. All patients and donors gave their informed consent before sampling.

4.1.3 Paper III

Clinical information was collected for all patients with MDS or AML with multilinear dysplasia and 20-29% marrow blasts treated with Azacitidine at the Karolinska, University Hospital. Biobank material based on previous bone marrow samplings for which the patients had given informed consent was used for targeted sequencing and methylation analysis.

4.2 STUDY DESIGN

4.2.1 Paper I

This was a prospective, open-label, multicenter interventional study conducted within the Nordic MDS group. The study was registered at clinicaltrial.gov as NCT01048034. Enrolled patients received six cycles of Azacitidine, 75 mg / m^2 for 5 consecutive days every 28 days, see Figure 7. Patients achieving transfusion independency terminated the study after six cycles. Patients who were still transfusion dependent after 6 cycles continued with another three cycles of Azacitidine, with the addition of Erythropoietin β 60 000 units / week, s.c. Efficacy assessment was performed after six cycles for all patients and after nine cycles for patients continuing with the combined treatment.

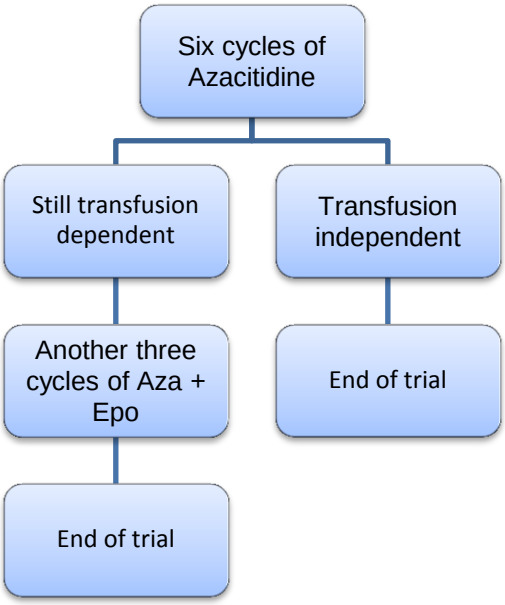

Figure 7: Study design in paper I

4.3 LABORATORY EXPERIMENTS

4.3.1 Cell sampling and sorting, paper I-IV

Bone marrow was obtained from patients and healthy donors. Mononuclear cells (MNCs) were separated by density gradient technique through Lymphoprep®. CD34+ cells were separated twice by using a MACS® magnetic labeling system, according to the manufacturers' protocols. Samples were either used directly for cell culture experiments or frozen as pellet in -80° C for later analysis of the DNA/RNA/Proteins.

4.3.2 Suspension cultures, paper II and IV

Cells were plated at a concentration of $1x10^6$ cells / ml in RPMI 1640-Glutamax with the addition of 10% fetal bovine serum. G-CSF (10 ng/ml), IL-3 (10 ng/ml) and stem cell factor (25 ng/ml) was added. Cells were cultured for 24h or 48h at 37°C in 5% CO_2. Azacitidine-treated cultures used Azacitidine, diluted in sterile H_2O filtered through 0.22 µm plastic syringe filter and added to the culture to final concentration of 1µM. Cells were harvested and analyzed for viability, and cell count.

4.3.3 Colony assays

To explore the effect of Azacitidine on colony growth, we exposed CD34+ normal and MDS progenitor cells to Azacitidine (0.05-10µM) for 24 hours. Cells were then washed once in PBS, re-suspended in fresh medium, mixed with 4 ml of MethoCult medium

GFH4434 and plated in triplicates on small Petri dishes. Dishes were incubated at 37°C and 5% CO2 for 14 days. Erythroid colonies (CFU-E and BFU-E) and myeloid colonies (granulocytic colonies, CFU-G, monocytoid colonies, CFU-M and mixed granulocytoid / monocytoid colonies, CFU-GM) were counted and a mean value was calculated for each position.

4.3.4 DNA extraction, paper I-IV

Genomic DNA was extracted using Gene Elute® genomic DNA extraction kit or by the AllPrep® extraction kit.

4.3.5 RNA extraction, paper IV

RNA was extracted using the AllPrep® extraction kit.

4.3.6 Protein extraction, paper II

Protein was extracted using the AllPrep® extraction kit.

4.3.7 Bisulphite modification, global methylation by LINE-1 and PCR, paper III

Genomic DNA samples were bisulfite treated using *EZ DNA Methylation Gold Kit*® according to standard protocol. Methylation specific assays for specific genes /LINE-1 regions, were designed for specific genomic regions using PyroMarQ® assay design software. Twenty ng of bisulfite treated DNA was amplified in 25µl PCR reactions using TaqStar® polymerase) through thermal cycling of 95°C 10 Min; 40 Cycle of 95°C; 20 sec 55°C and 30 Sec 72°C. PCR products were analyzed on *a PyroMark Q24 Pyrosequencer*® according to manufacturer's instructions. [254]

4.3.8 Assessment of mutations, paper I, III and IV

Patients were analyzed for 42 genes recurrently mutated in myeloid disorders using Haloplex™ target enrichment technology, followed by high throughput sequencing. The Haloplex™ target enrichment kit G9901A/B was designed using SureDesign™ wizard and we achieved 99.2% coverage of the 42 selected genes. All samples were individually barcoded during enrichment and sequenced using Illumina HiSeQ 2000 system at the Sci-Life lab, Stockholm, Sweden. Sequencing reads were mapped over Human genome 19 by Bowtie and the variants were called using SAMTOOLS. [255, 256] The minimum coverage to consider in the analysis was 300 reads and the allele should

have a minimum of 5% of reads. Sequence variations were annotated and functionally classified using ANNOVAR[257]. Variants previously reported as germline polymorphisms in the 1000 genome and the ESP5400 databases were excluded. [258, 259] Variants located in non-coding regions as well as synonymous variants were filtered out.

4.3.9 Illumina methylation array, paper II-IV

Five hundred ng of DNA from bone marrow MNC and CD34+ cells was processed using Illumina-supplied reagents and conditions at a core facility of the Karolinska Institute. After hybridization and scanning, the raw data files were quality checked in GenomeStudio. Data was imported into R v. 3.1 and pre-processed using the R BioConductor packages lumi, methylumi and minfi packages.[260-263] Identification of differentially methylated sites and regions were analyzed using the BioConductor packages limma, DMRCate and minifi.[264 261, 265] Pathway analysis was performed using GO-miner.[266]

4.3.10 Western blot, paper II

Protein concentration was determined by Bradford. Samples were boiled, and equal amounts of protein loaded on a 4-20% TrisHCK gel, separated by SDS-PAGE and transferred to nitrocellulose membrane, incubated in blocking buffer for 1 hour, probed with respective primary antibody overnight (H3K9ac rabbit polyclonal, ab1191; H3K27ac rabbit polyclonal, ab4729; H3 rabbit polyclonal, ab1791; and H4 mouse monoclonal loading control, ab31830; all from Abcam, diluted in 1:1000). After washing with PBST, secondary fluorescent signal was detected using LiCor® Odyssey infrared scanner. The Licor® Odyssey software version 3.0 was used for signal intensity calculations.

