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DNA METHYLATION AND HYPOMETHYLATING AGENTS IN HIGH-RISK MYELODYSPLASTIC SYNDROMES AND ACUTE MYELOID LEUKEMIA

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ABSTRACT

Epigenetic alterations are common in cancer. One example is aberrant hypermethylation of the promoters of tumor suppressor genes and hence silencing of gene expression. Azacitidine, a DNA hypomethylating drug, has been shown to prolong survival in patients with high-risk myelodysplastic syndromes (MDS) compared to conventional care regimens, and is now recommended as first-line therapy for patients not eligible for allogeneic stem cell transplantation.

Azacitidine has DNA hypomethylating properties and has been shown to re-induce expression of aberrantly silenced genes in various cell lines. However, its mechanism of action in primary hematopoietic progenitors *in vivo* is relatively unknown.

This thesis aimed to assess the prognostic value of DNA methylation in high-risk MDS and in *de novo* acute myeloid leukemia (AML), to evaluate the effect and feasibility of maintenance treatment with azacitidine in patients in complete remission (CR) after induction chemotherapy, and to study mechanisms of action of azacitidine in primary MDS and normal bone marrow (NBM) progenitors.

We show, for the first time, a correlation between promoter methylation patterns and outcome of induction chemotherapy in a cohort of 60 patients with high-risk MDS or AML following MDS (MDS-AML). Patients who were hypermethylated in the *E-cadherin* (*CDH*) promoter had lower CR rates than those without methylation (P=0.008). *CDH* methylation was also associated with shorter survival (P=0.003).

By contrast, in a material of 107 patients with *de novo* AML CDH methylation had no impact on survival or on outcome of induction chemotherapy. In fact, promoter hypermethylation of $P15^{ink4b}$, previously reported as a poor prognostic marker in MDS and MDS-AML, as well as genome wide promoter methylation corresponded to a better survival (P=0.001 and 0.005, respectively). Another novel finding was that *de novo* AML patients with a low degree of global DNA methylation had a poorer response to induction chemotherapy (P=0.005). These differences in the prognostic value of methylation status in MDS/MDS-AML and *de novo* AML suggest important differences in disease biology and response to treatment between the two entities.

Several mechanisms of action for azacitidine have been suggested, including induction of apoptosis, differentiation of blasts, histone modification, immunomodulation and DNA and RNA demethylation. However, the majority of data results from cell line experiments or from sequential bone marrow sampling during azacitidine treatment. In paper IV of this thesis we exposed primary MDS and normal bone marrow (NBM) progenitors to azacitidine *in vitro*. Interestingly, azacytidine caused marked up-regulation of gene expression in MDS but not in NBM CD34+ marrow cells. Compared to cell line experiments, induction of apoptosis as well as global and gene-specific hypomethylation was less pronounced in primary cells. Interestingly, azacitidine in doses up to 5 μM had no negative effect on proliferation in suspension cultures, and doses up to 0.5 μM even had a positive effect on colony growth. This is a useful finding since it may support the use of the drug for patients with low-risk MDS, and as maintenance after allogeneic stem cell transplantation.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which in the text are referred to by their roman numerals.

I. **Negative impact of DNA hypermethylation on the outcome of induction chemotherapy in older patients with high-risk myelodysplastic syndromes and acute myeloid leukemia following myelodysplastic syndrome**. M Grövdal, R Khan, A Aggerholm, P Antunovic, J Astermark, P Bernell, LM Engström, L Kjeldsen, O Linder, L Nilsson, A Olsson, J Wallvik, JM Tangen, G Öberg, SE Jacobsen, P Hokland, A Porwit and E Hellström-Lindberg. *Clinical Cancer Research* 2007;13(23):7107-12.

II. **Maintenance treatment with azacytidine for patients with high-risk myelodysplastic syndromes (MDS) or acute myeloid leukemia following MDS in complete remission after induction chemotherapy**. M Grövdal, M Karimi, R Khan, A Aggerholm, P Antunovic, J Astermark, P Bernell, LM Engström, L Kjeldsen, O Linder, L Nilsson, M Skov Holm, JM Tangen, G Öberg, SE Jacobsen, P Hokland, A Porwit and E Hellström-Lindberg. *British Journal of Haematology* 2010;150(3):293-302.

III. **Gene-specific and global methylation patterns predict outcome in patients with acute myeloid leukemia**. S Deneberg, M Grövdal, M Karimi, M Jansson, H Nahi, A Corbacioglu, V Gaidzik, K Döhner, C Paul, TJ Ekström, E Hellström-Lindberg and S Lehmann. *Leukemia* 2010;24(5):1-10.

IV. **Complex effects of azacitidine in primary myelodysplastic and normal bone marrow cell cultures.** M Grövdal, M Karimi, M Nikpour, L McGovern, K Luttropp, M Jansson, AM Forsblom and E Hellström-Lindberg. *Manuscript.*

CONTENTS

1 LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ATRA	All-trans retinoic acid
CCR	Conventional care regimens
CDH	E-cadherin
CDR	Commonly deleted region
CMML	Chronic myelomonocytic leukemia
CR	Complete remission
CRi	Complete remission with insufficient hematologica recovery
DFS	Disease free survival
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EMEA	European Medicines Agency
EPO	Erythropoietin
FAB	Frensh-British- American classification
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
G-CSF	Granulocyte colony-stimulating factor
HAT	Histone acteyltransferase
HDAC	Histone deacetylase
HIC	Hypermethylated in Cancer 1
IPSS	International prognostic scoring system
MDS- AML	AML following a previous episode of MDS
MDS	Myelodysplastic syndromes
MNC	Mononuclear cells
MPN	Myeloproliferative neoplasms
NBM	Normal bone marrow
OS	Overall survival
P15	$P15^{ink4b}$/CDKN2b
PCR	Polymerase chain reaction
RA	Refractory anemia
RAEB	Refractory anemia with excess blasts
RAEB-t	Refractory anemia with excess blasts in transformation
RARS	Refractory anemia with ring sideroblasts
RCMD	Refractory cytopenia with multilineage dysplasia
RNA	Ribonucleic acid
s.c.	Subcutaneous
WBC	White blood cells
WHO	World Health Organization

2 INTRODUCTION

2.1 Myelodysplatic syndrome (MDS)

2.1.1 *General background and epidemiology*

Myelodysplastic syndromes (MDS) constitutes a heterogeneous group of clonal malignant bone marrow diseases. The incidence of MDS varies between 4-10/100 000/year in different materials, and increases with age (Figure 1).[1-5] MDS is mainly a disease of the elderly. Median age at diagnosis is around 75 years with a slight male predominance.[1-5] Ninety percent of MDS cases are idiopathic (*de novo* MDS), while around 10% are defined as therapy-related MDS, e.g. arising as a result from previous exposure to cytotoxic drugs or radiotherapy.[6-8] Some studies have suggested an association between MDS and e.g. benzene, ionizing radiation, smoking and pesticides but results are inconsistent between studies.[9-11] Although familial cases have been reported, and MDS definitely is associated to congenital disorders such as Fanconi's anemia, MDS is in most cases not a hereditary disease.[12]

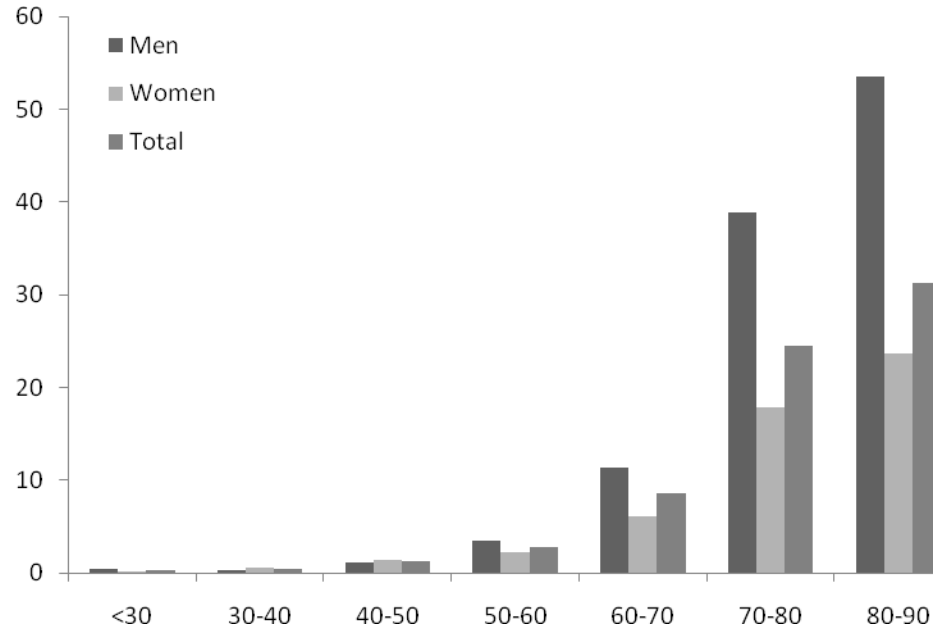

Figure 1: *The yearly incidence (per 100.000 individuals) of MDS according to age-groups and gender (Germing et al, Haematologica 2004)*[5]

2.1.2 *Clinical presentation*

The clinical presentation and the natural course of MDS vary largely among diagnostic subgroups and between individuals. An increasing number of patients are diagnosed though routine blood samples obtained when the patient consults their physician for non-MDS related problems. Symptoms from MDS are often secondary to the peripheral cytopenias caused by bone marrow failure. At diagnosis, a majority of the patients have some degree of anemia, which is usually macrocytic. Around 55% present with hemoglobin value <100 g/l.[13] Anemia-related symptoms (e.g. fatigue, dyspnea, worsening of angina pectoris and heart failure) constitute the most common clinical presentation of MDS. Other frequent symptoms include bleedings due to thrombocytopenia and platelet dysfunction, as well as infectious complications due to neutropenia. Bleeding and infection are also the major causes of death in MDS[14-17] Less common MDS-related symptoms include autoimmune diseases such as autoimmune hemolytic anemia and arthitis.[18,19] Around one third of patients eventually develop acute myeloid leukemia (AML), so-called MDS-AML.[13]

2.1.3 *Bone marrow morphology and cytogenetics*

The bone marrow of MDS patients is typically hypercellular. According to the WHO classification, at least 10% of the progenitors of one of the erythroid, myeloid and megakaryocytic lineage must show significant dysplasia for a diagnosis of MDS. Dysplasia is then graded into unilineage (one lineage) and multilineage (≥two lineages). Examples of dysplasia include nuclear abnormalities, poor granulation of the cytoplasm of granulocytes or ringed sideroblasts. The percentage of blast cells in bone marrow smears influences the classification of MDS and is achieved from counting at least 400 nucleated cells.[20] Around 50% of patients with MDS present one or more chromosomal aberrations. The most frequent finding is deletion of 5q (del5q), which alone or in combination, is found in close to 30% of the abnormal cases.[21] Genetic aberrations in MDS typically involve unbalanced loss or gain of genetic material (del5q, -7, +8 etc) but there are no MDS-specific abnormalities.[13,21] In secondary MDS, the type of cytogenetic aberrations are somewhat different and with a higher incidence of complex karyotypes, defined as ≥3 abnormalities.[22]

2.1.4 *Pathogenesis*

Since Knudson introduced his famous two-hit hypothesis in the early 70's, the accumulation of multiple genetic events has been considered necessary for a normal cell to transform into a cancer cell.[23] This multi-step theory is most likely valid also for MDS. Considering the heterogeneous nature of the disease, it is unlikely that a few genetic aberrations would explain the clinical picture for the whole group.

A few cytogenetic aberrations are recurring in MDS (e.g. del(5q), del(7q), del(20q), monosomy 5 and 7, and trisomy 8).[21] However, no aberration is pathognomonic for MDS and none of them is probably sufficient for the development of the disease. The genes involved in these aberrations are still basically unknown, with one exception; the deletion on chromosome 5q31 associated with low-risk MDS with del(5q) has been extensively studied. The genes proposed as putative candidates for the pathogenesis of the 5q- syndrome include *SPARC* and *RPS14* (see below 2.1.7.5). Since the remaining allele is not mutated in del(5q) MDS, haploinsufficiency has been suggested as a possible mechanism. The impact of point mutations is less well studied in MDS than in *de novo* AML. Frequent mutations that are strongly associated with prognosis in *de novo* AML (e.g. *NPM1*, *FLT-3* and *MLL*), are rarely found in MDS at diagnosis, but may appear at disease progression.[24-28] Their prognostic value in MDS is, however, less well defined. *JAK-2* mutations, a hallmark of myelo-proliferative diseases leading to constitutive thyrosine phosphorylation activity, can be found in subgroups of MDS patients typically presenting with elevated platelets, RARS-T (WHO mixed MDS/MPN) and occasionally in the 5q- syndrome.[20,29-32]

Epigenetic changes, i.e. heritable changes in gene expression without alteration in the DNA sequence, are a common feature of the cancer cell. Its role in MDS is discussed in detail below (see 2.3.2).

