General introduction and scope of this thesis
Chapter 1

GENERAL INTRODUCTION

Myelodysplastic syndromes

Myelodysplastic syndromes (MDS) are a heterogeneous group of malignant (oligo)clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis and dysplasia in one or more lineages. MDS predominantly affect older individuals with a median age at diagnosis of approximately 75 years and an estimated incidence of 75 per 100,000 persons aged ≥ 65 years.\(^1,2\) Patients suffer from varying degrees of anemia, neutropenia, and/or thrombocytopenia. Varying amounts of immature myeloid blasts can be present up till 20% of all nucleated bone marrow cells or peripheral blood leukocytes. Somewhat arbitrary, myeloid blast percentages of 20% and higher are defined as acute myeloid leukemia (AML) according to the criteria of the World Health Organization (WHO) in 2008, whereas previously patients with 20-30% blasts were considered to have MDS by French-American-British (FAB) criteria.\(^3,4\) MDS patients have on average a probability of about 30% of transformation to AML and therefore MDS is often considered as a pre-leukemic disease. However, most MDS patients die from the consequences of bone marrow failure rather than from AML.

MDS are sub classified according to the WHO-2008 classification based on the blast count in the bone marrow and peripheral blood, the type and number of cell lineages with dysplasia, the presence of ring sideroblasts, auer rods, deletion of the long arm of chromosome 5 (del(5q)), or defined cytogenetic abnormalities in case of absent dysplasia (MDS-U).\(^3\) The prognosis of MDS is highly heterogeneous and is not well predicted by the diagnostic subclass alone. Therefore, several prognostic scoring systems have been developed. The most commonly used prognostic system is the International Prognostic Scoring System (IPSS), which has recently been revised in the IPSS-R (Table 1A).\(^5,6\) Both scoring systems are based on cytogenetics, bone marrow blasts percentage, and cytopenias. By using the IPSS and the IPSS-R a distinction can be made between lower-risk MDS and higher-risk MDS, which is widely used for guidance in treatment decisions. Median survival rates of the natural course of the disease vary from 8.8 years in the very low IPSS-R risk group to 0.8 years in very high risk MDS (Table 1B).\(^6\) Many other factors associated with poor survival have been recognized, including older age, performance status, red blood cell transfusion dependency, bone marrow fibrosis, previous exposure to chemotherapy, mutations in \(TP53, EZH2, ETV6, RUNX1\) and \(ASXL1\), and DNA hypermethylation.\(^7-12\) Especially the insights in recurrent gene mutations and epigenetic alterations in MDS are rapidly evolving and change our view on diagnosis and prognosis. Overall, the prognosis of high-risk MDS has improved over the last 30 years.\(^13\) This improvement seems largely to be the result of improved supportive care, including erythropoiesis stimulating agents and granulocyte-colony stimulating factor (G-CSF), since especially the number of deaths due to infection or bleeding was reduced.\(^13,14\)
Table 1a. Revised International Prognostic Scoring System (IPSS-R) for myelodysplastic syndromes and clinical outcome

<table>
<thead>
<tr>
<th>Prognostic variable</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytogenetics (Table 1b)</td>
<td>Very good</td>
<td>—</td>
<td>Good</td>
<td>—</td>
<td>Intermediate</td>
<td>Poor</td>
<td>Very poor</td>
</tr>
<tr>
<td>BM blast, %</td>
<td>≤ 2</td>
<td>—</td>
<td>&gt; 2% - &lt; 5%</td>
<td>—</td>
<td>5%-10%</td>
<td>&gt; 10%</td>
<td>—</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>≥ 10</td>
<td>—</td>
<td>8 -&lt; 10</td>
<td>&lt; 8</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Platelets, x 10^9/L</td>
<td>≥ 100</td>
<td>50&lt;100</td>
<td>&lt; 50</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ANC, x 10^9/L</td>
<td>≥ 0.8</td>
<td>&lt; 0.8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 1b. Cytogenetic score for myelodysplastic syndromes in the IPSS-R

<table>
<thead>
<tr>
<th>Prognostic subgroups</th>
<th>Cytogenetic abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very good</td>
<td>-Y, del(11q)</td>
</tr>
<tr>
<td>Good</td>
<td>Normal, del(5q), del(12p), del(20q), double anomalies including del(5q)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>del(7q), +8, +19, i(17q), any other single or double independent clones</td>
</tr>
<tr>
<td>Poor</td>
<td>-7, inv(3)/t(3q)/del(3q), double abnormalities including -7/del(7q), complex: 3 abnormalities</td>
</tr>
<tr>
<td>Very poor</td>
<td>Complex: &gt; 3 abnormalities</td>
</tr>
</tbody>
</table>

Del, deletion; i, isochromosome; inv, inversion; t, translocation.