4.3.11 RNA sequencing, paper IV

The previously published single-cell tagged reverse transcription (STRT) protocol was applied using 10 ng total RNA as template and following minor modifications.[267] Forty-eight barcodes were used, the cell capture buffer contained 0.1% Triton X-100, 400 nM T30-VN-oligo and 2 µM TSO without magnesium chloride. All 48 cDNAs were pooled into one tube using 10% PEG-6000 and a final concentration of 0.9 M NaCl. Purified cDNA was first amplified using 14 cycles of PCR and later an additional 10 cycles to introduce a complete set of adapters for Illumina sequencing.

The ready library was size-selected using the sequential AMPure XP bead selection protocol (https://www.neb.com/protocols/ 1/01/01/size-selection-e6270) where 0.7× and 0.22× ratios were used.

Sequencing reads obtained from the STRT library were preprocessed by STRTprep[268] to (i) demultiplex by the sample barcodes, (ii) exclude redundant reads to reduce PCR bias by unique molecular identifier (UMI)[269], (iii) align the reads to human reference genome hg19 and spike-in RNA sequences by TopHat[270], (iv) quantitate the expression levels by 50 base pairs (bp) strand-specific windows sliding in 25 bp step, and (v) perform the basic quality check of the library and the sequencing. Next, we extracted the 50 bp windows with expression levels that showed significantly more fluctuation in the target samples (potentially relevant biological variation) than in the spike-in RNAs (technical variation), and identified and excluded outlier samples in the expression levels of the fluctuated regions by PCA. The fluctuated regions contain many regions regulated by various factors, not only by treatments but also by gender, age, and so on. We therefore also tested differential expression of the fluctuated regions between control and the treatment of the targets by SAMstrt[271]; we specified different permutation blocks for different patients as the paired statistics.

4.3.12 Chromatin immunoprecipitation followed by qPCR (ChIP-qPCR)

ChIP assay was performed using the iDeal® Chip-seq kit from Diagenode, using 250 000 CD34+cells per ChIP. Briefly, after 24h incubation with or without Azacitidine, cells were crosslinked for 8 min by adding formaldehyde (37 %) to the growth media to a final concentration of 1% at room temperature. Crosslinking was quenched by adding 2.5 M glycine to a final concentration of 125 mM, and cells were washed twice with ice cold PBS. Cell nuclei were extracted by resuspending cells in lysis buffer 1 (iDeal® ChiP-seq Diagenode) for 10 min at 4°C and lysis buffer 2 (iDeal® ChiP-seq Diagenode) for 10 min at 4°C. Chromatin was fragmented using the Bioruptor sonicator (Diagenode) for 25 min (30s pulses) to produce fragments 200-500 nt in size. Antibodies used were H3K9Me3 (Abcam, #ab8988), and H3 (Diagenode). Immunoprecipitates were collected using protein A coated magnetic beads from the iDeal® Chip-seq kit (Diagenode). Precipitated DNA was eluted by 30 min incubation on rotating wheel in RT, and crosslinking was reversed by overnight incubation at 65°C. The ChIP DNA was extracted with a PCR purification kit (MiniElute®,Qiagen). qPCR validation of enrichment using the primers specific for the selected genes. Each gene was assessed by ChIP qPCR for repressive mark H3K9me3 in three different

patients, technical triplicates were run 4-5 times per gene. GAPDH was used for positive control and Ct levels of GAPDH were unchanged upon treatment. For qPCR, 200nM primer (TaqCopenhage) and SybGreen® (Bio-Rad) on the CFX96 Touch® Real-Time PCR Detection System (Bio-Rad) was used. Normalized gene expression was calculated using the CFX manager software (Bio-Rad), and expression was normalized to the percentage of input.

5 RESULTS AND DISCUSSION

5.1 PAPER I

LIMITED CLINICAL EFFICACY OF AZACITIDINE IN TRANSFUSION-DEPENDENT, GROWTH FACTOR RESISTANT, LOW- AND INT-1 RISK MDS. RESULTS FROM THE NORDIC NMDSG08A PHASE II STUDY

Azacitidine has been confidently shown to prolong survival in high-risk MDS, while data on lower-risk MDS patients is limited to subgroup analysis and retrospective studies. This study aimed to evaluate the efficacy of Azacitidine treatment in a cohort of transfusion-dependent, epo-refractory patients with lower-risk MDS (IPSS low or int-1).

We enrolled 30 patients from 11 Nordic centers in a prospective study and defined the proportion of patients achieving transfusion independency as primary endpoint. All patients were refractory to EPO/G-CSF treatment or scored "low" probability for response according to a prognostic score model and had a transfusion need ≥4 units over 8 weeks. [99] Included patients had a median number of transfused units of 7 (4-14) during the 8 weeks preceding inclusion. Median platelet and ANC count pre-treatment was $220x10^9$/L (22-1468) and $2.1x10^9$/L (0.3-15.1), respectively. Severe thrombocytopenia (<30) and neutropenia (<0.5) was observed in 3 and 3 patients, respectively. Ten patients pre-terminated the study; five due to sustained cytopenia after start of treatment; two due to death (one sudden death and one neutropenic septicemia); two due to patient's wish and one due to investigators choice. Thirty-eight serious adverse events were reported in 18 patients with infection (n=28) being the most common. Nadir values after each cycle of Azacitidine were seen at week 3 for platelet count (median $130x10^9$/L) and at week 4 for neutrophil count (median $1.2x10^9$/L), respectively.

Twenty-four patients were evaluable for treatment with Azacitidine alone and 15 patients for Azacitidine+Epo. Transfusion independency was achieved in 5 patients (21%) after Azacitidine treatment alone, and in one additional patient after Azacitidine+Epo, see Figure 8. The response duration was relatively short with only 2 patients being transfusion independent for more than 6 months.

Recurrent mutations were found in all but three sequenced patients, rendering this lower-risk cohort a mutational frequency at least as high as in previous reports on MDS of all risk groups, see Figure 9.[35, 36] A majority of the patients carried a mutation in a splice factor gene and / or in one or several of the genes involved in epigenetic

regulation. None of the mutations predicted for a response to Azacitidine. Notably, mutations in two genes; *DNMT3A* (n=4), and *SF3A1* (n=3) were only observed in non-responders, which deserve further investigation.

In summary, Azacitidine can induce transfusion independency in patients with transfusion dependent, lower-risk MDS, but the response rate is lower in this cohort of documented EPO-G-CSF-refractory patients compared to previous reports of less well-controlled cohorts. Since toxicity is substantial, candidate patients for this treatment must be selected carefully. The combination of Azacitidine and Epo can be effective in rare cases.