Several studies have showed increased apoptosis (programmed cell death, see below 2.4), of hematopoietic progenitors in the bone marrow of MDS patients.[33] This explains the clinical picture in MDS with hypercellular bone marrow and peripheral cytopenia. It is, however, intriguing and a topic for ongoing research how a malignant clone with increased apoptosis can gain a growth advantage over normal cells. The exact mechanisms underlying increased apoptosis in MDS are unclear but seem to involve dysregulation of both the extrinsic pathway via death receptors (e.g. FAS and TNF-α) and the intrinsic apoptotic pathways (e.g. mitochondria and Bcl-2 family). Up-regulation of death receptors as well as pro-apoptotic members of the Bcl-2 family is seen in MDS.[34-36] Erythroid progenitors in RARS patients show a spontaneous leakage of cytochrome c from the mitochondrial intermembrane space and subsequent activation of caspases.[37] P53 is a pro-apoptotic protein and mutations in the *TP53* gene are common in cancer and usually correlate to poor prognosis and poor response to treatment.[38,39] In MDS *TP53* mutations are seen in around 10% of the patients and mainly in high-risk disease and therapy-related cases.[40,41] Also in MDS *TP53* mutations relates to poor outcome and resistance to therapy.[24,42,43]

In a subgroup of MDS patients (see 2.1.7.4) the disease mechanism seems to be immune mediated, similar to that in aplastic anemia. MDS bone marrow precursors are in these cases the target of cytotoxic T-cells and natural killer (NK) cells. The hypothesis is supported by observations that therapeutic depletion of T-cells may improve colony

growth from bone marrow mononuclear cells (MNC) in MDS patients, while not affecting normal bone marrow.[45-47] Moreover, several studies have reported on oligoclonal expansion of T-lymphocytes in MDS.[48-50]

The interaction between the bone marrow stroma and hematopoietic stem cells is crucial for the survival and the function of the stem cells. The interaction consists of both cell-to-cell adhesion and secretion of cytokines from the stromal cells.[51] In MDS the stroma seems to have decreased ability to support normal hematopoietic stem cells.[52]

2.1.5 *Classification*

The French-American-British (FAB) classification of MDS was presented in 1982.[53] It was based exclusively on bone marrow morphology and patients were classified into subgroups according to the amount of blasts in the bone marrow and the existence or not of ring sideroblasts. The WHO classification was first published in 2001 and then updated in 2008.[20] This is now the most widely used classification system for MDS. The number of dysplastic lineages is of importance. Patients with dysplasia in more than one lineage are due to their poorer prognosis separated from refractory anemia (RA) and refractory anemia with ring sideroblasts (RARS), and classified as refractory cytopenia with multilineage dysplasia (RCMD). Patients with a del(5q) as the sole cytogenetic aberration and marrow blasts <5% form a separate entity. The FAB-subgroup refractory anemia with excess of blasts in transformation (RAEB-t) disappeared in the WHO classification when the cut-off for bone marrow blasts for a diagnosis of AML was reduced from 30 to 20%. The reason was the similar prognosis and response to treatment between RAEB-t and AML. Chronic myelomonocytic leukemia (CMML) was transferred from the MDS classification to a new subgroup; mixed MDS/MPN. The full WHO classification of MDS is shown in Table 1.

2.1.6 *Prognosis*

Prognosis in MDS varies a lot. Some patients live for many years with only moderate anemia whereas others are transfusion dependent at diagnosis. The risk of leukemic transformation is very high in some patients and almost absent in some. Since 1997 the international prognostic scoring system (IPSS) has been the most widely used prognostic tool for patients with MDS, serving as a basis for therapeutic decisions.[13] It is based on different risk groups derived from the number of cytopenias, percentage of bone marrow blasts and type of cytogenetic aberration. Patients are grouped into one of four prognostic groups; low, intermediate-1, intermediate-2 and high risk, with increasing risk of leukemic transformation and poorer survival. In studies, the low and intermediate-1 subgroups are often referred to as low-risk MDS and the intermediate-2 and high as high-risk disease. The IPSS is

described in detail in Table 2. Furthermore, the WHO classification system, as well as transfusion dependency provides additional help in prognostication and is included into a new prognostic scoring system, the WPSS.[54] Moderate to severe bone marrow fibrosis is also associated with significantly poorer prognosis.[55]

Table 1: *The 2008 WHO classification of myelodysplastic syndromes*[20]

Disease	Blood findings	Bone marrow findings
Refractory cytopenias with Unilineage dysplasia (RCUD) Refractory anemia (RA) Refractory neutropenia (RN) Refractory thrombocytopenia (RT)	Unicytopenia or bicytopenia[a] No or rare blasts (<1%)	Unilineage dysplasia; >10% of the cells of the affected lineage are dysplastic <5% blasts <15% of the erythroid precursors are ring sideroblasts
Refractory anemia with ring sideroblasts (RARS)	Anemia No blasts	Erythroid dysplasia only <5% blasts ≥15% of ring sideroblasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenias No or rare blasts (<1%) No Auer rods <1x10^9 monocytes/L	Dysplasia in >10% of the cells of two or more myeloid lineages <5% blasts No Auer rods ± ring sideroblasts
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenias <5% blasts No Auer rods <1x10^9 monocytes/L	Unilineage or multilineage dysplasia 5-9% blasts No Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenias 5-19% blasts ± Auer rods[b] <1x10^9 monocytes/L	Unilineage or multilineage dysplasia 10-19% blasts ± Auer rods[b]
Myelodysplastic syndrome unclassified (MDS-U)	Cytopenias No or rare blasts (<1%) No Auer rods	Unequivocal dysplasia in <10% of cells in one or more myeloid lineages <5% blasts
MDS associated with del(5q)	Anemia No or rare blasts (<1%) Platelet count usually normal or increased	Normal to increased number of megakaryocytes with hypolobated nuclei <5% blasts Isolated del(5q) cytogenetic abnormality No Auer rods

[a] Bicytopenia may occasionally be observed. Cases with pancytopenia should be classified as MDS-U [b] If the diagnostic critera for MDS are fulfilled and Auer rods are present, the patient should always be categorized as RAEB-2

Table 2: *The international prognostic scoring system*[13]

Prognostic variable	**0 points**	**0.5 points**	**1.0 point**	**1.5 points**
Number of cytopenias[a]	0-1	1-2	-	-
Karyotype[b]	Good	Intermediate	Poor	-
Bone marrow blasts (%)	<5	5-10	-	11-20
Risk group	**Score**	**Median survival (years)**	**25% AML evolution (years)**	
Low	0	5.7	9.4	
Intermediate-1	0.5-1.0	3.5	3.3	
Intermediate-2	1.5-2.0	1.2	1.1	
High	≥2.5	0.4	0.2	

[a] Cytopenias defined as hemoglobin <100 g/L, platelet counts <100 x 10^9/L and absolute neutrophil counts <1.8 x 10^9/L [b] Good: Normal, -Y, del(5q), del(20q); Poor: Complex karyotype (≥3 abnormalities) or chromosome 7 abnormalities; Intermediate: Not fulfilling criteria for good or poor.

2.1.7 *Treatment*

The clinical picture in MDS varies from mild, asymptomatic cytopenia to severe transfusion dependency and rapid leukemic transformation, and the expected survival times range from a few months to several years. A small subgroup of MDS has an almost normal life expectancy. Accordingly, therapy for the disease has to be individualized depending on symptoms, risk group, and age. Whereas some patients will not require any treatment, others will need intensive treatment with chemotherapy and also be eligible for allogeneic stem cell transplantation.

2.1.7.1 Transfusion therapy

A majority of patients with MDS will develop transfusion-dependent anemia at some time, and more than 50% have severe anemia already at diagnosis.[13] Hence, red blood cell transfusion is the most common therapy in MDS. Anemia is associated with increased morbidity and mortality, and with decreased quality of life, which improves with measures that increase the hemoglobin level.[17,56-58] The hemoglobin target of transfusion therapy has to be adapted to the individual needs of each patient depending on symptoms and co-morbidity.

2.1.7.2 Iron chelation therapy

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

transfusion dependency from early childhood, it is well known that iron overload will cause organ damage (liver, heart, pancreas) and eventually organ failure and death, and that treatment with iron chelators decreases both morbidity and mortality.[59-61] Excess iron is seen also in organs of heavily transfused MDS patients but there is still no clear evidence showing benefit of chelation therapy in MDS. A retrospective study shows that patients with higher ferritin levels have increased risk of death but whether this is due to the iron overload *per se* or just reflects a more severe disease is not clear.[17] Improved erythropoiesis and reduced transfusion requirements have been reported after iron chelation therapy.[62,63] Studies showing better survival in chelated patients are not prospective or randomized, and the results may reflect physicians inclination to avoid chelation therapy in patients expected to have poorer survival.[64] One could argue that the effect might be different in individuals exposed to excess iron in a period when the organs are still growing (thalassemia) compared to those exposed adulthood (MDS patients). The recommendation for MDS, however, is to start iron chelation therapy (desferrioxamine, deferasirox or deferiprone) in MDS patients with an expected survival of more than 2 years after around 24 units of red blood cells or when serum ferritin reaches 1000-2000 µg/L. A special subgroup that always should be considered for iron chelation is candidates for allogeneic stem cell transplantation.[65-68]

2.1.7.3 Growth factors

Several studies have shown improved erythropoiesis in MDS patients treated with subcutaneous (s.c.) injections of recombinant human erythropoietin (EPO) in doses between 30,000 and 60,000 U/week. Meta-analyses show response rates between 16-82% depending on diagnostic subgroup.[69.70] Responses are rare in MDS with excess of blasts. RA patients respond better than patients with RARS and patients with unilineage dysplasia respond better than those with multilineage dysplasia.[71] Addition of granulocyte colony-stimulating factor (G-CSF) significantly improves the erythroid response and is recommended for patients not responding to EPO alone and for transfusion-dependent RARS patients up-front.[72-74] Response rates correlate with endogenous levels of serum-EPO and with the degree of transfusion need. These parameters are included into a predictive model for response developed by Hellström-Lindberg et al. (Table 3).[75] A number of studies have shown improvement of quality of life by treating the anemia of MDS patients with EPO ± G-CSF.[73-75] Several mechanisms of action have been suggested for growth factor therapy in MDS, e.g. inhibition of apoptosis in MDS progenitors, increased maturation of dysplastic blasts and stimulation of the remaining normal erythro-poiesis.[37,76,77]

Table 3: *Predictive model for response to EPO and G-CSF.*[75]

Variable	Value	Score
Transfusion need	<2 U	0
	≥2 U	1
Serum EPO	<500 U/L	0
	≥500 U/L	1
Predictive group	**Score**	**Response rate**
Good	0	74%
Intermediate	1	23%
Poor	2	7%

2.1.7.4 Immunosuppression

A small subgroup of patients with low-risk MDS can benefit from immuno-suppressive treatment with anti-thymocyte globulin (ATG) ± cyclo-sporine-A (CyA). Response rates are around 30% and the duration of response varies in different materials. Hypocellular bone marrow, age below 60 years, and HLA DR15 positivity constitute positive predictive factors for response.[78-81]

2.1.7.5 Immunomodulatory drugs

Lenalidomide, an analogue to thalidomide, was approved by the US Food and Drug Administration (FDA) in 2006 for the treatment of low-risk MDS with a karyotype including del(5q). The approval in the US was based on one study showing major erythroid response in 67%, and complete cytogenetic response in 45% of patients with low-risk MDS and del(5q) with or without other abnomalities.[82] However, the MDS hematopoietic stem cells seem to be resistant to treatment, as shown by a recent publication.[83] In MDS patients without del(5q) the response rates are much lower; 26% major erythroid response and 19% complete cytogenetic response, and response durations are shorter.[84] Response to lenalidomide is also observed in high-risk MDS and AML with a karyotype including del(5q), but response rates are lower and treatment should more be seen as an induction regimen.[85,86] The most common side effects of the drug are thrombocytopenia and neutropenia. Lenalidomide did not gain approval by the European Medicines Agency (EMEA) due to safety concerns based on reports of clonal evolution and higher rates of leukemic transformation than expected in patients who fail to achieve sustained remission.[87,88] Thalidomide induces few responses in MDS and cannot be recommended as treatment of the disease.[89,90] The mechanisms of lenalidomide include anti-angiogenetic effects, effects on cell adhesion, modulation of T-cell immune response and induction of apoptosis in tumor cells.[91] In vitro studies show that lenalidomide inhibits growth of MDS del(5q) progenitors but does not affect the growth of normal cells.[92]

Haploinsufficiency of one or several genes expressed in the commonly deleted region (CDR) of MDS del(5q) has been postulated as a possible mechanism in del(5q). Restoration of gene expression levels could, accordingly, be a possible mode of action of lenalidomide. Several genes within the CDR have been investigated. The expression of *SPARC*, a gene coding for a protein with anti-proliferative, anti-angiogenetic and anti-adhesive effects, is down-regulated in del(5q) MDS progenitors and is up-regulated by lenalidomide treatment.[92] Another gene of interest is *RPS14*, encoding for a component of the 40S ribosomal subunit. In vitro blocking of the gene in normal bone marrow progenitors by RNA interaction techniques led to impaired erythroid differentiation but preserved megakaryocytic differentiation, mimicking the del(5q) syndrome. Increased expression of the gene in MDS cells by lentiviral techniques improved erythroid maturation.[93] The gene is also up-regulated by lenalidomide treatment.[94]

2.1.7.6 Chemotherapy

There is insufficient data for the recommendation of low dose chemotherapy such as e.g. hydroxyurea in MDS. In CMML-1, on the other hand, hydroxyurea is the first-line option to reduce high peripheral white blood cells (WBC).[95] The use of low-dose (10-30 mg/m^2/day s.c.) cytosine arabinoside (ara-C) induces response in around 30% of patients, but does not influence survival or leukemic transformation rate.[96,97] Until the introduction of hypomethylating drugs (see below, 2.3.3.1) the only therapeutic option for patients with high-risk MDS, not eligible for allogeneic hematopoietic stem cell transplantation, was induction chemotherapy, as used for AML. Around 50% of patients achieve complete remission (CR) with this therapy.[98-102] Parameters indicating a more proliferative disease, such as high WBC and high lactate dehydrogenase (LDH), as well as complex karyotype correlates to lower probability for CR.[98,101,102] However, CR durations are short, most often less than a year, and there is no evidence for cure from this treatment.[98-100,102,103] Conventional consolidation courses, autologous stem cell transplantation, interleukin-2 maintenance or G-CSF priming does not improve CR duration.[99,100,102,104,105] Today, considering the positive survival data with hypomethylating agents, the rationale for induction chemotherapy is uncertain in patients where it cannot be followed by allogeneic stem cell transplantation. It might be considered in otherwise fit patients with high-risk disease and very rapidly increasing blasts, or after failure to hypo-methylating drugs.