Acute myeloid leukemia

AML is a malignant disorder of hematopoietic stem- and progenitor cells that is characterized by the (oligo)clonal expansion of myeloid blasts in bone marrow, blood, and other tissues (e.g. skin, gingiva), and a block in differentiation leading to cytopenias. AML is a heterogeneous disease that is classified according to the WHO-2008 classification. This classification incorporates the impact of genetic abnormalities in addition to morphologic features and previous exposure to radiotherapy or chemotherapy (Table 2). Although children can also be affected, AML is primarily a disease of older individuals with a median age at diagnosis of 65-70 years and an estimated
Table 2. WHO-2008 classification of acute myeloid leukemia

<table>
<thead>
<tr>
<th>AML with recurrent genetic abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML with t<a href="q22;q22">8;21</a>; RUNX1-RUNX1T1</td>
</tr>
<tr>
<td>AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11</td>
</tr>
<tr>
<td>APL with t(15;17)(q22;q12); PML-RARA</td>
</tr>
<tr>
<td>AML with t(9;11)(p22;q23); MLLT3-MLL</td>
</tr>
<tr>
<td>AML with t(6;9)(p23;q34); DEK-NUP214</td>
</tr>
<tr>
<td>AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1</td>
</tr>
<tr>
<td>AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1</td>
</tr>
<tr>
<td>Provisional entity*: AML with mutated NPM1</td>
</tr>
<tr>
<td>Provisional entity*: AML with mutated CEBPA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AML with myelodysplasia-related changes</th>
</tr>
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<tbody>
<tr>
<td>Therapy-related myeloid neoplasms</td>
</tr>
<tr>
<td>Acute myeloid leukemia, not otherwise specified</td>
</tr>
<tr>
<td>AML with minimal differentiation</td>
</tr>
<tr>
<td>AML without maturation</td>
</tr>
<tr>
<td>AML with maturation</td>
</tr>
<tr>
<td>Acute myelomonocytic leukemia</td>
</tr>
<tr>
<td>Acute monoblastic/monocytic leukemia</td>
</tr>
<tr>
<td>Acute erythroid leukemia</td>
</tr>
<tr>
<td>Acute megakaryoblastic leukemia</td>
</tr>
<tr>
<td>Acute basophilic leukemia</td>
</tr>
<tr>
<td>Acute panmyelosis with myelofibrosis</td>
</tr>
</tbody>
</table>

* Newly described entity at the time of WHO-2008 criteria that should be considered in the classification. AML, acute myeloid leukemia; t, translocation; inv, inversion.

incidence of 17 per 100,000 persons aged ≥ 65 years (Figure 1).\(^\text{15,16}\) Older AML patients, for practical purposes usually defined as 60 years and older, generally have a poorer prognosis compared to younger patients, with 5-year survival rates of 8-19% in patients aged 60 years and older versus 33-53% in adults younger than 60 years.\(^\text{17,18}\) This difference in outcome between young and old patients may be explained by the fact that older patients more often have unfavorable disease characteristics in addition to an increased incidence of comorbidities.\(^\text{19}\) The adverse disease characteristics include adverse cytogenetic abnormalities, higher rates of secondary AML, including previous MDS, higher incidence of multidrug resistance and different gene expression profiles.\(^\text{17,20,21}\)

Cytogenetic- and molecular alterations are important predictors for the response to therapy. Our understanding about these genetic features is rapidly evolving. The European LeukemiaNet (ELN) has proposed a risk stratification that distinguishes four risk groups based on cytogenetic alterations and molecular alterations (Table 3).\(^\text{22,23}\) Median overall survival in patients younger than 60 years was 11.5, 1.2, 2.1, and 0.8 years in favorable, intermediate-I, intermediate-II, and adverse risk groups, respectively.\(^\text{24}\) Median overall survival in patients older than 60 years was considerably shorter with 1.6, 0.9, 0.9, and 0.5 years, respectively in the various risk groups.\(^\text{24}\)
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Figure 1. Incidence of acute myeloid leukemia by age group
Data have been derived from the National Cancer Institute (Surveillance, Epidemiology, and End Results, 2008-2012, http://seer.cancer.gov/).