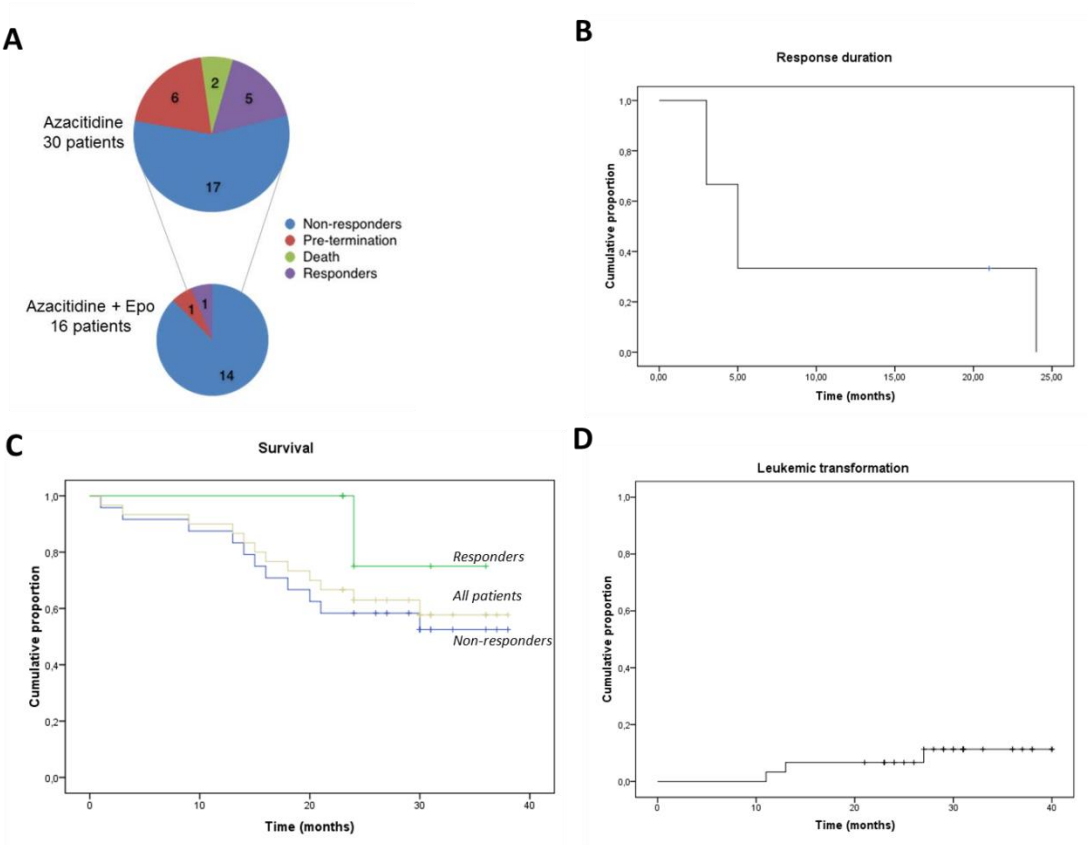

Figure 8 A - Outcome after Azacitidine as monotherapy and the combined treatment of Azacitidine and Erythropoietin (Epo). Response defined as transfusion independence. B – Duration of response. C – Overall survival. D – Leukemic transformation

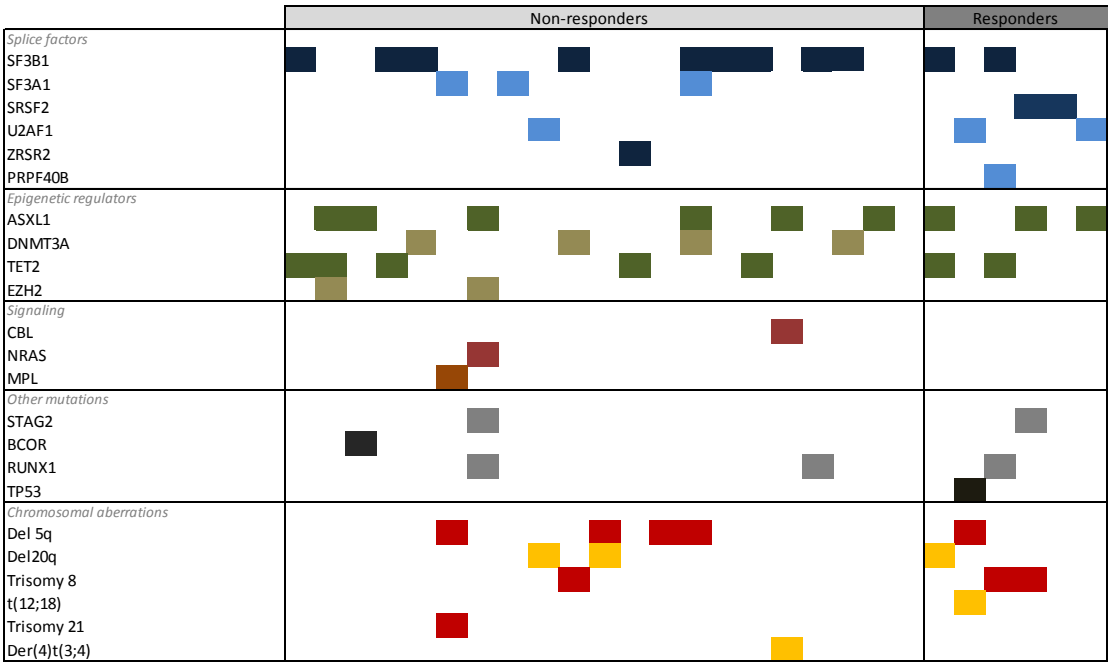

Figure 9: Spectrum of mutations and cytogenetic aberrations in responders and non-responders

5.2 PAPER II

AZACITIDINE INDUCES PROFOUND GENOME-WIDE HYPOMETHYLATION IN PRIMARY MYELODYSPLASTIC BONE MARROW CULTURES BUT MAY ALSO REDUCE HISTONE ACETYLATION.

This study evaluated the effect of Azacitidine in primary bone marrow progenitors from MDS patients. We studied the effect on growth, colony formation, DNA methylation and histone acetylation.

In the cell suspension assays, we observed that doses up to 5 µM did not affect cell growth or viability. Actually, the absolute number of cells per ml was higher in Azacitidine treated positions than in controls in 1/10 NBM samples and 4/10 MDS samples. Neither was there any inhibitory effect on colony formation after exposure to Azacitidine in doses up to 0.5µM for 24 hours. In fact, the number of colonies (especially erythroid colonies) increased by > 70% with lower doses of Azacitidine in 5/9 MDS samples and 5/13 NBM samples. This may suggest a direct stimulatory effect of Azacitidine on erythropoiesis. The seemingly non-toxic effect of the drug on normal bone marrow progenitors is a useful finding since it may support the use of the drug as maintenance after allogeneic stem cell transplantation without toxicity on donor hematopoiesis.