2.1.7.7 Allogeneic stem cell transplantation

As allogeneic transplantation of hematopoietic stem cells is the only potentially curative option in MDS, all newly diagnosed patients should be considered for this option.[106] However, due to the high median age at diagnosis

in MDS, only a minority of patients will be eligible. Allogeneic stem cell transplantation with conventional myeloablative conditioning regimens is usually only considered for patients up to about 60 years of age. Reduced intensity conditioning is tolerated even for older patients, but is also associated with a higher relapse rate.[107] With myeloablative conditioning regimens overall survival (OS) is between 30-50%, and relapse rates 20-40%.[108-110] Due to the high transplant related mortality in MDS (30-40%)[108-110] the timing of transplant is of great importance. For patients with high-risk disease and thus short expected survival, allogeneic stem cell transplantation is recommended as a part of first-line treatment, usually following hypomethylating agents or induction chemotherapy. Decision-making is much more difficult for patients with lower-risk disease, who may have a good quality life for several years with only supportive care and growth factors, but who eventually may die from disease. Patients that have achieved CR before transplantation have a much better outcome than those transplanted with resistant or relapsing disease.[109,110]

2.1.7.8 Epigenetic therapies (see below: 2.3.3)

2.2 Acute myeloid leukemia (AML)

AML can either arise *de novo,* or secondary following a history of previous MDS, myeloproliferative neoplasms, or previous anti-cancer treatment with cytotoxic drugs or radiation therapy. The criteria for AML include ≥20% bone marrow blasts, and the occurrence of Auer rods is pathognomonic for the diagnosis. Table 4 shows the classification of AML according to WHO.[20] The incidence of AML is about 4/100,000/year with the majority of cases being *de novo* disease.[111] With the exception of older patients with AML following MDS (MDS-AML) and <30% bone marrow blasts, in whom the therapy of choice is hypomethylating drugs, and patients with AML FAB M3 (acute promyelocytic leukemia (APL)), a subgroup with particularly good prognosis and where all-trans retinoic acid (ATRA) should be added to the induction chemo.[112] Therapy for AML consists of induction chemotherapy followed by consolidation courses. Patients with high-risk disease and in some cases also those with intermediate risk, should, if possible, be subject to allogeneic stem cell transplantation already in first remission while the rest of patients are transplanted only after relapse and in second remission.[113]

2.2.1 *Differences between de novo AML and MDS-AML*

MDS-AML and *de novo* AML, apart from being two heterogeneous entities, show major differences. The median age is higher in MDS-AML than in *de novo* AML, and older MDS-AML patients tolerate intensive treatment less well. Prognosis is worse in MDS-AML than in *de novo* AML.[114] Among patients with *de novo* AML the rate of cytogenetic aberrations is comparable

with MDS-AML, around 50%, but the type of aberrations differ. The typical aberration in *de novo* AML is a balanced translocation whereas in MDS-AML most commonly a loss of genetic material is seen. The same aberration can also have different prognostic value. A normal karyotype is e.g. associated with good prognosis in MDS, whereas in *de novo* AML it correlates to intermediate or poor prognosis depending on the co-existence or not of molecular aberrations.[24,115,116] The prognostic importance of genetic aberrations in *de novo* AML is shown in Table 5.

Table 4: *The 2008 WHO classification of acute myeloid leukemias*[20]

Disease	Description
AML with recurrent genetic abnormalities	1. AML t(8;21)(q22;q22) *RUNX1/RUNX1T1* 2. AML with inv(16)(p13;q22) or t(3;3)(p13;q22) *CBFB-MYH11* 3. APL t(15;17)(q22;q21) *PML-RARA* 4. AML with t(9;11)(p2;q23) *MLLT3-MLL* *5.* AML with t(6;9)(p23;q34) *DDEK-NUP214* *6.* AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2) *RPN1-EVI1* 7. AML with t(1;22)(p13;13) *RBM15-MKL1*
AML with myelodysplasia related changes	Patients who have had a prior MDS or MPD that transforms to AML.
AML and MDS, therapy related	Patients that have had prior chemotherapy and/or radiation and that subsequently develop AML or MDS.
AML not otherwise categorized	Patients with AML that do not fall into any of the categories above. 1. AML with minimal differentiation 2. AML without maturation 3. AML with maturation 4. Acute myelomonocytic leukemia 5. Acute monoblastic and monocytic leukemia 6. Acute erythroid leukemia 7. Acute megakaryoblastic leukemia 8. Acute basophilic leukemia 9. Acute panmyelosis with myelofibrosis

Table 5: *Genetic aberrations and prognosis in de novo AML*[116]

Risk group	Type of genetic aberration
Favorable	APL with t(15;17)(q22;q12); *PML-RARA*
	inv(16)(p13.1;122) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	Normal karyotype and mutated *NPM1* without *FLT3-ITD*
	Normal karyotype and mutated *CEBPA*
Intermediate	Normal karyotype and mutated *NPM1* and *FLT3-ITD*
	Normal karyotype and wild-type *NPM1* and *FLT3-ITD*
	Normal karyotype and wild-type *NPM1* without *FLT3-ITD*
	t(9;11)(p22;q23); *MLLT3-MLL*
	Cytogenetic aberrations not classified as favorable or adverse
Adverse	inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
	t(6;9)(p23;q34) *DDEK-NUP214*
	t(v;11)(v;q23); *MLL* rearranged
	-5 or del(5q); -7; Abnl(17p); Complex karyotype

2.3 Epigenetics and epigenetic therapies

2.3.1 *General background on epigenetics*

The term epigenetic refers to changes in gene expression without any alteration in the actual DNA sequence. It plays an important role during embryogenesis and during cell differentiation. It is the explanation why cells of a multi-cellular organism that all carry an identical genome (DNA sequence) can differentiate to cells with very diverse and specialized function.[117,118]

Epigenetic modulation of gene expression is also a hallmark of carcinogenesis.[119] The field has gained increasing interest during the last decade, especially when drugs with epigenetic modulating properties were approved for the treatment of cancer patients. There are three principally different ways of epigenetic modulation of gene expression; DNA methylation, histone modifications and RNA inter-actions.

2.3.1.1 DNA methylation

DNA methylation is the most studied epigenetic mechanism of gene expression regulation and refers to the addition of a methyl group at position 5 on cytosine nucleosides of the DNA. DNA methylation occurs predominantly at sites where the cytosine is followed by a guanine residue (CpG).[120] CpG sites are unevenly distributed through the genome. Regions that are particularly rich in CpGs are called CpG-islands and are often located downstream to or in close proximity to gene promoters. In contrast to most

CpGs of the genome, which normally are methylated, CpG-islands are normally unmethylated. An unmethylated promoter usually allows for gene expression and methylation of the promoter causes silencing of the gene.[121] The exact mechanism by which promoter methylation causes silencing of the gene is somewhat unclear but could include direct interference with the binding of transcription factors, blocking of the transcription machinery by the recruitment of methyl-binding domain proteins to methylated DNA, or alteration of chromatin structure.[122]

DNA methylation is catalyzed by DNA methyltransferases (DNMTs), enzymes that transfer a methyl group from a methyl donor to the cytosine residue of the DNA. In mammals there are four DNMTs. DNMT1, with high affinity for semi-methylated DNA, maintains the methylation pattern during replication while DNMT3a and DNMT3b are mainly responsible for *de novo* methylation. DNMT2 does not methylate DNA to any significant extent but seems to be involved in methylation of small RNAs.[123,124]

2.3.1.2 Histone modification

Histones are proteins forming the nucleosomes around which the DNA is wrapped to form the chromatin. Each nucleosome consists of two copies each of histone H2A, H2B, H3 and H4. Depending on how tight the DNA is packed around the nucleosomes the chromatin will be either condensed (heterochromatin) and inactive as e.g. during mitosis, or relaxed (euchromatin) and available for transcription.[125] H3 and H3 carry “tails” (the amino-terminal end of these proteins) that can be modified by e.g. acetylation and methylation, modifications that are responsible for the switch between heterochromatin and euchromatin and thereby influence gene expression. The acetylation status of histones is determined by the relative activities of histone acetyltransferases (HAT) and histone deacetylases (HDAC).[126] DNA is negatively charged and is detached from the nucleosomes when acetyl groups are transferred by HAT from actyl-CoA to neutralize the positively charged lysine residuals of the histone tails. The acetylated state of histones is accordingly associated with eu-chromatin and the deacetylated state with heterochromatin.[127]

2.3.1.3 RNA interference

In 2006 Drs. Andrew Z. Fire and Craig C. Mello were awarded with the Nobel Prize in Physiology or Medicine for their work in the field of RNA interferance.[128] Apart from being a physiologic cellular mechanism to regulate gene expression, RNA interference is also used as a method to inhibit the expression of specific genes *in vitro*. RNA interference starts with the cleavage of long double stranded RNA by an endonuclease, Dicer. The product, short interfering RNAs (siRNAs) have an overhang of two unpaired nucleotides on each of the 3’ ends. The anti-sense strand of the

siRNA then forms a complex (RISC) with a protein with RNase activity (Ago2) and guides the complex to the target RNA, which is cleaved. After cleavage, the target RNA lacks the 5' cap and the 3' poly-A tail, elements responsible for RNA stability, and is accordingly further degraded and the corresponding RNA can no longer be synthesized.[129]

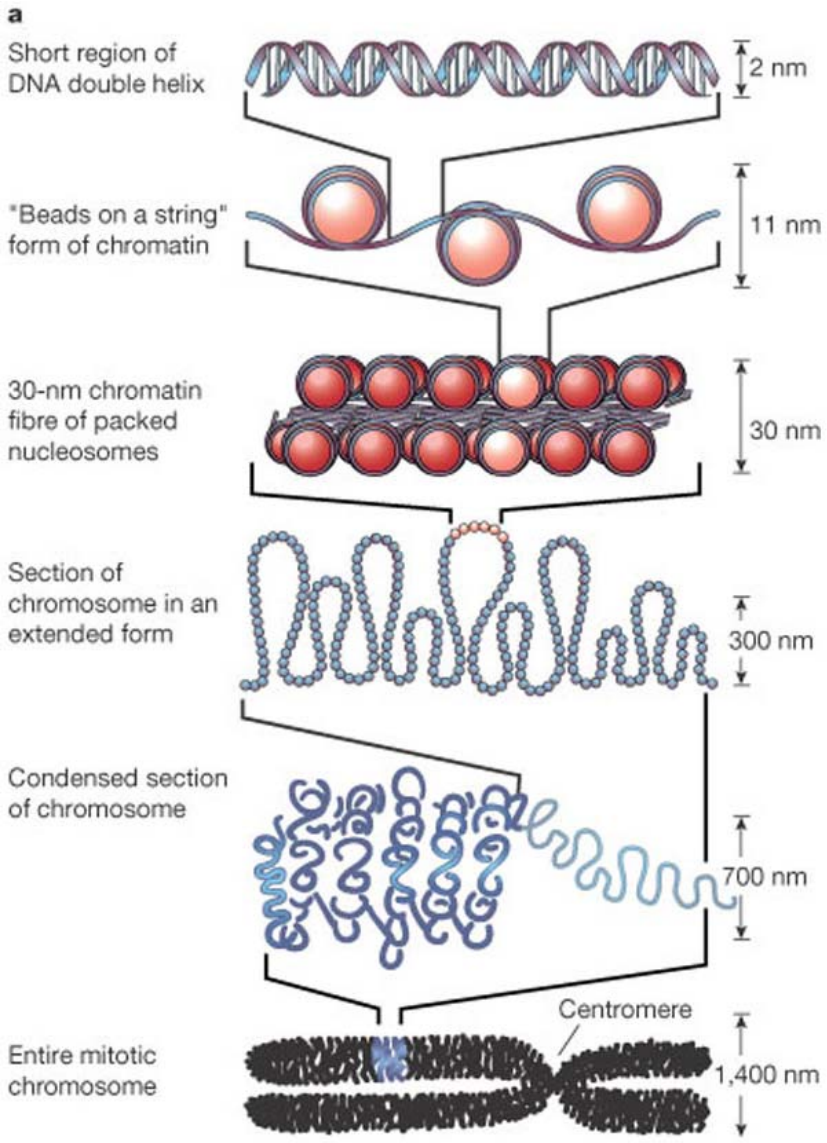

Figure 3: *Chromatin structure (Felsenfeld & Groudine, Nature 2003)*[125]