Table 3. Current stratification of molecular genetic and cytogenetic alterations, according to recommendations of the European LeukemiaNet

<table>
<thead>
<tr>
<th>Risk Profile</th>
<th>Subsets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Favorable</td>
<td>t<a href="q22;q22">8;21</a>; RUNX1-RUNX1T1</td>
</tr>
<tr>
<td></td>
<td>inv(16)[p13.1q22] or t[16;16][p13.1;q22]; CBFB-MYH11</td>
</tr>
<tr>
<td></td>
<td>Mutated NPM1 without FLT3-ITD (normal karyotype)</td>
</tr>
<tr>
<td></td>
<td>Biallelic mutated CEBPA (normal karyotype)</td>
</tr>
<tr>
<td>Intermediate-I†</td>
<td>Mutated NPM1 and FLT3-ITD (normal karyotype)</td>
</tr>
<tr>
<td></td>
<td>Wild-type NPM1 and FLT3-ITD (normal karyotype)</td>
</tr>
<tr>
<td></td>
<td>Wild-type NPM1 without FLT3-ITD (normal karyotype)</td>
</tr>
<tr>
<td>Intermediate-II</td>
<td>t<a href="p22;q23">9;11</a>; MLLT3-KMT2A</td>
</tr>
<tr>
<td></td>
<td>Cytogenetic abnormalities not classified as favorable or adverse</td>
</tr>
<tr>
<td>Adverse</td>
<td>inv(3)[q21q26.2] or t<a href="q21;q26.2">3;3</a>; GATA2–MECOM (EV11)</td>
</tr>
<tr>
<td></td>
<td>t<a href="p23;q34">6;9</a>; DEK-NUP214</td>
</tr>
<tr>
<td></td>
<td>t<a href="v;q23">v;11</a>; KMT2A rearranged</td>
</tr>
<tr>
<td></td>
<td>−5 or del(5q); −7; abn(17p); complex karyotype§</td>
</tr>
</tbody>
</table>

† This category includes all cases of AML with a normal karyotype except for those included in the favorable subgroup; most of these cases are associated with a poor prognosis, but they should be reported separately because of the potential different response to treatment.

§ A complex karyotype is defined as three or more chromosomal abnormalities in the absence of one of the World Health Organization–designated recurring translocations or inversions — t[8;21], inv[16], t[16;16], t[9;11], t[v;11](v;q23), t[6;9], and inv(3)/t[3;3]. About two thirds of patients with AML with a complex karyotype have a mutation of TP53, a deletion of TP53, or both. TP53 alterations in AML rarely occur outside a complex karyotype.
Pathophysiology of myelodysplastic syndromes and acute myeloid leukemia

Normal hematopoiesis and etiology of MDS and AML
The normal hematopoietic system is shown to be organized in an extensive hierarchy. On top of the hierarchy reside hematopoietic stem cells, predominantly remaining in a quiescent state, and capable of self-renewal and generating both myeloid and lymphoid lineages of blood cells during lifetime. Long-term quiescent hematopoietic stem cells give rise to short-term stem cells, progenitors of different cell lineages, and differentiated cells, each expressing a specific set of surface proteins. Analogous to this normal hierarchy, MDS and AML cells are organized in a hierarchy as well, with malignant hematopoietic stem cells on top of it. Multiple alterations in a hematopoietic stem cell result in dysplasia and ineffective hematopoiesis in the case of MDS and in the accumulation of abnormal immature myeloid blasts in the case of AML. In some cases, MDS or AML can be related to leukemogenic factors such as exposure to radiotherapy/chemotherapy (“therapy-related”), or benzene. Further, MDS or AML can be related to genetic predisposition, including Down’s syndrome, Fanconi anemia, and familial mutations such as anomalies of CEBPA and RUNX1. However, these risk factors account for only a small number of observed cases and most myeloid malignancies seem to arise spontaneously. In the following paragraphs, the current understandings and theories about the development of MDS and AML will be summarized.