Assessment of global methylation by pyrosequencing of LINE-1 repetitive elements showed a significant decrease in methylation after incubation with 1µM of Azacitidine

for 24h in samples from NBM as well as for MDS (p=0.001 and 0.02, respectively). No further hypomethylating effect was observed with higher doses. There were 32 155 significantly differentially methylated sites in untreated MDS vs. untreated NBM, see Figure 10. The majority (89%) of the sites were more methylated in MDS than in NBM. Azacitidine at 1 μM for 24h induced a marked genome-wide reduction of methylation levels in CD34+ MDS progenitors and induced significant alterations at 15 531 examined sites, whereof 96% were less methylated after Azacitidine treatment. Importantly, the demethylating effect was not promoter specific but included to an equal degree non-promoter CpG-sites. Interestingly, only 1% of the sites that were aberrantly methylated in untreated MDS vs NBM, changed by Azacitidine treatment. In these 239 sites, Azacitidine restored the methylation level towards that of NBM. Unexpectedly, only 3% (8/239) corresponded to gene promotors which suggests that other effects than re-expression of genes silenced by aberrant DNA hypermethylation are activated by Azacitidine. NBM progenitors also became demethylated but the difference did not reach statistical significance which indicates differential effects on normal and MDS progenitors.

The effect of Azacitidine on histone modifications has been sparsely investigated. Our initial hypothesis was that H3K9ac and H3K27ac (markers of active chromatin) would increase after Azacitidine treatment due to chromatin activation within previously silenced promoter regions. By contrast, we showed a decrease of histone acetylation after Azacitidine treatment. H3K9ac decreased in eight of eight examined patients from which we had enough sample material (average decrease 48%, $P<0.05$) and H3K27ac decreased in five of six examined patients (average 35%, $P=0.15$) as assessed by western blot analysis. This pattern was not observed in normal CD34+ cells, which remained largely unaffected or showed slightly increased acetylation. H3 and H4 were run as loading controls and showed stable and similar loading, which ensures that the effects did not reflect alterations in global H3/H4 ratio. We hypothesize that the observed pattern may be due to interplay between DNA methylation and chromatin structure; if gene silencing by DNA methylation is lost, histone modifications will keep the chromatin repressed. Upregulation of histone deacetylase by demethylation after Azacitidine treatment, and hence, a global reduction of histone acetylation could be one reason for resistance or incomplete clinical response to Azacitidine. Interestingly, we found that the gene encoding for the histone deacetylase enzyme *HDAC4* became hypomethylated in MDS progenitor cells upon Azacitidine treatment. Thus, it is

possible that increased *HDAC4* levels are contributing to the observed reduction of H3K27ac and H3K9ac.

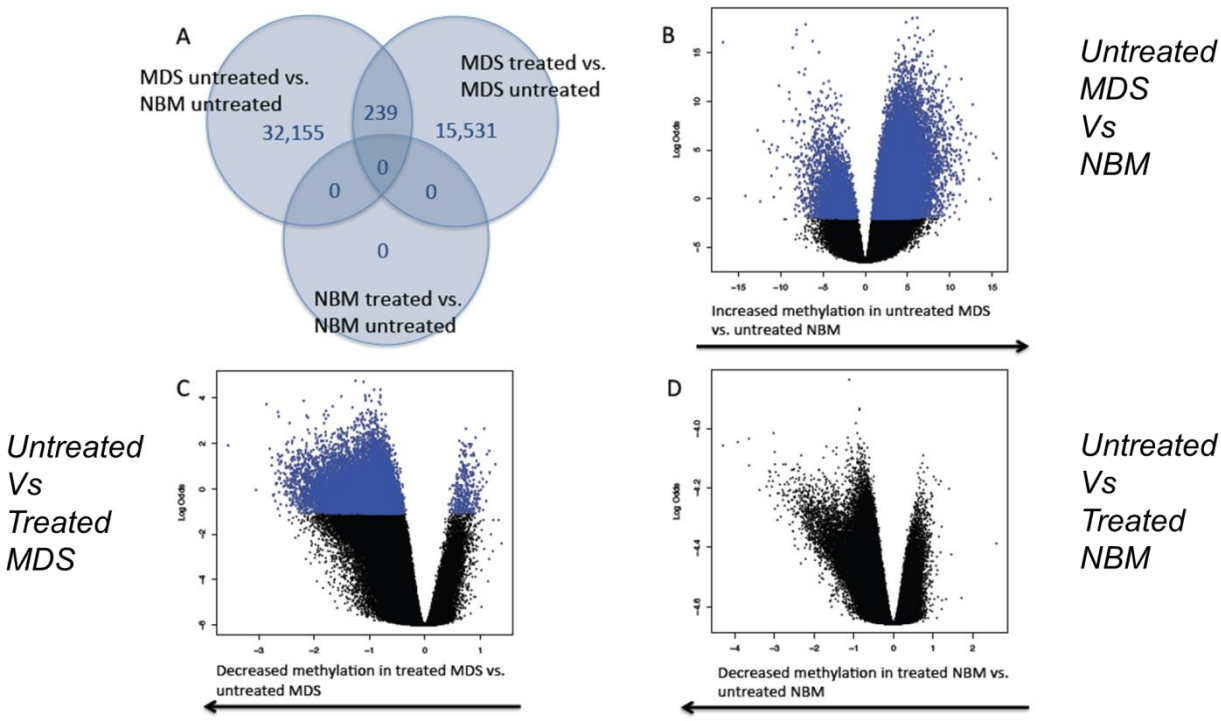

Figure 10: Genome-wide DNA-methylation (a–d) (a) Venn diagram showing number of differentially methylated genes between MDS vs. NBM and treated vs. untreated cells (b) Volcano plot showing difference in methylation untreated MDS vs untreated NBM. Blue dots represent data above the significance level. (c) Volcano plot showing difference in methylation between treated MDS vs untreated MDS. Blue dots represent data above the significance level. (d) Volcano plot showing difference in methylation treated NBM vs untreated NBM. No blue dots, that is, no data reached the significance level.

5.3 PAPER III

MUTATIONS IN HISTONE MODULATORS AND HOXA5 METHYLATION LEVELS PREDICT SURVIVAL IN AZACITIDINE TREATED MDS PATIENTS

Only around 50% of patients respond to treatment with Azacitidine, and at least one third of patients show no response at all or even disease progression. Since it usually takes 6 months to define a non-responder, it is clinically highly relevant to identify predictive factors for response. This study aimed to identify clinically relevant predictors for the efficacy of Azacitidine treatment at three different levels: basic clinical parameters, mutational status, and methylation profiles.

We first evaluated standard clinical parameters and identified disease duration as a factor associated with poor response to treatment and shorter survival estimated from

start of Azacitidine therapy. Cytogenetic risk profile was associated with survival but not with response, see Figure 11. High ANC counts did not affect response rates but were associated with shorter survival. There was a trend towards lower response rates and shorter survival for transfusion-dependent patients, in analogy to our findings in paper I.