2.3.2 *Epigenetics in cancer with focus on MDS and AML*

Carcinogenesis involves gradual accumulation of genetic as well as epigenetic aberrations during disease progression. Typical epigenetic changes in both solid tumors and in hematological malignancies include genome wide (global) hypomethylation and promoter hypermethylation and hence silencing of e.g. tumor suppressor genes.[119,130-132] The pattern of epigenetic alterations (promoter methylation best studied) is tumor-type specific.[133,134] It is not understood why e.g. certain gene promoters get methylated in one type of cancer but not in another and in most cases a causative role of the epigenetic alteration in the development of cancer has not been demonstrated. Cancer is also associated with imbalance in the histone modification pattern by e.g. increased histone acetylation due to impaired activity of HATs, or over expression of HDACs as well as recruitment of HADCs by fusion proteins produced from chromosomal translocations, as e.g. PML-RARα in APL.[135,136]

Aberrant promoter hypermethylation has been described in both MDS and in AML. Commonly hypermethylated genes in both MDS and AML include $P15^{ink4b}$ (*P15*), E-cadherin (*CDH*), Hypermethylated in Cancer 1 (*HIC*), and Estrogen receptor (*ER*).[137,138] However also clear differences in the methylation pattern between *de novo* AML and MDS or MDS-AML have been shown using microarray assays to analyze methylation in bone marrow progenitors. Patients with MDS and MDS-AML show a more extensively aberrant methylation pattern than *de novo* AML. Moreover, the epigenetic changes tend to affect different chromosomal regions in *de novo* AML and MDS/MDS-AML.[139]

In MDS, promoter hypermethylation increases with progression towards AML.[140,141] Hypermethylation of e.g. *P15*, *CDH*, *HIC* and *ER* in different combinations, has in MDS been shown to correlate with an increased rate of leukemic transformation and poor survival.[137,142,143] In one of the papers included in this thesis we show a strong negative correlation between *CDH* methylation and the ability for patients with high-risk MDS and MDS-AML to achieve CR on induction chemo-therapy.[144] In AML, one study show improved survival among patients with methylated *ER* promoter.[145] Regarding the impact of *P15* and *CDH* methylation in AML results are somewhat contradictory as studies have shown association with both poor and good prognosis.[146-150] The inconsistency in results regarding the role of methylation in AML treatment may reflect different proportions of MDS-AML vs. *de novo* cases in these studies. In the third study of this thesis, encompassing a cohort of *de novo* AML only, we show better survival in *P15* methylated patients and higher CR-rates in patients with low global methylation levels.[151]

2.3.3 *Epigenetic therapies*

2.3.3.1 Hypomethylating drugs

Azacitidine (5-azacitidine; Vidaza®) and decitabine (5-aza-2'-deoxycitidine; Dacogen®) are two structurally related drugs with DNA hypomethylating properties. Azacytidine was approved by the FDA in 2004 for the treatment of patients with myelodysplastic syndromes, and Decitabine for the same indication in 2006. Since 2009, azacitidine is approved also in Europe and in most other parts of the world. The drugs are related to cytarabine, a cytotoxic drug that has been used e.g. in the treatment of AML for many years. First synthesized in 1964, azacitidine was also used, in higher doses in the treatment of AML but was soon abandoned due to considerable toxicity, mainly gastrointestinal.[152] Both azacitidine and decitabine act as cytosine analogs. After phosphorylation, azacitidine can be incorporated into DNA as well as RNA whereas decitabine will be incorporated solely into DNA. They exert their hypomethylating effect by inhibition of DNMTs, thereby blocking the addition of methyl groups to the new DNA strand during mitosis.[153] Their exact mechanism of action *in vivo* is not known but *in vitro* studies on myeloid cells (mainly cell line experiments) have shown a broad spectrum of effects including DNA hypomethylation, re-expression of aberrantly silenced genes, induction of apoptosis, immunomodulation, differentiation of blasts, histone modifications and inhibition of RNA methylation.[154-163] The fact that several courses usually are required to achieve a response suggests that the clinical effect is not merely cytotoxic but probably also associated with epigenetic alterations in the cancer cells.[164]

The approval in the US followed a randomized study on azacitidine vs. best supportive care for MDS patients, showing prolonged time to the composite endpoint of leukemic transformation or death in the azacitidine arm. Positive effect was seen also in patients with high-risk MDS, a group where so far no other therapy than allogeneic stem cell transplantation had proved to alter the natural course of the disease and actually improve survival.[164] The effect on survival was later confirmed in a subsequent large randomized trial on high-risk MDS and MDS-AML, where azacitidine was compared to physician's choice of conventional care regimen (CCR) (supportive care only, low-dose cytarabine or intensive induction chemotherapy). The two-year survival was 50.8% in the azacitidine treated group compared to 26.2% in the CCR group with an overall prolongation of survival of 9 months. Azacitidine was beneficial also in the group with poor risk cytogenetic, and patients with monosomy 7 or del(7q) responded particularly well.[165]. Later publications using the same material have shown a significant survival advantage also in subgroups; patients above or younger than 75 years, and WHO MDS vs. WHO AML.[166,167] The effect of azacidine has been studied in different clinical situations in a large number of phase I and II trials. A positive effect was observed in patient with low-risk

MDS and cytopenia.[168,169] Effects in high-risk MDS were also seen in clinical trials using decitabine, including two randomized phase III trials. However, these studies did not show statistically significant improvement in OS.[170,171] Overall response rates with hypomethylating agents are usually around 50% but CR-rates are much lower, 10-20%.[164,165,170,171] However, CR does not seem to be required for improved survival.[172] Methylation of the *P15* promoter decreases during treatment but the results are contradictory regarding the association between promoter pre-treatment methylation status and response to hypomethylating agents.[173,174]

Hypomethylating agents has also been studied as part of the conditioning regimen for patients with relapse after allogeneic stem cell transplantation, and as maintenance after allogeneic SCT.[175-179] Several phase II studies have evaluated the effect of azacitidine in combination with other drugs including lenalidomide, thalidomide and HDAC inhibitors.[180-184] In these studies it is difficult to separate the effect of azacitidine from other drugs and immune effects such as the graft vs. host effect. One of the papers included in this thesis is a phase II study on azacitidine given as maintenance therapy as an attempt to prolong the otherwise short CR duration for patients with high-risk MDS and MDS-AML in CR after induction chemotherapy.[185]

There are no large studies on the efficacy of hypomethylating agents in pure *de novo* AML material. However subgroup analyzes from MDS studies including MDS-AML or studies on AML cohorts including both MDS-AML and *de novo* cases show a positive effect also in patients with AML.[166,186]

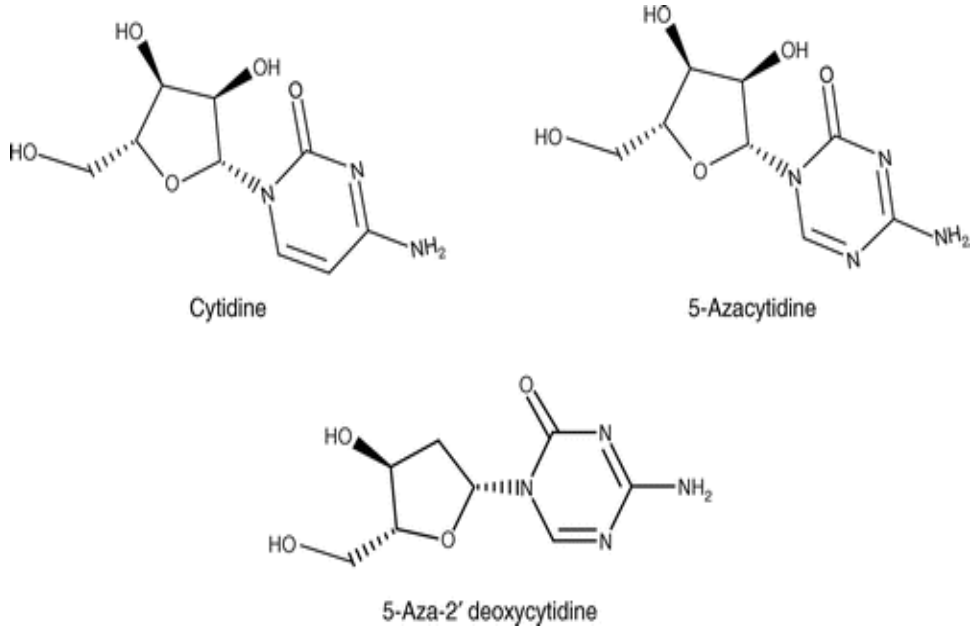

Figure 4: *Structure of cytosine analogs (O'Dwyer & Maslak, Exp Opin Pharmacother 2008)*[152]

2.3.3.2 Histone deacetylation inhibitors

Inhibitors of histone deacetylase are quite new in cancer treatment. However, members of the first generation of HDAC inhibitors (short fatty acids, e.g. valproic acid and butyric acid) have been used to induced transcription of HbF in patients with sickle cell anemia for several years.[187] Suberoylanilide hydroxamic acid (SAHA; vorinostat; Zolinza®), a member of another group of HDAC inhibitors, was recently approved for the treatment of cutaneous T-cell lymphomas.[188] HDAC inhibitors bind to a Zn^{2+} ion, critical for the enzymatic activity of HDACs, and different HDAC inhibitors have varying potency and specificity to specific HDAC isoenzymes.[189,190] HDAC inhibitor treatment *in vitro* leads to rapid increase in acetylated histones and changed chromatin structure. The mechanisms of action of HDAC inhibitors are not fully understood. It has been suggested that the effect in cancer might be due to restoral of aberrant changes in chromatin structure and thus re-expression of aberrantly silenced genes involved in e.g. apoptosis and cell cycle control. Most certainly however, the effect of HDAC inhibitors is not solely related to chromatin structure but involve also other non-histone related mechanisms.[191]

The effect of HDAC inhibitors have been evaluated in several clinical phase I and II trials, both as single therapy and in combination with hypomethylating agents. A problem has been dose-limiting gastrointestinal and sedative side effects.

Phase I and II studies have evaluated valproic acid, originally an anti-epileptic drug, as single therapy or in combination with ATRA in mixed MDS and AML. Most studies show response rates of 20-30%, the majority being only hematologic improvement and with few PR and CR.[192-196] The addition of ATRA does not seem to increase the response [192,193] and combinations with hypomethylating agents show response rates similar to hypomethylating agents alone.[182,184,197]

Vorinostat (SAHA) has also been evaluated in clinical phase I trials in MDS and AML. In a study on vorinostat in single therapy 7/31 (22%) patients with AML responded with 2 CR and 2 CRi.[198] Preliminary reported phase I studies of the combination vorinostat and hypomethylating drugs for patients with MDS and AML, have shown promising results with an overall response rate of 83% (53% CR or CRi) for the combination vorinostat and azacitidine.[199,200]

2.4 Apoptosis

Apoptosis is a form of programmed cell death. It is an energy dependent controlled process and it does not cause inflammation which is different from cell death in necrosis.[201] Apoptosis is essential in embryonic development and in protection of the organism by removal of cells that are damaged from e.g. viruses, mutations, toxic drugs or radiation.[202] Morphologically apoptosis

is characterized by cell shrinkage, condensation of nucleus and chromatin, break down of DNA, nuclear fragmentation, formation of apoptotic bodies and finally phagocytosis by macrophages.[203] Apoptosis can be triggered either via the extrinsic or the intrinsic pathway. These pathways, however, are not totally independent from each other. The extrinsic pathway includes interaction between so-called death receptors and their ligands and subsequent activation of caspases. The stimulus can be e.g. binding of Fas ligand to Fas receptor, withdrawal of growth factor stimulus, or T-cell mediated. The intrinsic pathway includes activation of mitochondria and release of cytochrome c, as a response to e.g. DNA damage. The apoptosis pathways are controlled by a wide number of pro- (e.g. Bid and Bax) and anti-apoptotic proteins (e.g. Bcl-2 and BclXL).[204] In tumorogenesis, dysregulation of apoptosis and disturbances of cell cycle regulation is considered necessary for tumor progression.[205] Mutation of the *P53* gene, coding for a key pro-apoptotic protein and important for the induction of apoptosis in response to DNA damage, is common in cancer and often associated with poor prognosis and poor response to chemotherapy.[206] Over expression of the anti-apoptotic protein Bcl-2 is also well known in several tumor types.[207] Apoptosis in the pathogenesis of MDS is discussed in section 2.1.4.

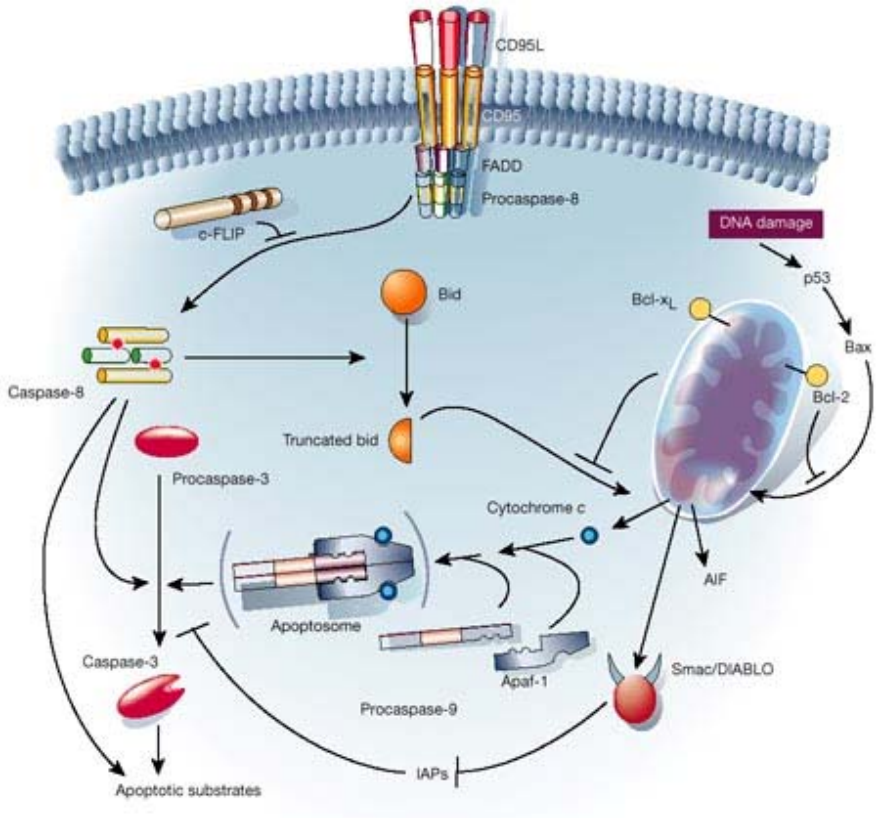

Figure 5: *Apoptotic pathways. Extrinsic pathway – Death receptor in cell membrane. Intrinsic pathway - Mitochondria (Hengartner, Nature 2000)*[204]

3 AIM OF THE THESIS

The aim of this thesis was to elucidate the role of DNA methylation in high-risk MDS and also in AML, thereby improving the possibility to better patients for the optimal therapy, and evaluate mechanisms and utility of the hypomethylating drug azacitidine. Specific aims were:

I. To investigate the correlation between promoter methylation and the outcome of induction therapy in patients with high-risk MDS and MDS-AML.