Gene mutations in MDS
The pathophysiology of MDS has not been fully elucidated. Nevertheless, it has become clear that recurrent gene mutations and chromosome alterations play key roles in the pathogenesis and progression of MDS. Two recent large cohort studies revealed that 78-90% of MDS patients have at least one oncogenic gene mutation with an average of 3 mutations per case. More than 60 mutated genes have been identified in MDS patients, however, only a small number of genes is recurrently mutated in more than 5-10% of MDS patients. These genes are involved in DNA methylation (TET2, DNMT3A, IDH1/2), histone modification (ASXL1, EZH2), transcriptional regulation (RUNX1), RNA splicing (SF3B1, SRSF2, U2AF1), and the transcription of various genes, including tumor suppressor genes (TP53). Whole-genome sequencing studies and single cell studies further revealed that founder clones of AML secondary to MDS were already present in MDS, indicating that both MDS and secondary AML are (oligo)clonal diseases. Because of the heterogeneity in the clonal architecture in different patients, as determined by variant allele frequencies, it is likely that there are many genetic paths that can lead to the development of MDS rather than a fixed set of changes.

Epigenetic alterations in MDS
Besides genetic mutations, it is also increasingly recognized that epigenetic alterations play a key role in the development of MDS and the progression to AML. These epigenetic changes may also in part explain the large clinical heterogeneity of MDS that seems not to be well explained solely by the relative limited number of recurrent gene mutations that were identified. Epigenetic alterations are heritable changes that alter gene expression without changes to the DNA sequence
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itself, including DNA methylation, DNA hydroxymethylation, and histone modifications, such as methylation, acetylation, phosphorylation, ubiquitinylation, and sumoylation. Epigenetic mechanisms are fundamental for biological processes such as gene expression, differentiation, and imprinting. Theoretically, epigenetic changes are reversible and are therefore an attractive target for therapeutic intervention. In MDS, especially aberrant DNA methylation has been often described. DNA methylation occurs by the covalent addition of a methyl group to a cytosine base within a CpG dinucleotide by a DNA methyl transferase (DNMT). DNMT1 maintains the methylation status during cell division while DNMT3A and DNMT3B are responsible for de novo methylation. About 60% of gene promoter regions contain clusters of CpG dinucleotides called ‘CpG islands’ that are found to be hypomethylated in actively transcribed genes such as housekeeping genes and tumor suppressor genes. Hypermethylation of a promoter region results in transcriptional silencing and is associated with aging and cancer. Notably, MDS and MDS-related AML appear to have more extensive aberrant methylation patterns than de novo AML, and increased promoter hypermethylation in MDS has been linked to progression to AML. In MDS, promoter hypermethylation of specific cancer-related genes such as CDKN2A and genes of the WNT signaling pathway has often been described. A recent genomewide study indicates that promoter hypermethylation in MDS is more widespread involving thousands of genes, suggesting that the epigenetic involvement in MDS is more complicated and not limited to methylation-mediated silencing of tumor suppressor genes alone. Although mutations in genes involved in DNA methylation are detected in about 50% of MDS patients, aberrant methylation was observed in all tested MDS samples in the studies of Jiang et al. and Figueroa et al. This suggests that also still unknown factors influence methylation patterns, such as altered transcriptional networks that change accessibility for DNA methylation and combinations of mutated genes that have synergistic epigenetic effects.

Alterations in the hematopoietic niche in MDS
Not only hematopoietic cells but also the bone marrow micro-environment, the so called ‘niche’, is likely to be involved in the pathogenesis of MDS. The bone marrow niche consists of many cell types including mesenchymal stem- and progenitor cells, osteoblasts, osteoclasts, CXCL12-abundant reticular (CAR) cells, and endothelial cells. These cells express various adhesion molecules and secrete factors that regulate hematopoiesis by contributing to maintenance, self-renewal, and differentiation of hematopoietic stem- and progenitor cells. Many alterations in MDS stromal cells have been described, including diminished growth capacity and altered expression of adhesion molecules and molecules involved in the interaction with hematopoietic cells. These alterations may not only be a consequence of altered hematopoietic cells, but might also contribute to MDS development. For example, a mouse model has been reported that develops MDS-like disease after disturbance of the niche by selective deletion of Dicer1 in osteoprogenitors. Moreover, a recent publication indicated that co-injection of stromal cells of MDS patients together with primitive MDS cells in immunocompromised mice was considerably more effective than MDS cells alone or co-injection with normal stromal cells for achieving long-term engraftment of MDS cells. These data suggest that the MDS niche is important for propagation of the MDS clone. Also in the development and/or maintenance of AML a role of the
hematopoietic niche has been implicated. Several alterations have been described in mesenchymal stromal cells of AML patients, such as aberrant gene expression, altered cytokine production, and reduced capacity to support hematopoietic progenitors.