The second part of the study evaluated the impact of mutational status on response and survival. Recently, *Traina et al* reported a study of around 40 higher-risk patients and showed better response rates for patients carrying a *TET2* and/or *DNMT3A* mutation in a cohort of MDS-patients.[246] In our material of 90 patients, there was only a trend towards higher response rates in patients with epigenetic mutations including histone modulator mutations and DNA methylation mutations. Unexpectedly, while mutations in genes encoding DNA methylation enzymes had no impact on survival, patients presenting with mutations in the group of genes encoding histone modulators (ASXL1, EZH2, MLL) showed significantly better survival (p=0.01), a finding that retained significance in the multivariate analysis. *ASXL1*-mutation alone showed a trend towards prolonged survival in our material, contrasting the results from the study by *Traina* where *ASXL1* was reported as a negative marker, possibly due to the lower proportion of higher-risk MDS patients in the *Traina* study. In addition to *ASXL1* and *EZH2*, other well-known adverse genetic events, such as *RUNX1* and *TP53* mutations, and the number of mutations were neutralized as adverse prognostic factors by Azacitidine treatment.

Thirdly, we examined the role of global DNA methylation by analyzing genome-wide DNA methylation in MNCs using Illumina methylation array 450k. By studying differentially methylated regions (DMRs) we could identify specific differences in the pre-treatment profiles between responding and non-responding patients. The DMRs were strongly enriched for genes involved in development and differentiation pathways. Six *HOX*-genes were differentially methylated of which *HOXA5* was the most significant. By comparing our data with methylation data from different maturation stages in normal hematopoiesis, we showed that non-responders had a *HOXA5* methylation pattern closer to that of progenitor cells while responders resembled more differentiated cells, see Figure 12. Furthermore we could demonstrate that patients with higher methylation level of *HOXA5* had a longer survival compared to lower-methylation (p=0.03).

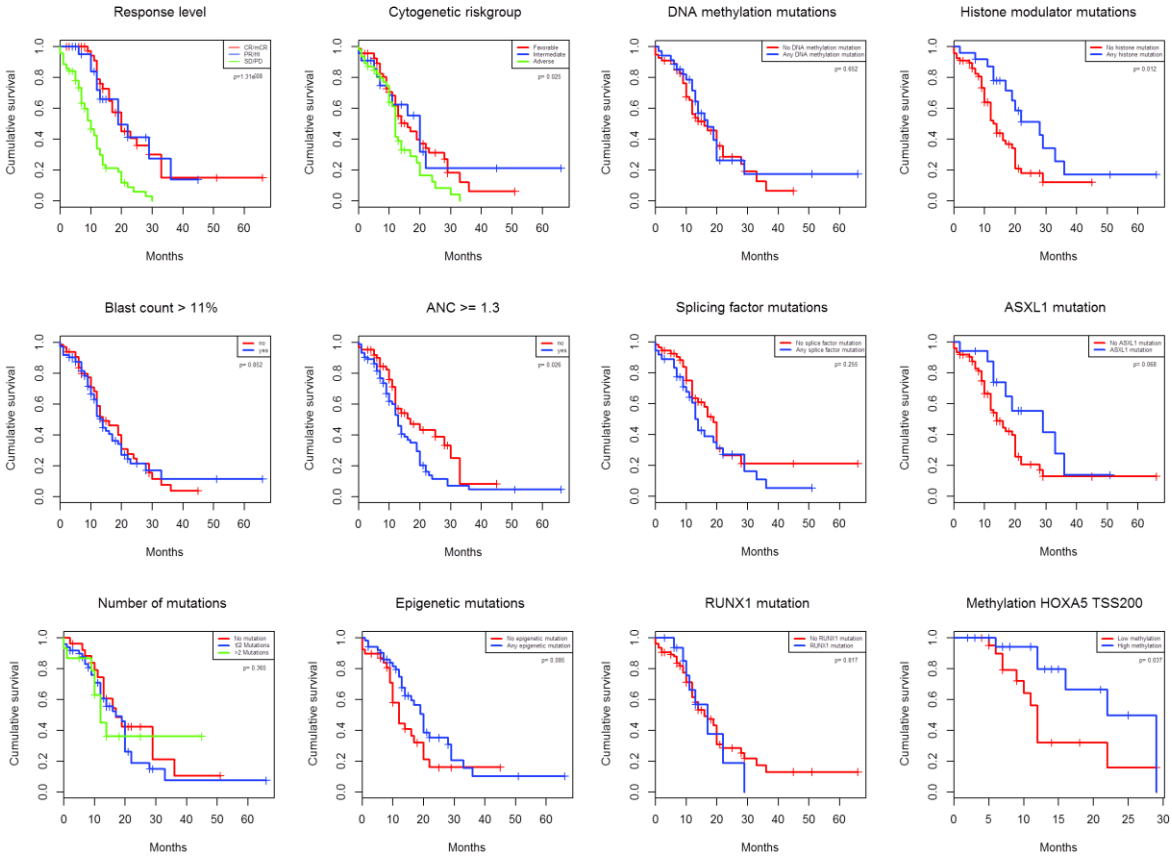

Figure 11: Survival after start of Azacitidine stratified for pre-treatment parameters and response

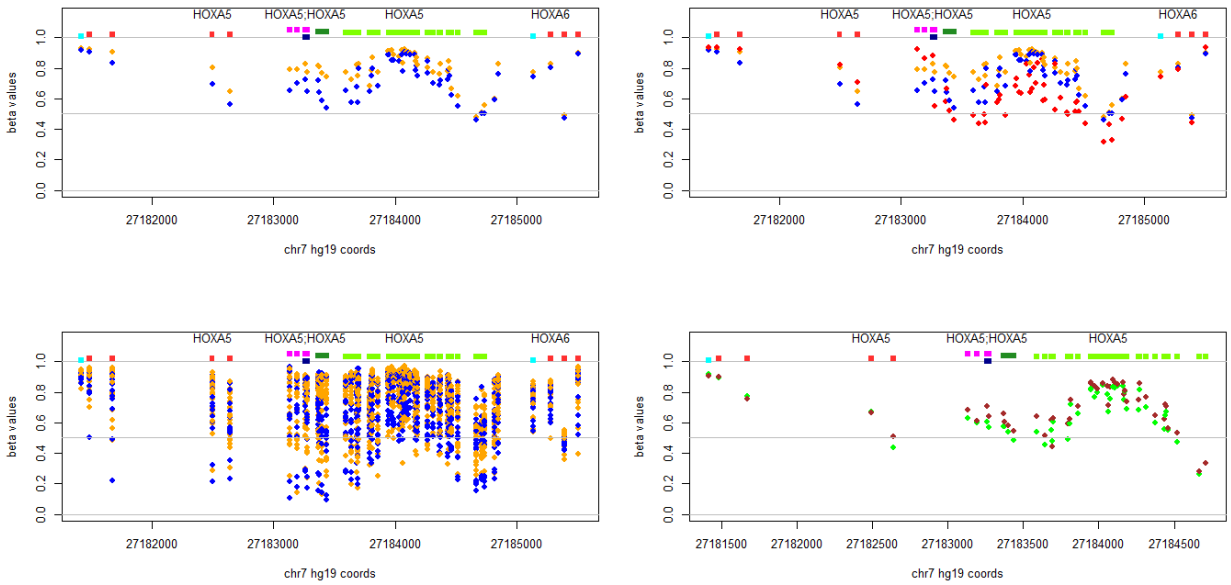

Figure 12: DNA methylation levels at the *HOXA5* locus. Squares represent gene location with light green=TSS-1500; Dark green=TSS-200; Red=Gene body; Magenta=1st Exon; Dark blue=5'UTR; Cyan=3'UTR and diamonds represent sample values. A=Median methylation level of responders illustrated with orange diamonds (MNCs) and non-responders with blue diamonds (MNCs). B=Added CD34+ cells with red diamonds. C=All patients. D=Normal bone marrow with PMN illustrated with brown diamonds and CMP with green diamonds.