II. To evaluate the use of azacitidine as maintenance therapy for patients in CR after induction chemotherapy with the hypothesis that it could prolong the otherwise short CR duration.

III. To study the prognostic impact of promoter methylation as well as global DNA methylation in a *de novo* AML population and compare these results to the pattern observed in high-risk MDS and MDS-AML.

IV. To assess the mechanisms of action of azacitidine in primary MDS and normal bone marrow progenitors.

4 MATERIAL AND METHODS

4.1 Patients

4.1.1 *Paper I and II*

Sixty consecutive patients with intermediate-2 and high-risk MDS, CMML with >10% blasts, and AML following an established MDS phase were eligible for the protocol. Patients should be deemed fit for at least one course of standard induction chemotherapy and were excluded if they were considered candidates for potentially curative regimens, including AML-like induction chemotherapy followed by intensive consolidation chemotherapy or allogeneic stem cell transplantation. Hence, the study was designed mainly for older patients or patients with multiple risk factors. Initial diagnosis as well as verification of CR was assessed at the central hematopathology unit at Karolinska University Hospital. For diagnosis, the WHO 2001 criteria were applied. Cytogenetic analyzes were done locally at each center by standard techniques and the findings were classified according to the IPSS into good, intermediate or poor prognostic karyotypes.

4.1.2 *Paper III*

The study population consisted of two cohorts. 1. *De novo* AML; a total of 107 previously untreated AML patients diagnosed at the Karolinska University Hospital at Huddinge were included in the study. Patients with APL or a previous history of MDS were excluded. 2. A cohort of 60 patients with Intermediate-2 or high-risk MDS, or AML following MDS, who were included in the above-described clinical study. For diagnosis and classification of CR and progression in the AML cohort, WHO criteria were applied. Chromosome banding and mutation analyzes of *FLT3*, *NMP1*, *CEBA* and *NRAS* were performed using standard techniques.

4.1.3 *Paper IV*

For this *in vitro* study, we used bone marrow samples from patients with high-risk MDS or CMML-2 and from healthy controls. Patients were previously untreated with chemotherapy and hypomethylating agents and were sampled at the time of diagnosis or at progression from low-risk to high-risk MDS. All patients and normal controls gave their informed consent before sampling.

4.2 Study design and treatment

4.2.1 *Paper I and II*

Induction chemotherapy consisted of a standard DA 2+7 regimen: daunorubicin 60 mg/m^2 days 1 and 2 and cytarabine 150 mg/m^2 days 1 to 7. Patients that did not respond to the first course were given a second induction course if judged medically appropriate. Criteria for CR were <5% bone marrow blasts, stable hemoglobin >100 g/l, WBC >1.5x10^9/l with normal differential count, and platelets >100x10^9/l.

Patients achieving CR were treated with azacitidine 60 mg/m^2 s.c. 5/28 days until disease progression or unacceptable toxicity. The initial dose was 75 mg/m^2 but the protocol was amended due to several observations of grade IV neutropenia in the first 5 enrolled patients. Further reduction of the azacitidine dose was also allowed to avoid hospitalization and severe cytopenias.

Bone marrow for methylation analysis was sampled before induction chemotherapy, at CR and then 6, 12 and 24 months after CR.

4.2.2 *Paper III*

This was a retrospective study on two cohorts of patients. Treatment of the MDS cohort is described in 4.2.1. For the *de novo* AML cohort, induction chemotherapy including cytarabine and an anthracycline, was given according to national guidelines for acute myeloid leukemia. Patients who achieved CR were given standard consolidation courses according to national guidelines or study protocol, or underwent allogeneic stem cell transplantation. The bone marrow samples used in this study were obtained at diagnosis, prior to the first induction course.

4.3 Cell sampling and cell sorting (Paper I-IV)

Bone marrow was obtained from patients and normal controls. Mono-nuclear cells (MNC) were separated by density gradient technique through Lymphoprep. When the yield of MNCs was sufficient, CD34+ cells were purified by magnetic cell sorting using the CD34 Progenitor Isolation Kit according to the manufacturer's guidelines. For paper IV MNCs or CD34+ cells were used directly for suspension cultures whereas for paper I-III cells were stored as pellet in -80°C. For the Nordic multicenter study (paper I and II), bone marrow was shipped to a central laboratory (DHL <24 hours service) in order to avoid the effect of local separation techniques and storage factors. Cell line experiments were done on the P39 myeloid cell line that was originally derived from a patient with CMML.

4.4Suspension cultures (Paper IV)

Cells were plated at a concentration of $1x10^6$ cells/ml (P39 experiments $0.25x10^6$/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 to cultures of primary cells. Cells were cultured for 24 hours at 37°C and in 5% CO_2, prior to adding azacitidine. Azacitidine was diluted in sterile H_2O, filtered through 0.22 µm plastic syringe filter, and added to the cultures in final concentrations from 0.05 to 10 µM. Cells were incubated for 24, 48 and in some cases 72 hours and cell count and viability was checked with Trypan blue. Cells were harvested and analyzed for viability, cell count, apoptosis and DNA methylation. Gene expression profiling was done on cells cultured for 24 hours with and without azacitidine 0.5 µM.

4.5 Colony assays (Paper IV)

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 (1 ml/dish) on small Petri dishes. Dishes were incubated at 37°C and 5% CO_2 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.

4.6 DNA extraction (Paper I-IV)

Genomic DNA was extracted from MNC and CD34+ cells using QiAmp DNA mini kit (paper I and II) or Gene Elute Genomic DNA extraction kit (paper III and IV) according to manufacturer's guidelines. The amount of DNA was measured by spectrophotometry and DNA was stored at -20°C.

4.7 RNA extraction (Paper IV)

RNA for gene expression profiling was extracted from around 0.5 x 10^6 cells using the RNeasy plus mini kit according to the manufacturer's guidelines. The final elution step was repeated twice with 30 and 20 µl respectively of the elution buffer. The RNA was stored in the elution buffer at -80°C.

4.8 Bisulfite modification (Paper I-IV)

During bisulfite modification of DNA cytosine residues are deaminated to uracil but methylated cytosine remains unchanged. This will give methylated and unmethylated DNA different properties, which can be assessed by

different methylation assays (see below). For paper I-III this was done manually as previously described.[208] In brief 2.5 µg of DNA were denaturated in NaOH for 15 minutes at 37°C followed by the incubation in sodium bisulfite at 55°C for 16 hours. Thereafter, DNA was recovered using the GeneClean II kit, desulfonated in NaOH and precipitated in ethanol. For paper IV bisulfite modification of DNA was done using the EZ methylation gold kit according to the manufacturer's instructions.

4.9 Polymerase chain reaction (PCR) (Paper I-III)

PCR specific for bisulfite-reacted *P15*, *CDH* and *HIC* promoters was carried out in a final volume of 25 µl containing 50 to 100 ng of bisulfite modified DNA, 1x TEMPase PCR buffer I with 1.5 mM $MgCl_2$, 0.2 mM cresol red and 12% sucrose, 0.2 mM dNTP, 0.5 µM each forward and reverse primers,[137] and 0.75 units of TEMPase HotStart DNA polymerase. The polymerase was activated by incubation at 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 55°C (*P15* and *HIC*) or 48°C (*CDH*) for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. PCR was performed in a PX2 Thermal cycler and PCR products were examined by electrophoresis in a 2.5% agarose gel.

4.10 Promoter methylation by DGGE (Paper I-III)

In the denaturing gradient gel electrophoresis (DGGE) methylated and unmethylated DNA can be separated on a denaturing gel due to their different denaturing properties from the bisulfite treatment described above.[209] 15-20 µl of the PCR product (see 4.8) were loaded to a 10% denaturant/ 6% polyacrylamide - 70% denaturant/ 12% polyacrylamide double gradient gel. A fully methylated control and an unmethylated control were also loaded to each gel. Gels were run for 270 minutes in 1x Tris acetate/ EDTA buffer kept at a constant temperature of 58°C for *P15* or 55°C for *CDH* and *HIC*. Gels were then stained in 1x Tris acetate/ EDTA buffer containing ethidium bromide (2 µg/ml) and photographed under UV illumination. Methylated DNA denaturate later during the electrophoresis. Samples where bands were seen under the level of the unmethylated control were classified as methylated.

4.11 Promoter methylation analysis by melting curve analysis (Paper III)

Methylation specific melting curve analysis was performed for *P15*, *CDH* and *HIC* using the same PCR product as for DGGE. After bisulfite modification of DNA (see 4.7), methylated and unmethylated DNA will have different melting temperatures depending on their difference in CG content. We performed the melting curve analysis as previously described by Guldberg et al.[210]

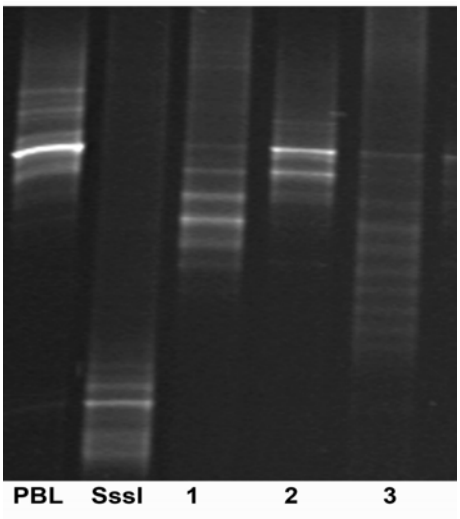

Figure 6: *Example of DGGE results. PBL: Unmethylated control/ DNA from peripheral blood lymphocytes from healthy controls. SssI: Positive control/ in vitro methylated. 1, 2 and 3: Patient samples. Patient 1 methylated. Patient 2 unmethylated. Patient 3 methylated.*

4.12 Promoter methylation analysis by pyrosequencing (Paper III and IV)

Pyrosequencing technology was applied to analyze the methylation levels at specific CpG-sites in *p15*^{ink4b} and *CDH* promoters (Paper III and IV) and LINE-1 repetitive elements (Paper IV). Primer sequences for *P15* and *CDH* are available in pyrosequencing assay data base (http://techsupport.pyrosequencing.com). LINE-1 elements methylation was analyzed as previously described.[211] HotStar Taq polymerase and HotStar Taq master mix kit were used to amplify 1.5 µl of bisulfite treated DNA. Cycling conditions were: 95°C for 15 min, 40 x (95°C for 20 s, 53°C for 20 s and 72°C for 20 s), 72°C for 10 min and then 4°C. Epi Tect PCR control set was used as 100% methylated, 50% methylated and unmethylated controls for the reactions. More than 10% change in the mean degree of methylation of the examined CpG-sites, treated samples vs. untreated control, was considered physiologically significant.

4.13 Global methylation by LUMA (Paper III and IV)

Luminometic Methylation Assay (LUMA) was used to measure global DNA methylation.[212] In brief, 500 ng of genomic DNA was incubated in parallel reactions with a pair of enzymes with endonuclease activity (recognition sequence CCGG). One (*Hpa*II) is unable to cut DNA if the cytosine is methylated (C_{met}CGG) whereas the other (*Msp*I) cuts both methylated and unmethylated DNA. Both *Msp*I and *Hpa*II leave 5' CG-overhangs after cleavage, which are filled in a polymerase extension assay during stepwise addition of dCTP and dGTP where inorganic pyrophosphate is released and converted to ATP. ATP is then used by luciferase to convert luciferin to oxyluciferin, a step where a small amount of visible light, proportional to the ATP produced, can be detected and measured. A ratio between the results from the *Hpa*II and *Msp*I reaction is then calculated. If DNA is completely unmethylated the *Hpa*II/*Msp*I ratio would be 1.0 and for 100% methylated DNA the ratio would approach 0. The assay was carried out

in duplicates. More than 5% change in the *Hpa*II/*Msp*I ratio, treated samples compared to un-treated controls, was considered a physiologically significant alteration.

4.14 Whole genome "promoter" methylation by Illumina (Paper III)

Bisulfite modified DNA from a total of 20 samples was evaluated for genome wide promoter methylation using the Illumina Infinitum HumanMethylation27 bead array. This analysis was made at the core facility of the Karolinska Institute as previously described.[213] This detects the methylation level of 27.578 individual CpG sites spread across the promoter regions of 14.495 genes.