Preleukemic clonal hematopoiesis and mutations in AML
AML can be distinguished in ‘secondary AML’, progressed from previous MDS, chronic myelomonocytic leukemia, or myeloproliferative neoplasms, and in ‘de novo AML’, without apparent preceding disease. Still, it is believed that virtually all cases of AML are preceded by premalignant proliferation of a hematopoietic clone. Presumably, multiple mutations are acquired in hematopoietic cells of healthy persons over life-time. Some of these mutations are so-called driver mutations that encompass enhanced survival or expansion, resulting in clonal hematopoiesis. These preleukemic clones are thought to be prone to additional mutations leading to AML. Three large population-based sequencing studies indicated that preleukemic clonal hematopoiesis is indeed present in about 2-4% of the general population and that the incidence of mutations that have been related to AML increases with age: 6% of persons 60 to 69 years had AML-like mutations; 10% of persons 70 to 79 years; 12% of persons 80 to 89 years; and 18% of persons 90 years or older. Clonal expansion in the absence of cytopenia or dysplastic hematopoiesis is recently named ‘clonal hematopoiesis of indeterminate potential’ (CHIP), analogous to monoclonal gammopathy of undetermined significance and monoclonal B-cell lymphocytosis. Mutations in healthy persons with CHIP were most frequently found in DNMT3A, TET2, or ASXL1, and were associated with an 11-13 times increased risk of hematologic malignancies. Other recurrent mutations in AML such as FLT3, NPM1 and IDH1 mutations were not detected in the healthy persons, supporting the idea that these mutations are cooperating mutations that occur in a later stage and are important for disease progression.

Secondary AML may arise from different mutation patterns compared to de novo AML. A recent mutational analysis indicated that mutations in SRSF2, SF3B1, U2AF1, ZRS2, ASXL1, EZH2, BCOR, or STAG2 were almost exclusively associated with secondary AML, while mutations in NPM1, 11q23-rearrangements and CBF-rearrangements were specific for de novo AML patients. The ‘secondary-type’ mutations could also be detected in some patients that were clinically described as having ‘de novo AML’ or ‘therapy-related AML’, and were in all of these patients associated with persistence of the mutation in remission, lower remission rates, and decreased event-free survival suggesting an MDS-like pre-stage.

Gene mutations in clinically defined de novo AML are most frequently found in FLT3 (56%), NPM1 (54%), DNMT3A (50%), IDH1 or IDH2 (19%), TET2 (18%), RUNX1 (18%), TP53 (16%), NRAS (16%), and CEBPA (14%). Unlike other types of cancer, AML genomes have relatively few mutations with an average of 13 gene mutations of which on average 5 are found in genes recurrently mutated in AML. Like in MDS, the paucity of genetic mutations seems not to fully explain the variation in AML phenotypes. With in addition the observation that mutations in DNA methylation-related genes and chromatin remodeling genes occur in 44% and 30% of patients,
respectively, it is likely that also in the pathophysiology of AML disturbed epigenetic regulation is of importance.

**Leukemic stem cells and relapses**

The cell of origin of AML is generally considered to be an altered hematopoietic stem cell, as gene expression profiles and phenotypes of AML stem cells are more resembling normal hematopoietic stem cells than normal progenitors.\(^{66,67}\) The disease maintaining AML stem cells may however not only originate from a normal stem cell, but may also develop from malignant progenitor cells that re-acquire self-renewal capacity.\(^{68-70}\) In either way, it has become clear that functionally different cells exist within the genetically different (pre)leukemic clones, varying from quiescent leukemic stem cells to fast-dividing progeny and more committed cells.\(^{28}\) The genetic and functional heterogeneity of AML cells not only points out the difficulty in targeting the disease in general and to achieve a clinical remission following chemotherapy treatment; it also offers explanations to the high frequency of relapses after initial response to therapy. Firstly, a relapse may be initiated by a rare population of quiescent stem cells within the malignant clones that is insensitive to conventional antiproliferative therapy, which targets the bulk of fast-dividing cells.\(^{28}\) Secondly, minor genetically variant clones present at diagnosis may be resistant to therapy and get the opportunity to expand in the altered environment (clonal selection).\(^{71-73}\) Thirdly, after successful therapy, new mutations may occur in the vulnerable pre-leukemic clones resulting in a relapse.\(^{74,75}\) Hence, to effectively target hematopoietic malignancies, the tumor heterogeneity forms a major challenge and direction for current and future research.