5.4 PAPER IV

IN VITRO EXPOSURE TO AZACITIDINE INDUCES DEMETHYLATION AND INCREASED GENE EXPRESSION IN PRIMARY MDS PROGENITOR CELLS.

As demonstrated in paper II, Azacitidine has a clear demethylating effect on primary MDS cells during cell culture. The effect on gene expression is however still unclear. In this paper we investigated the impact of *in vitro* exposure to Azacitidine on both DNA methylation and gene expression in primary MDS cells.

Bone marrow samples from 11 consecutive patients with a clinical indication for Azacitidine treatment, i.e. MDS with IPSS int-2 or high, CMML with >10% blasts or AML with multilinear dysplasia and <30% blasts, were collected. After separation of CD34+ cells, samples proceeded to cell culture freshly without any freezing / thawing step in between. Since there is no consensus regarding culture conditions for studying the Azacitidine-effects, we cultured cells with or without Azacitidine, and harvested cells after 0, 24 and 48 hours of culture in order to study the impact of different exposure time and to study if the culture procedure itself alters the epigenome and transcriptome. The culture conditions were identical to those described in paper II including the same dose, 1µM of Azacitidine.

Sample material allowed assessment of differences in methylation status between samples cultured with or without Azacitidine for 24h (n=9), samples cultured without treatment for 0h or 24h (n=4), samples cultured without treatment for 24h or 48h (n=2) and samples cultured with Azacitidine for 24h or 48h (n=4). DNA methylation was studied using the Illumina 450k platform. We observed no differences in global methylation pattern when comparing samples cultured without Azacitidine for 0h and 24h or for samples cultured for 24h and 48h without Azacitidine, see Figure 13. When comparing samples cultured with or without Azacitidine for 24h, we could, as expected, reproduce the same methylation pattern as was seen in the cohort used for paper II.

By comparing sampled cultured for 24h with or without Azacitidine, we identified a large number (n=65 769) of differentially methylated probes (DMPs). The vast majority of these were less methylated in the Azacitidine samples. Furthermore, we identified between 94 and 1822 DMRs, depending on DMR calling algorithm used. Pathway analysis of the genes associated to the DMRs showed a strong enrichment for biological processes involved in cellular differentiation and development.

Due to low RNA concentration, samples from 6 of the patients underwent a concentrating procedure by SpeedVac ™. The concentrated samples clustered together in a principal component analysis and were separated from the non-concentrated samples, probably reflecting RNA degradation during the concentration procedure, and these samples were excluded from further analyses. Hence, gene expression could be evaluated in paired samples from 4 patients, comparing 24h Azacitidine vs. 24h control, 2 patients comparing 24 h vs. 48 control and 2 patients comparing 24 h vs. 48 h Azacitidine.

Comparing treated and untreated cells cultured for 24 h, we observed an increase in total polyadenylated RNA in the Azacitidine samples in all four patients. A total number of 3855 transcripts, corresponding to 2136 genes, were differentially expressed between treated and non-treated cells (Figure 14A). The vast majority of these (n=3850) were upregulated. Pathway analysis of the upregulated genes showed enrichment of a variety of pathways including translation, cellular component disassembly and RNA processing. Of the upregulated genes, 104 were transcription factors and 24 were epigenetic regulators. Comparing samples with and without Azacitidine for 24 and 48 hours did not show any significant skewedness in gene expression, see Figure 14B and 14C.

Interestingly, DMRs and genes with increased expression showed very limited overlap. Moreover, the percentage of the total number of transcripts upregulated in the selected DMR-associated genes was 73%, as compared to 77% for the whole transcriptome. Hence, no direct relation between genes associated with DMRs and increased gene expression was observed. By selecting the promotor-related probes we could see a general slight decrease (9.6%) in methylation level (using beta level as output from the Illumina array) but there was no correlation between mean methylation level and relative change in gene expression. It is unclear if the promotor demethylation is causing increased gene expression.

To further explore the reasons for the observed discrepancy, we next designed primers for the promoter regions of three genes (*Glrx3, AKAP12* and *NUP210)* associated with demethylated regions in the Azacitidine samples without increase in gene expression. ChIP was performed on samples cultured for 24 h with or without Azacitidine, from the same individual patients that we obtained RNA seq results from. We used the repressive chromatin mark H3K9me3, whereafter qPCR for the three genes was performed. Two genes were neither transcribed at all in the control samples nor in the treated samples, and one was transcribed in the control sample with preserved

transcription in the treated sample. *Glrx3 i*ncreased repressive chromatin mark H3K9me3 by on average 5 fold, *AKAP12* by 4.2 fold, and *NUP210* by 2.6 fold on average, in the three different patients assessed. Azacitidine is thus affecting the chromatin inducing an increase in repressive mark, providing a potential explanation why these genes do not gain increased expression despite demethylation.