4.15 FISH (Paper IV)

Fluorescence in situ hybridization (FISH) is widely used to detect genetic aberrations with specific DNA probes both for whole chromosomes and short sequences. Cells from azacitidine treated and untreated cultures (see 4.3) were collected and spread onto slides by cyto-centrifugation. Probes were selected according to the patient's known specific aberrations at diagnosis, centromer probe CEP8 and 7q31/7 centromer probe. The manufacturer's protocols were used. Signals were counted in 20-200 cells depending on yield from the cultures.

4.16 Gene expression profiling (Paper IV)

RNA quality and concentration were measured using an Agilent 2100 bioanalyzer and Nanodrop ND-1000, respectively. cDNA was generated with the GeneChip Whole Transcript (WT) cDNA Synthesis and Amplification kit (Affymetrix) using 300 ng total RNA and labeled using the GeneChip WT Terminal Labeling Kit (Affymetrix) according to the manufacturer's specifications. Labeled cRNA was hybridized to oligonucleotide probes on a GeneChip Human Gene 1.0 ST array (Affymetrix) containing 764,885 probes representing 28,869 genes, and washed according to manufacturer's guidelines. Image analysis was performed using Affymetrix Command Console and the arrays were scanned using a GeneChip Scanner 3000 (Affymetrix). Pre-processing was performed using Affymetrix Expression Console (Summarization: PLIER, Background correction: PM-GCBG, Normalization: Global Median). The number of highly significant differences between untreated and azacitidine treated MDS were too low to allow for any proper pathway analysis to be done.

4.17 Flow cytometry for apoptosis (Paper IV)

Cells ($0.5x10^6$/ position) from suspension cultures (see 4.3) were harvested at 24 and 48 hours for analysis of the apoptotic cell fraction by FACS. The cells were washed once in PBS and re-suspended in the flourochrome solution (0.1% sodium citrate (wt/v), 0.1% Triton X-100 (v/v) and propidium iodide 50 mg/l in distilled H_2O). Cells were then incubated at 4°C for 1 hour and then analyzed on a FACS Calibur cytometer using CellQuest Pro software. Propidium iodide stains DNA and accordingly cells with low amount of DNA (apoptotic cells) will be weekly stained.[214]

5 RESULTS AND DISCUSSION

5.1 Paper I: Negative effect of DNA hypermethylation on the outcome of intensive chemotherapy for patients with high-risk myelodysplastic syndromes or acute myeloid leukemia following myelodysplastic syndromes

Sixty patients were enrolled in the study. Baseline characteristics of the patients are shown in Table 6. CR was achieved in 24 patients (40%), which is in line with previous studies of induction chemotherapy in similar groups of patients.[99-102] The CR rate was higher in patients with MDS (59%) and CMML (50%) than in patients with MDS-AML (30%). However, these differences in CR rates were not statistically significant (P=0.07). Previous studies have shown that pre-treatment variables indicative of a more proliferative disease are associated with a lower CR rate.[99-102] In this study, a high WBC correlated negatively to CR (P=0.03).

Table 6: *Baseline characteristics of 60 enrolled patients*

	N=60 N (%)	Median (Range)
Sex		
Male	40 (67)	
Female	20 (33)	
Age		68 (54-83)
Diagnosis (WHO)		
MDS	17 (28)	
MDS-AML	37 (62)	
CMML	6 (10)	
Cytogenetic risk group (IPSS)		
Good	25 (42)	
Intermediate	10 (17)	
Poor	17 (28)	
Not done/unsuccessful	8 (13)	
Hemoglobin (g/L)		95 (62-122)
WBC (10^9/L)		4.3 (0.8-90.9)
Platelets (10^9/L)		65 (5-381)
S-LDH (µkat/L)		4.8 (1.9-27)
Bone marrow cellularity (%)		90 (30-100)
Blasts in bone marrow (%)		23 (6-98)
$CD34^+$ in bone marrow (5%)		10 (1-82)

The main focus of this paper was to evaluate the impact of promoter methylation on outcome of induction chemotherapy. Moreover, we wanted to assess the relation between promoter methylation and a set of previously known risk factors in MDS. Bone marrow samples for methylation analyzes of *P15*, *CDH* and *HIC* were obtained from 50 patients. In 23/50 the yield of MNCs was too low to allow for further CD34+ sorting. However, there was a high concordance between methylation results from MNCs and CD34+ cells (P<0.001). Only one patient showed *P15* in MNCs but not in CD34+ cells. Overall, 55% of the patients showed hypermethylated P15, whereas 37% and 22% were hypermethylated at the *CDH* or *HIC* promoters, respectively. These frequencies are comparable to previous methylation results in MDS materials.[137]

Promoter hypermethylation in MDS is reported to increase with disease progression and to be more frequent in higher age groups.[142,151] In our cohort, promoter hypermethylation was more frequent in MDS-AML compared to MDS or CMML (P=0.02). Methylation also increased with increasing bone marrow blasts and CD34+ cells in the bone marrow. (P=0.007 and 0.04). Methylation of all three genes was more common in older patients (P=0.02). Interestingly, there was no difference in methylation status between cytogenetic risk groups (Table 8).

Table 8: *Methylation status in relation to other pre-treatment variables*

	P15 methylated			P15 + one methylated gene			All three genes methylated		
	Yes	**No**	**P**	**Yes**	**No**	**P**	**Yes**	**No**	**P**
Sex (m/f)	*19/7*	*12/9*	*0.36*	*11/4*	*16/12*	*0.34*	*5/1*	*24/15*	*0.40*
Age[1]	*70.3±6.3*	*67.4±5.6*	*0.12*	*71±6*	*68.2±5.6*	*0.07*	*74.2±6.5*	*68.4±5.4*	**0.02**
Diagnosis[2]	*18/8*	*10/11*	*0.15*	*13/2*	*13/15*	**0.02**	*5/1*	*21/18*	*0.22*
Cytogenetics[3]	*10/12*	*5/16*	*0.20*	*6/5*	*7/21*	*0.13*	*1/4*	*13/23*	*0.65*
Hb[1]	*97.3±9.6*	*93.5±16.0*	*0.33*	*96.1±10.7*	*95.9±14.6*	*0.97*	*96.8±9.4*	*95.1±13.3*	*0.76*
WBC[1]	*12.0±13.7*	*11.3±18.2*	*0.89*	*13.1±14.5*	*9.9±16.1*	*0.52*	*12.4±10.9*	*11.8±16.8*	*0.93*
Platelets[1]	*95.3±84.6*	*104.9±105.5*	*0.73*	*75.1±70.8*	*106.8±97.6*	*0.29*	*75.8±50.1*	*98.4±93.0*	*0.57*
LDH[1]	*5.7±4.0*	*6.8±0.5*	*0.50*	*4.8±2.2*	*6.5±5.0*	*0.31*	*5.1±2.7*	*6.5±5.0*	*0.59*
Cellularity (%)[1,4]	*81.0±21.3*	*80.0±24.0*	*0.90*	*75.6±6.0*	*80.0±21.6*	*0.56*	*80.8±30.7*	*80.0±21.7*	*1.00*
Blasts (%)[1,5]	*37.2±28.7*	*27.7±22.5*	*0.24*	*49.0±31.1*	*25.0±20.3*	**0.007**	*49.0±32.2*	*30.8±25.7*	*0.16*
CD34+ (%)[1,6]	*28.0±29.6*	*10.3±7.8*	**0.04**	*33.8±31.7*	*11.9±11.2*	**0.01**	*36.8±33.3*	*19.1±23.3*	*0.16*

[1]Mean ± SD [2]Diagnosis AML vs. not AML [3]Cytogenetic group poor vs. not poor [4]Bone marrow cellularity
[5]Blasts in bone marrow [6]CD34+ phenotyping of bone marrow cells

A key finding of in this paper was that promoter hypermethylation of *CDH* (P=0.008) or *P15* plus at least one other gene (P=0.05) was associated with poor outcome of induction chemotherapy. In fact, no patient with all three genes methylated achieved CR (P=0.03). *P15* methylation status alone showed no impact on outcome (Figure 7). Previous studies have reported that

promoter hypermethylation of e.g. *P15* is associated with poor prognosis.[137,141-143] This study is the first to show a correlation between methylation status and the outcome of induction chemotherapy in patients with high-risk MDS.

When this study was designed in 2002, primary therapy for high-risk and transformed MDS in the Nordic countries was induction chemotherapy or supportive care only. Today, in the light of results showing a survival advantage for patients treated with azacitidine compared to conventional care regimens,[165] the place for induction chemotherapy in high-risk MDS is unclear. Future studies are warranted to evaluate a possible benefit of intensive chemotherapy in subgroups of patients with high-risk MDS, e.g. those with rapidly proliferating disease, in which there may not be enough time to await the effect of hypomethylating drugs. Our results suggest that patients with hypermethylated *CDH* should not be offered intensive chemotherapy. If these results are reproduced in future studies, examination of promoter methylation status could be one way to select patients for optimal therapy. Whether pre-treatment of patients with hypomethylating agents could lead to a better response to conventional chemotherapy would also be worth analyzing in a prospective study.

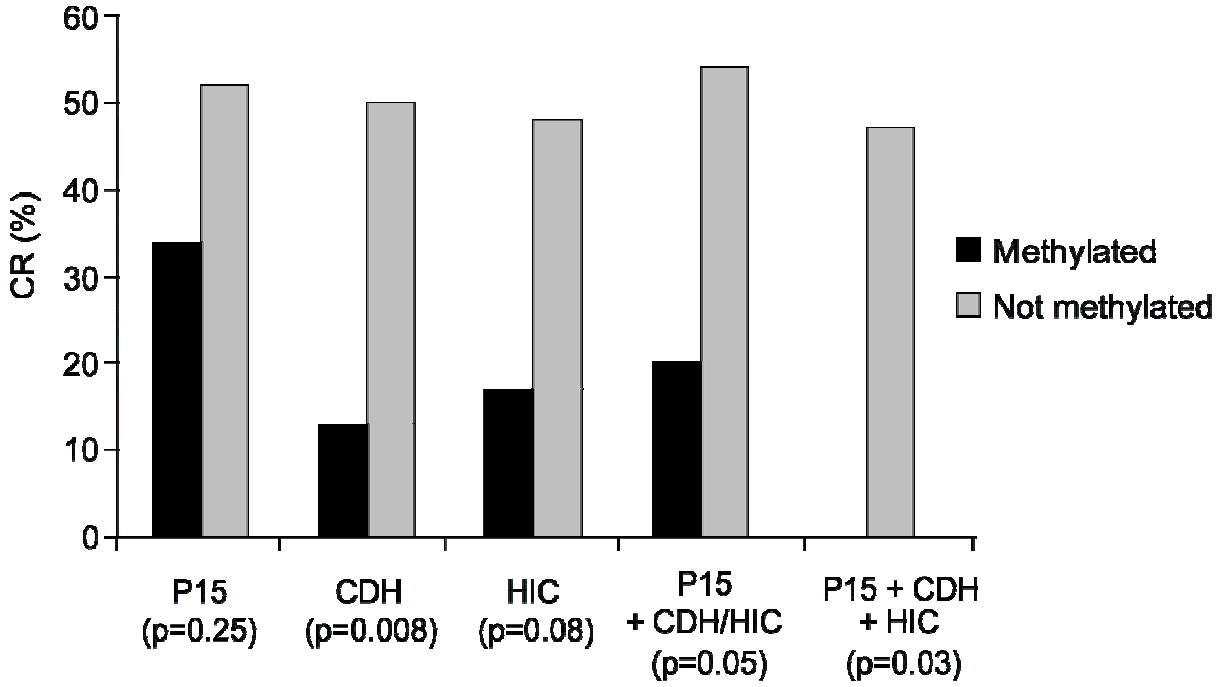

Figure 7: *Percentage of CR in patients with and without methylation of P15, CDH, HIC, P15 plus at least one other gene, or of all three genes*

5.2 Paper II: Maintenance treatment with azacytidine for patients with high-risk myelodysplastic syndromes (MDS) or acute myeloid leukemia following MDS in complete remission after induction chemotherapy

Based on the positive results of azacitidine in high-risk MDS from the study by Silverman et al,[164] we hypothesized that maintenance treatment with azacitidine given to patients with CR after induction chemotherapy could prolong the usually short CR duration. [98-100,102,103] Twenty-four of 60 enrolled patients achieved CR. One patient underwent allogeneic stem cell transplantation and was taken off the study. The remaining 23 patients (median age 70 years) started maintenance therapy (see 4.2.1). Last follow up was at August 1st, 2008, 24 months after the last patient achieving CR. Median follow up time was 20.0 months (4.5-50.3).

This paper was the first to report on feasibility and effects of maintenance therapy with hypomethylating agents after intensive chemotherapy. Azacitidine maintenance was well tolerated and only one patient stopped azacitidine due to an adverse event. The most frequent adverse events were grade 3 or 4 neutropenia or thrombocytopenia, reported at any time point in 43.5% and 30.5% of the patients, respectively. However, dose reduction was allowed and only 22% (grade 3) and 8% (grade 4) of the courses were associated with neutropenia of this magnitude. Thrombocytopenia grade 3 was reported after only 9.5% of the courses and grade 4 thrombocytopenia was not observed. Interestingly, hemoglobin levels rose during the first courses of azacitidine in 16/23 patients. In the whole cohort hemoglobin was significantly higher before cycle 4 than before cycle 1 (P=0.02). Potential explanations include both slow natural recovery after chemotherapy and a direct positive effect of azacitidine on erythropoiesis.

Median CR duration (13.5 months) and median OS (20.0 months) for the 23 patients treated with azacitidine maintenance is not clearly different from previous studies on induction chemotherapy in high-risk MDS or MDS-AML. [98-100,102,103] However, many of these studies included younger patients and two studies on older patients including both de novo AML and MDS-AML, showed OS of only 9 and 10 months respectively.[215,216] Although, the study was not powered for subgroup analysis, no difference in CR duration was seen between diagnostic or cytogenetic subgroups, or in patients with different *P15* methylation status. A low platelet count before induction chemotherapy was associated with shorter CR duration (Figure 8).

As dose reduction of azacitidine was allowed to avoid severe cytopenias and hospitalization, one explanation to lack of clear benefit of azacitidine maintenance could be insufficient dosing. However, there was no difference in CR duration or OS between patients treated with doses above or below the median dose in the study. As few patients with any other promoter than *P15* methylated ever reached CR, another explanation might be that those that could have benefited

the most from the maintenance therapy never reached that stage of the study. Interestingly 4/5 patients with a karyotype including +8 reached CR and all 4 showed CR duration and a survival well above the median for the whole maintenance group. The only two patients who were hypermethylated at the *CDH* promoter and reached CR had very short CR-durations, 4 and 6 months respectively. *CDH* methylation remained a negative prognostic marker for OS in the whole cohort of 60 patients (4 months vs. 9.3 months, P=0.003).

This study shows that azacitidine treatment is feasible following intensive chemotherapy. Whether there are subgroups of patients that can benefit from azacitidine maintenance remains to be further studied.

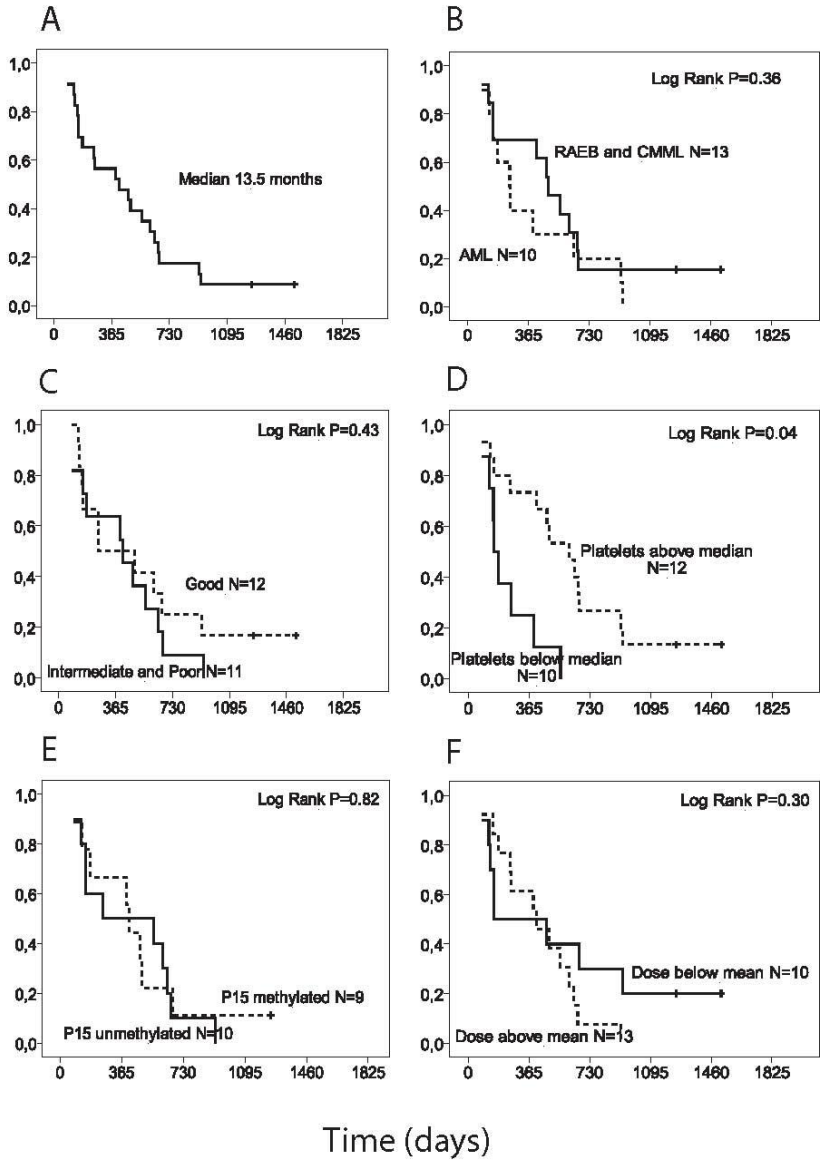

Figure 8: *CR duration in subgroups A) All 23 maintenance patients B) AML vs. RAEB and CMML C) Cytogenetics IPSS Good vs. Intermediate or Poor D) Pre-induction platelets above or below the median E) P15 methylated vs. unmethylated F) Mean dose for the patient above vs. below median for the whole group*

5.3 Paper III: Gene-specific and global methylation patterns predict outcome in patients with acute myeloid leukemia

Many previous studies on AML include both *de novo* AML and AML following MDS. It is know that e.g. certain cytogenetic aberrations have different prognostic implications in *de novo* AML and MDS-AML. Moreover, response to therapy differs between the two diseases. Based on our finding in paper I, we wanted to evaluate the impact of DNA methylation in a cohort of AML excluding MDS-AML.

Ninety-eight patients with *de novo* AML and 9 with AML secondary to previous chemotherapy (for easier legibility referred to as *de novo* AML) were included in this retrospective study. Median age was 65.4 years (23-85). Baseline characteristics are shown in Table 9. CR was achieved in 75% of the patients which is higher than in most studies of induction chemotherapy in high-risk MDS and MDS-AML, including paper I in this thesis (median age 68 years, 54-83) where the CR rate was only 40%. Allogeneic stem cell transplantation was performed in 24 patients while 41 received standard consolidation courses. Sixteen patients had reduced intensity consolidation courses due to treatment toxicity.

Table 9: Base line characteristics of 107 non-MDS AML patients

Patient characteristics	
Age (years)	65 (23-85)[1]
Bone marrow blasts (%)	68 (20-98)[1]
WBC count (x10^9/L)	22 (0-368)[1]
Female/Male	66/41
Cytogenetic risk group (low/intermediate/high)	5/77/25
CR rate	80 (75%)
FABsubtype	
M0	8 (7%)
M1	30 (28%)
M2	30 (28%)
M4	21 (20%)
M5	8 (7%)
M6	2 (2%)
Not defined	8 (7%)
Molecular analysis (number positive/number analyzed)	
FLT3-ITD	29/95
FLT3-TKD mutation	10/94
NPM1 mutation	34/93
FLT3-ITD negative and NPM1 mutated	18/93
CEBPA mutation	7/95
NRAS mutation	4/86

[1]median (range)

The frequency of promoter hyper-methylation, assessed by DGGE, was higher in the *de novo* AML cohort compared to the MDS material, 66% for *P15*, 66% for *CDH* and 51% for *HIC*. However, in the MDS and MDS-AML cohort a higher degree of methylation was seen in the more advanced patients. Hence a higher portion of methylated patients could be expected in the AML cohort. The results from DGGE were validated by melting curve analysis (*P15*, *CDH* and *HIC*) and pyrosequencing (*P15*) in a subset of patients with good concordance, 100% and 79% respectively.

Unlike in the MDS cohort, no association between methylation status of *P15*, *CDH* or *HIC* and response to induction chemotherapy was observed in *de novo* AML. Moreover, patients who were hypermethylated in the *P15* promoter had longer disease free survival (DFS) as well as OS than unmethylated patients. In studies on MDS *P15* methylation status is universally associated with poor prognosis,[137,142,143] while studies on *P15* methylation and prognosis in AML show conflicting results.[146-150] The results in previous AML studies might reflect a varying proportion of patients with MDS-AML in many as well as the use of different methylation assays. *CDH* methylation, which was the strongest negative prognostic marker in the MDS material did not correlate to outcome in the *de novo* AML cohort. Instead, promoter hypermethylation of *P15* as well as increased genome wide promoter methylation (Illumina HumanMethy-lation27 bead chip array) was associated with better DFS and OS in the *de novo* AML material.

Next we performed a comparison of global methylation assessed by LUMA and examination of 27.578 mainly promoter-associated CpG sites by Illumina HumanMethylation27. The analysis confirmed the typical inverse correlation between global methylation and promoter methylation in cancer. Patients with low global methylation by LUMA showed methylation at a significantly larger number of examined CpG sites by Illumina (P=0.003).

Interestingly, CR rates were higher in patients with low global methylation assessed by LUMA (P=0.05). The difference retained significance also in the multivariate analysis, when other known risk factors were entered. However, global methylation had an impact on the outcome of induction chemotherapy only in patients <65 years and this did not translate to a difference in survival. Whether AML patients with high global methylation could benefit from hypomethylating agents before induction chemotherapy remains to be investigated.

Differences in methylation pattern between high-risk MDS/MDS-AML and de novo AML was previously reported by Figueroa et al.[139] We could show that also the prognostic value of methylation patterns differs between the two entities. These findings suggest that MDS-AML and de novo AML should be studied separately, at least in clinical trials of DNA methylation and hypomethylating agents

5.4 Paper IV: Complex effects of azacitidine in primary myelodysplastic and normal bone marrow cell cultures

Understanding the cellular mechanisms of azacitidine is essential for well-designed future studies of combination therapies, and for optimal clinical use of the drug. A variety of mechanisms have been suggested, mostly from cell line experiments or sequential sampling during azacitidine treatment.[154-163] We wanted to assess the effects of azacitidine on primary bone marrow progenitors from patients with high-risk MDS and from normal controls *in vitro*. We exposed MNC and CD34+ progenitors to azacitidine in suspension cultures and evaluated its effects on cell growth, colony formation, apoptosis, methylation, and gene expression.

First we assessed the effect of azacitidine on cell growth and colony formation. Interestingly, azacitidine in doses up to 5 μM did not affect cell growth or viability in the suspension cultures. Actually, the absolute number of cells per ml was higher in azacitidine treated positions than in controls in 1/10 NBM and in 4/10 MDS. Neither was there any inhibitory effect on colony formation after exposure to azacitidine in doses up to 0.5 μM for 24 hours. In fact, the number of colonies (especially erythroid colonies) increased by >70% with the lower doses of azacitidine in 5/9 evaluable MDS and 5/13 NBM. This is in line with our findings in paper II where the hemoglobin level during azacitidine treatment rose in a majority of the patients, whereas thrombo-cytopenia and neutropenia was common. It may suggest a direct stimulatory effect of azacitidine on erythropoiesis.[185] The seemingly un-toxic effect of the drug on normal bone marrow progenitors is a useful finding since it may support the use of the drug for patients with low-risk MDS and as maintenance after allogeneic stem cell transplantation.[177,179]

Azacitidine did induce apoptosis also in our experiments on primary bone marrow progenitors, but to a much lesser degree than in previously reported cell line experiments from our group (Figure 9).[156] The difference in apoptosis between control and azacitidine treated positions was not significant for MDS progenitors, which may partly be explained by the well-described spontaneous apoptosis in these cultures.[217]

Also the hypomethylating effect was less pronounced in MDS cultures than previously reported in cell line experiments. Promoter methylation for *P15* and *CDH* was not affected by incubation with azacitidine. Global methylation assessed by LUMA showed no consistent pattern in samples from 5 NBM and only a slight decrease after azacitidine treatment in cells from 5 MDS patients (P=0.19). Assessment of global methylation by pyrosequencing for LINE-1 repetitive elements showed a significant decrease in methylation after incubation with 1 μM azacitidine for 48 h in samples from NBM as well as MDS (P=0.001 and 0.02, respectively). No further hypomethylating effect was observed with higher dose (2 μM).

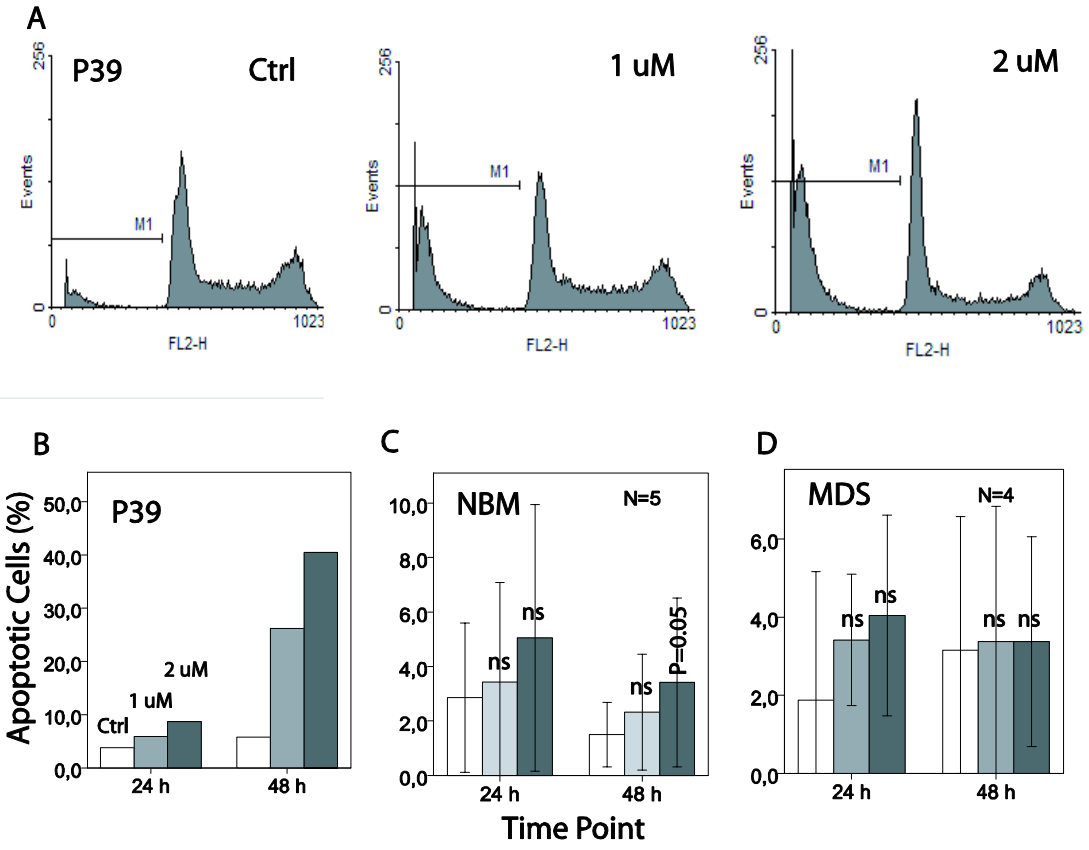

Figure 9: Apoptotic effects of azacitidine:
A) P39 cell line, 48 hours. Histograms showing increasing apoptotic proportion with increasing dose of azacitidine
B) Percentage of apoptotic cells in P39 cell line with and without azacitidine
C) Percentage of apoptotic cells in normal controls with and without azacitidine
D) Percentages of apoptotic cells in MDS with and without azacitidine.