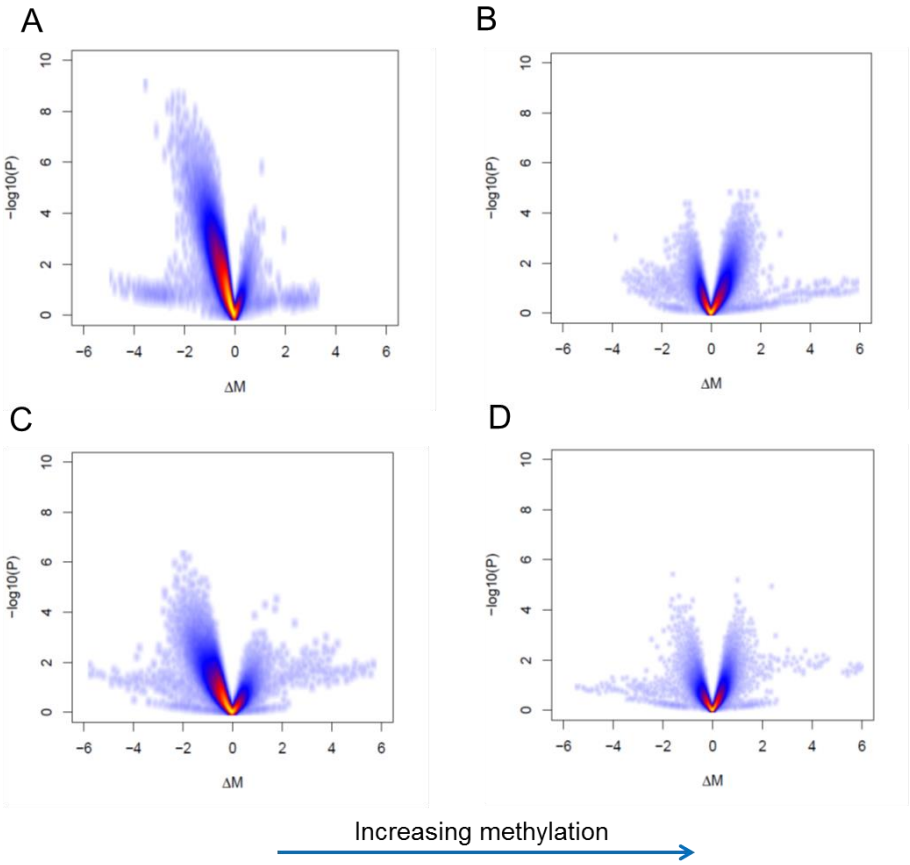

Figure 13: Volcano plot illustrating differences in methylation for the comparisons: A). Azacitidine vs. control after 24 h of culture B) Azacitidine cultured for 48 h vs. 24 h C) Azacitidine cultured for 48 h vs. control cultured for 48 h D) Control cultured for 24 h vs no culture (0h)

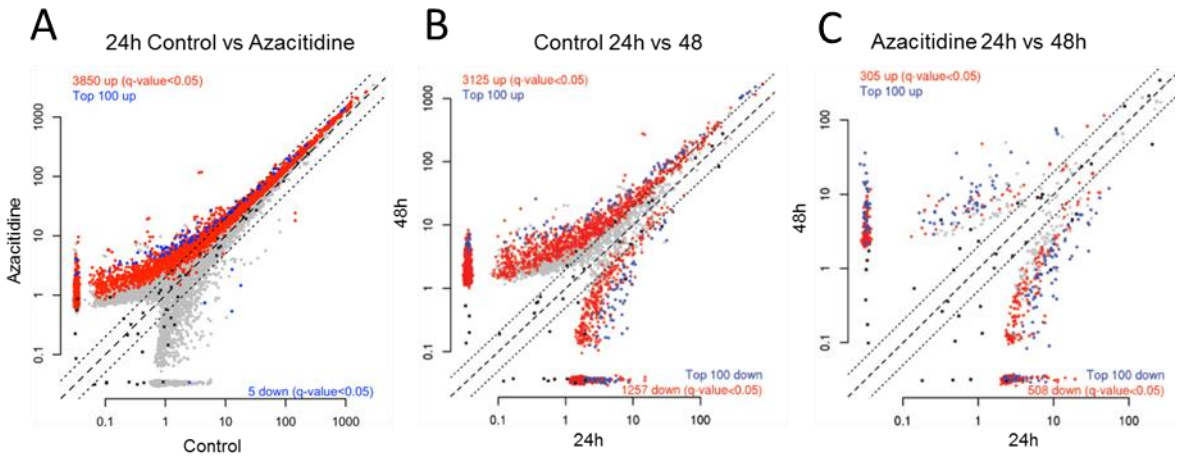

Figure 14: Illustration of differences in gene expression, comparing samples A) cultured for 24 h with or without Azacitidine; B) samples without Azacitidine, cultured for 24 h vs 48 h; and C) samples with Azacitidine, cultured for 24 h vs 48 h . Red dots indicate statistically significant values (FDR<0.05) and blue dots indicate top 100 up / down regulated.

6 FUTURE PERSPECTIVES

Azacitidine has changed the way we treat patients with higher-risk MDS and has resulted in prolonged survival and improved quality of life for a proportion of these patients. However, despite the progress made with the advent of Azacitidine, the prognosis for patients with higher risk MDS is still very poor why improved treatment for this group of patients is highly warranted. The clinical experience of Azacitidine is rapidly increasing but many clinical questions remain to be scrutinized; can predictive markers discriminating responding from non-responding patients be identified allowing physicians to avoid Azacitidine treatment in patients without clinical benefit; what is the optimal dosing regimen and administration route; what is the role of Azaciditine before and after stem cell transplantation and what drug combinations can improve outcome.

Azacitidine has also opened a new field of epigenetic treatment which has just recently started and which will be in focus of cancer research for a long time. This field demands a broader collaboration between basic epigenetic researchers and clinical andtranslational researchers but also calls for knowledge in various other fields like immunology, genetics, and pharmacology.

Still, more than 50 years since Azacitidine was first used in hematological malignancies, the mechanism of it´s action is largely unknown. Although several mechanisms, and most importantly the demethylating effect, have been described, the essential processes involved in the Azacitidine-effect are yet to be explored. A deeper understanding of these mechanisms is crucial in order to develop better drugs and find successful combinations with other drugs. The demethylating effect is maybe still the best clue to understand the pharmacodynamics of Azacitidine and further studies on this field are warranted using methods with higher sensitivity and using improved bioinformatic tools for translating methylation data into biological models. However, several other areas of research should be in focus and most importantly the effects on chromatin structure, direct effects on RNA, effects on the micro-environment and the immune system.

7 SAMMANFATTNING PÅ ENKEL SVENSKA

Myelodysplastiskt syndrom (MDS) är en grupp elakartade blodsjukdomar som har sitt ursprung i de blodbildande stamcellerna i benmärgen. MDS drabbar ca 400 svenskar varje år. Förekomsten ökar med stigande ålder och medianåldern vid diagnos är drygt 70 år. Sjukdomen är ovanlig före 50 års ålder. Symtomen vid MDS är relaterade till brist på en eller flera typer av blodceller och det ger symtom i form av trötthet, infektionsbenägenhet och blödningar. Hos ungefär en tredje del av patienterna går sjukdomen över i en akut leukemi. Sjukdomen kan uppträda med ett mer långsamt förlopp (lågrisk MDS) eller som en mer aggressiv sjukdom som påminner om en leukemi (högrisk MDS).

Mekanismerna som gör att sjukdomen uppträder är ofullständigt kända. Vi vet att skador på arvsmassan (DNA) bidrar till sjukdomens uppkomst. Skadorna kan uppträda dels genom stora strukturella förändringar på kromosomerna t.ex. kan delar av kromosom 5 eller 7 falla bort, eller som mutationer på enskilda gener d.v.s. den genetiska koden ändras på en specifik plats i arvsmassan. Man har idag identifierar ett 40-tal gener där man har sett mutationer som kan kopplas till MDS-sjukdomen. Vilka gener som har skadats vid MDS har betydelse för prognosen och utvecklingen av sjukdomen men detaljerad kunskap om detta och hur mutationerna bidrar till uppkomsten av sjukdomen är fortsatt ofullständig.