We found marked differences in the effect of azacitidine on gene expression between NBM and MDS. Only 100/3,174 (3%) genes that were deregulated by azacitidine in MDS were up-regulated. In NBM however, the vast majority of deregulated genes (1,114/1,396; 81%) were down-regulated. In this material the number of significant differences in gene expression between untreated and azacitidine treated MDS samples was too low to allow for pathway analysis. However, we particularly looked for genes involved in the regulation of hematopoiesis, cell cycle control, differentiation and apoptosis, where there was a baseline difference in gene expression between untreated NBM and MDS and where azacitidine treatment of MDS cells restored expression towards the level in NBM. We found three genes involved in the regulation of the P53 pathway, *ATM, MDM2* and *TP53BP1.* Interestingly none of these showed promoter hypermethylation before azacitidine incubation, indicating that up-regulation was promoted by alternative mechanisms. Also others have reported

a lack of correlation between changes in gene expression level after azacitidine and promoter methylation patterns pre-treatment.[159]

Our results, together with recent data from other groups, suggest that the mechanisms of azacitidine are complex, that they involve more than one cellular function and that they possibly are not related only to the DNA hypo-methylating effects. Alternative mechanisms include histone modifications and inhibition of RNA methylation.[159,160]

6 FUTURE PERSPECTIVES

Until quite recently the therapeutic options for patients with myelodysplastic syndromes, especially those with high-risk disease were limited and prognosis was dismal. In the last decade both hypomethylating agents and lenalidomide have shown substantial positive effects and are used in routine clinical practice. Histone deacetylation inhibitors and thrombopoietin agonists are being evaluated in clinical trials with promising results. The exact mechanisms of action of these drugs are still not known, and are most probably complex. However, for hypomethylating agents and histone deacetylation inhibitors it is probable that they involve epigenetic modification. Future studies are warranted to elucidate their way of action in order to use the drugs in an optimal way in combination therapies and to be able to better select patients for the optimal treatment.

Epigenetic changes in the MDS and AML stem cell will probably in the future be useful for prognostication and possibly also for the selection of therapy. However, standardization of techniques as well as a wider investigation of the patterns of epigenetic aberrations and their association with e.g. prognosis is necessary before this becomes a reality in the clinic.

7 CONCLUSIONS

Based on these studies it can be concluded that:

- Hypermethylation of the *E-cadherin* promoter is associated with poor outcome of induction chemotherapy in high-risk myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) following MDS (MDS-AML). This finding, if reproduced in future studies, may have significant impact on study design as well as clinical management of patients with MDS.

- Maintenance therapy with azacitidine after induction chemotherapy is feasible and has no negative effects on hemoglobin levels.

- There is no clear effect of azacitidine maintenance after induction chemotherapy on duration of complete remission or overall survival, compared to historical materials. Some findings suggest a possible benefit in subgroups such as those with trisomy 8.

- In *de novo* AML, hypermethylation of the $P15^{ink4b}$ promoter, as well as genome wide promoter hypermethylation, is associated with better disease-free survival and overall survival. However, global hypomethylation is associated with low complete remission rates.

- Methylation patterns are of prognostic importance in both high-risk MDS/MDS-AML and in *de novo* AML, but their prognostic value is completely different. This suggests that high-risk MDS/MDS-AML and *de novo* AML should be studied separately.

- Azacitidine, also in high doses, has limited toxicity on normal bone marrow progenitors. In fact, clonogenic assays suggest a possible direct stimulatory effect of azacitidine on bone marrow progenitors. This supports the use of azacitidine maintenance after allogeneic stem cell transplantation to reduce the risk of relapse.

- The intracellular effects of azacitidine are complex. Compared to cell line experiments, the drug induces less apoptosis and hypomethylation in primary MDS progenitors. Its effect on gene expression is strikingly different in MDS compared to normal bone marrow progenitors.

8 SAMMANFATTNING PÅ ENKEL SVENSKA

Myelodysplastiskt syndrom (MDS) är en grupp av elakartade tumörsjukdomar som utgår från de blodbildande stamcellerna i benmärgen. MDS drabbar mellan 4 och 10 av 100 000 invånare per år. Förekomsten ökar med stigande ålder. Medelåldern vid diagnos är ca 75 år och MDS är relativt ovanligt före 50 års ålder. Symtomen vid MDS är relaterade till brist på en eller flera typer av blodkroppar. Merparten av patienterna har uttalad brist på röda blodkroppar och det ger symtom som t.ex. trötthet, dålig kondition eller yrsel. Brist på blodplättar kan ge blödningsbesvär och brist på vita blodkroppar ger ökad risk för infektioner. Sjukdomsbilden varierar från långsamt tilltagande brist på blodkroppar och så småningom behov av blodtransfusioner (lågrisk-MDS) till snabbt förlöpande sjukdom med övergång till akut myeloisk leukemi (AML) och död (högrisk-MDS).

För att en normal cell ska övergå till att bli en cancercell krävs flera förändringar inne i cellen. Exakt vad som orsakar MDS är inte känt. En orsak kan vara uppkomst av fel på de blodbildande cellernas arvsmassa (genetiska fel). Detta yttrar sig bl.a. i kromosomavvikelser, t.ex. förlust av delar av eller hela kromosomer. Kromosomer finns i cellkärnan och är uppbyggda av tätt packat DNA, vår arvsmassa. Epigenetiska förändringar i cellen bidrar också. Detta innebär att själva DNA-sekvensen är oförändrad men uttrycket av olika gener ändå är ändrat. Detta kanske t.ex. genom att ökad mängd metylgrupper (små kemiska strukturer innehållande en kolatom och tre väteatomer) har kopplas till DNA i den del av en gen som reglerar om genen är aktiv eller inte, vilket stänger av genen (promotor hypermetylering). Aktiviteten av en gens uttryck kan också regleras av att DNA i kromosomen är olika hårt packat kring s.k. histonproteiner (histonmodifiering).

För patienter med högrisk-MDS och de med tidigare MDS som övergått till AML (MDS-AML) har det tills nyligen inte funnits några riktigt bra behandlingsalternativ eftersom de flesta patienter är för gamla för att tolerera höga doser av cellgifter eller allogen stamcellstransplantation, den enda potentiellt botande behandlingen vid MDS. Måttliga doser cellgifter kan tolereras relativt högt upp i åldrarna och har i flera studier visats kunna trycka tillbaka MDS-sjukdomen hos ca 50 % av patienter med högrisk-MDS. Problemet i samtliga dessa studier har dock varit att sjukdomen kommer snabbt tillbaka, oftast inom ett år. Någon effekt på överlevnad har inte kunnat visas på denna typ av behandling.

Efter att stora studier visat god effekt, relativt lite biverkningar och förlängd överlevnad vid behandling av patienter med högrisk-MDS med läkemedlet azacytidin, blev detta läkemedel godkänt i USA 2004 och i Europa i början av 2009. Detta läkemedel anses verka på epigenetisk nivå. Dess exakta verkningsmekanism är inte känd men man vet

att läkemedlet blockerar aktiviteten hos DNA metyltransferaser, enzymer som kopplar metylgrupper till DNA, och därigenom ger minskad DNA-metylering. En möjlig verkningsmekanism som föreslagits är att azacytidin tar bort felaktigt påkopplade metylgrupper från DNA och därigenom slår på felaktigt avstängda gener.

I det första arbetet (Paper I) ville vi studera hur metyleringsmönstret av tre gener (P15, E-cadherin och HIC) relaterade till effekt av cellgiftsbehandling av patienter med högrisk-MDS eller MDS-AML. 60 patienter inkluderades i studien och behandlades med cellgifter. Vi tog prover från benmärgen före och efter behandlingen, dels för att kontrollera behandlingseffekt och dels för att studera metyleringsmönstret för de tre ovanstående generna. 24 patienter (40%) svarade på cellgifterna, d.v.s. man kunde inte längre se sjukdomen i benmärgen. Det viktigaste fyndet i den här studien var att patienter som hade ökad metylering av E-cadherin eller av mer än en av ovanstående gener hade mycket liten chans att svara på cellgiftsbehandlingen. Ingen av patienterna som hade ökad metylering på alla tre generna svarade. Denna studie var den första där ett sådant samband kunde visas. Detta innebär att man i framtiden möjligen bör kontrollera metyleringsmönstret hos patienter där man överväger cellgiftsbehandling och inte utsätta patienter med ökad metylering för sådan tuff behandling då deras chans att svara ändå är mycket liten.

I det andra arbetet (Paper II) studerade vi effekten av att ge azacytidin till 23 patienter från ”Paper I” som svarat på cellgiftsbehandling med förhoppning att kunna förlänga den annars korta tiden till återfall. Denna studie var den första där azacytidin gavs på detta sätt. Behandlingen tolererades väl men medeltiden till återfall (13,5 månader) och medelöverlevnaden (20,0 månader) var tyvärr inte tydligt längre än i tidigare studier av cellgiftsbehandling utan efterföljande azacytidin i denna typ av patienter. En bidragande orsak kan möjligen vara att patienterna i vår studie var något äldre (medel 68 år) än i de flesta tidigare studier. I vissa undergrupper, t.ex. de med 3 kopior av kromosom 8, sågs långvariga behandlingssvar men antalet sådana patienter var för få för att kunna dra säkra slutsatser.

Efter att ha sett koppling mellan DNA-metyleringsmönster och effekt av cellgiftsbehandling i delarbete I ville vi studera om detta gällde också för patienter med akut leukemi utan tidigare blodsjukdom (de novo AML). I det tredje arbetet studerade vi metyleringsmönstret hos 107 sådana patienter. Till skillnad från i arbete I på MDS/MDS-AML såg vi ingen koppling mellan metylering av E-cadherin och prognos hos patienter med de novo AML. Däremot hade patienter med ökad metylering av P15 bättre överlevnad. Ökad metylering av denna gen har i flera studier på MDS visat sig vara associerat med sämre prognos. Vi tittade också på metyleringsnivå över hela DNA, dvs. inte bara enstaka gener, och såg att patienter med låg grad av metylering svarade sämre på cellgiftsbehandling. Metyleringsmönster är således kopplat till prognos både vid högrisk-MDS/MDS-AML och vid de novo AML men betydelsen av förändringarna skiljer sig. I många studier på AML blandar man MDS-AML och de novo AML. Våra resultat pekar mot att de båda grupperna bör studeras separat.

Målet med det sista arbetet (Paper IV) var att studera hur azacytidin påverkade benmärgsceller från patienter med högrisk-MDS och från friska individer för att på så sätt få bättre kunskap angående läkemedlets verkningsmekanismer. Vi odlade celler i laboratoriet och tillsatte azacytidin till flaskorna. Vi jämförde sedan obehandlade med azacytidinebehandlade benmärgsceller, både från MDS patienter och friska kontroller. Azacytidin visade sig ha förvånansvärt liten effekt på tillväxten av både MDS-celler och normala celler i odlingarna. När vi kontrollerade cellernas förmåga att bilda kolonier på odlingsplattor efter azacytidin-behandling sågs snarast ökad sådan förmåga. Att läkemedlet verkar orsaka ganska liten skada på normala celler är viktig kunskap när man nu börjat använda azacytidin som behandling efter allogen stamcellstransplantation i syfte att minska risken för eller behandla återfall, en situation där man vill orsaka så liten skada som möjligt på de donerade friska cellerna. Vi såg viss generell minskning av DNA-metylering men den genspecifika metyleringen förblev oförändrad. Hur azacytidine påverkade uttrycket av 28 869 studerade gener varierade stort mellan MDS och normala celler. För ett fåtal särskilt intressanta gener där uttrycket ändrades av azacytidinbehandling kontrollerade vi också metylering. Samtliga var dock ometylerade redan innan azacytidinbehandlingen. Slutsatsen blir att verkningsmekanismen för azacytidin sannolikt är komplex och inte enbart relaterad till DNA-metylering, utan också förändrar uttryck av gener genom andra mekansimer.

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