Utöver genetiska skador bidrar även epigenetiska skador till uppkomsten av sjukdomen. Epigenetik innebär att cellen har egenskaper som nedärvs till dotterceller utan att själva arvsmassan påverkas. Denna information, som ger cellen en specifik signatur och identitet, regleras genom bl.a. DNA metylering och histonmodifikationer och har stor betydelse vid utmognaden av blodbildande celler från stamceller till färdiga blodceller. Vid MDS har man visat att det föreligger en ökad DNA metylering jämfört med friska celler vilket sannolikt medverkar till att utmognaden vid MDS är störd.

Azacitidine är ett cellgift som har visat sig förlänga överlevnaden vid MDS vilket man kunnat konstatera genom att i stora studier slumpmässgt lotta patienten till att få antingen Azacitidine eller konventionell behandling t.ex. blodtransfusioner eller klassiska cellgifter. Effekten av Azacitdine är i stora delar oklar men man tror att Azacitidine har en epigenetisk effekt då man sett att DNA metyleringen minskar till följd av att Azacitidine hämmar de enzym som leder till DNA metylering.

I vår första studie ville vi undersöka om Azacitidine utöver att ha effekt på högrisk MDS, även kunde ha effekt vid lågrisk MDS. Vi valde ut patienter som hade en uttalad blodbrist till följd av sjukdomen och krävde regelbundna blodtransfusioner. Dessa patienter hade tidigare behandlats med Epo, ett hormon som stimulerar blodbildningen, utan att ha effekt. Dessa patienter behandlades med Azacitidine i 6 månader. De patienter som fortsatt behövde blodtransfusioner fick ytterligare 3 månaders behandling med tillägg av Epo. Vi behandlade 30 patienter varav 6 efter behandling inte längre behövde blodtransfusioner. Det uppstod 38 fall av allvarliga biverkningar, varav den vanligaste orsaken var infektion vilket krävde inläggning på sjukhus. Sammanfattningsvis var studien en besvikelse där endast en mindre andel av patienterna hade nytta av behandlingen medan antalet allvarliga biverkningar var relativt stor och vår konklusion är att behandlingen inte ska ges rutinmässigt till dessa patienter men kan övervägas i särskilda tillfällen.

I vårt andra och fjärde arbete har vi studerat hur celler från patienter med MDS reagerar på att i laboratorium exponeras för Azacitidine och jämförde med celler från friska försökspersoner. Vi kunde till att börja med konstatera att Azacitidine, trots att det är ett cellgift, i låga doser motsvarande de man ger till patienter, inte har någon avdödande effekt på vare sig MDS-celler eller friska celler. Tvärtom kunde man vid s.k. koloniexperiment där celler får växa i ett odlingsmedium under två veckors tid, att låga doser Azacitidine snarar stimulerade tillväxt av MDS celler. Det faktum att Azacitidine inte har någon avdödande effekt på MDS-celler i dessa doser gör att vi inte tror att behandlingseffekten av Azacitidine orsakas av en cellgiftseffekt utan att den har andra orsaker t.ex. epigenetisk påverkan. Vi undersökte därefter effekt på DNA metylering genom en så kallad metyleringsarray som undersöker metyleringen på 480 000 platser i avsmassan. Vi jämförde MDS celler med friska celler och kunde konstatera att MDS cellerna hade en högre metyleringsnivå på många av generna. Vidare kunde vi konstatera att de MDS-celler som odlats tillsammans med Azacitidine hade en lägre metyleringsnivå på en stor del av de undersökta platserna jämfört med de MDS-celler som odlats utan Azacitidine och således minskar Azacitidine metyleringen. Vi undersökte också en annan epigenetisk mekanism: histon acetylering och kunde något förvånande konstatera att histonacetyleringen minskar vilket innebär ett minskat uttryck av gener tvärtemot den förväntade effekten av minskad metylering. Möjligen kan minskad histonacetylering leda till att MDS-celler är motståndskraftiga mot Azacitidine vilket skulle tala för att dessa patienter också behöver läkemedel inriktade mot att öka

histonacetyleringsnivån. I vårt fjärde arbete upprepade vi de odlingsförsök som låg till grund för arbete två. Vi kunde återigen se att Azacitidine minskade metyleringen. Vi kunde också se att uttrycket av gener ökade vilket är förväntat som ett resultat av minskad metylering. Förvånande nog verkar det inte vara specifikt de gener som får minskad metylering som också får ökat genuttryck vilket man skulle förvänta sig. Möjligen föreligger det indirekta effekter t.ex. direkt påverkan på RNA eller påverkan på s.k. transkriptionsfaktorer som i sin tur påverkar genuttrycket. En annan effekt vi såg var att Azacitidine verkar få kromatinet att sluta sig vilket vi kunde mäta genom att studera en ytmarkör på kromatinet som kallas H3K9me3. Denna ytmarkör avspeglar ett slutet kromatin där gener inte kan uttryckas och Azacitidinbehandlade celler hade högre signal av H3K9me3 än obehandlade celler. Vi kan konstatera att de epigenetiska effekterna av Azacitidin är komplexa och ytterligare studier som studerar olika epigenetiska aspekter behöver genomföras.

I vårt tredje arbete letade vi efter faktorer som kan hjälpa oss att förutse vilka patienter som kommer att svara på behandling med Azacitidine. Ungefär hälften av alla patienter svarar på behandlingen och inga tillförlitliga studier har kunnat förklara vilka patienter som har nytta av behandlingen. Många patienter får således behandlingen i onödan. Vi sammanställde all tillgänglig information från journaler på alla de 134 patienter som fått Azacitidine vid MDS på Karolinska sjukhuset. Vi kunde konstatera att varken blodprover, benmärgsprov eller kromosomskador d.v.s. de undersökningar som görs på alla patienter med sjukdomen, ger information om vilka patienter som svarar på behandlingen. Vidare genomförde vi en DNA sekvensering på de gener som kunnat kopplas till sjukdomen på 90 av patienterna och undersökte om mutationer i dessa gener kan förutse vilka som svarar på behandlingen. Vi fann då att de patienter som hade mutationer i någon av de tre gener som påverkar histoner levde längre än de patienter som inte har någon sådan mutation. Detta kan vara ett viktigt verktyg för läkare som behandlar patienter med MDS då förekomsten av dessa mutationer skulle ökar chansen för att Azacitidine kommer att vara verkningsfullt. Vi undersökte också metylering med en metyleringsarray beskriven ovan på 42 patienter och sökte efter skillnader i metyleringsmönster mellan de patienter som svarade och de som inte gjorde det. Vi fann att metyleringsnivåerna skiljde sig på 200 gener och att en stor andel av dessa gener var sådana som styrde utmognadsprocessen av blodbildande celler. Vi jämförde vidare våra resultat med metyleringsnivå på sorterade MDS-stamceller och benmärg från friska försökspersoner och kunde konstatera att de patienter som inte

svarar har ett metyleringsmönster som mer liknar stamceller medan de patienter som svara har ett metyleringsmönster som mer liknar utmognade celler. Slutligen kunde vi konstatera att metyleringsnivån på den gen som skilde sig tydligast mellan de patienter som svarade och de som inte svarade, *HOXA5*, har betydelse för överlevnaden, där patienter med högre metyleringsnivå har en bättre överlevnad.

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