**Conventional treatment of MDS and AML**

**Lower-risk MDS**

The treatment approach in MDS patients is largely based on the risk profile. Lower-risk MDS patients (considered as IPSS low- or intermediate-1-risk) have a relatively favorable prognosis of 2-12 years, depending on age.\(^{5}\) However, it should be noted that this is substantially shorter than the life expectancy of healthy individuals. For example, a 76 year old has a normal life expectancy of 11 years, and in case of MDS with IPSS low- or intermediate-1 risk, the life expectancy is 3.9 or 2.4 years, respectively.\(^{5}\) For lower risk MDS, treatment is mainly aimed at minimizing symptomatic cytopenias and transfusion dependency to optimize quality of life and survival.\(^{76-78}\) Therapies include growth factors (erythropoietin, G-CSF), lenalidomide for 5q- syndrome, antithymocyte globulins with or without ciclosporin (especially in young patients with HLA DR15 genotype), red blood cell- and platelet transfusions, iron chelation, and in certain cases antibiotics. In case of treatment failure and/or poor risk cytogenetics, allogeneic hematopoietic cell transplantation (allo-HCT) should be considered.\(^{77,78}\) Further, new drugs are currently studied in patients with lower-risk MDS, including ACE-536 (Luspatercept) and pacritinib.

**Fit higher-risk MDS and AML patients**

Higher-risk MDS patients (considered as IPSS intermediate-2- and high-risk), have a poor prognosis without treatment and therefore the treatment goal is preferably to alter the natural
course of the disease by prolonging survival and preventing progression to AML. Higher-risk MDS and AML are treated similarly, except for acute promyelocytic leukemia, which usually responds very well on all-trans retinoic acid-based therapy. For decades, the standard treatment of higher-risk MDS and AML consisted of high-dose induction chemotherapy followed by consolidation chemotherapy and, optimally, followed by allo-HCT. High-dose induction chemotherapy usually consists of cytarabine-antracycline combinations. In MDS, induction therapy induces complete remission in about 55% of the patients. Remission rates in AML are about 60-85% in patients younger than 60 years and 40-60% in patients older than 60 years, depending on the cytogenetic risk group. To reduce the relapse risk after initial remission, post-remission therapy is generally administered, which can include additional cycles of chemotherapy or allo-HCT. The optimal post-remission strategy for various patient groups remains the subject of continuous research and debate. Because allo-HCT provides a potential graft-versus-leukemia effect that may eradicate occult leukemia cells, it is considered to be the therapy with the highest curative potential. A recent post-hoc analysis of four large trials indicates that allo-HCT might even be the preferred option in older (>60 years) AML patients, especially in patients with intermediate- or adverse risk cytogenetics. Until recently, it was thought that the higher the treatment dosage, the better the chance of long-term remission and survival. Patients were therefore preferentially treated with myeloablative conditioning, consisting of total body irradiation with >10 Gy or busulfan doses >8 mg/kg. However, recent reports indicated that reduced-intensity conditioning results in similar outcome and lower non-relapse mortality compared to myeloablative conditioning in patients aged 35-60 years. With both conditioning types 5-year relapse-free survival is about 50%. Also in the setting of reduced-intensity regimens, allo-HCT is an intensive therapy and the relapse risk should be balanced with the risk of treatment complications. Early treatment-related mortality rates of 10-20% after reduced-intensity conditioning and 20-30% after myeloablative conditioning have been reported. Even if a patient is cured, he or she can suffer from long-term complications such as chronic graft-versus-host disease and secondary malignancies, which reduce the life expectancy with about 30% compared to the general population.

Unfit higher-risk MDS and AML patients
Since the majority of higher-risk MDS and AML patients is older than 65 years and often suffering from comorbidities and frailty, many patients are unfit for intensive chemotherapy and allo-HCT. Conventional treatment options for these patients are limited. They include low-dose cytarabine (20 mg twice daily by subcutaneous injection for 10 days every 4-6 weeks), hydroxyurea and best supportive care with transfusions and antibiotics as needed. Low-dose cytarabine has widely been used (until recently) in high-risk MDS and AML patients and resulted in remission rates of 18% and a better overall survival compared to hydroxyurea and best supportive care in a clinical trial (1-year survival rates of about 25% versus 8%, p=0.0009). However, patients with adverse cytogenetics did not benefit.
Hypomethylating agents

Recently, the hypomethylating agents azacitidine and decitabine have become available for the treatment of higher-risk MDS and AML. They offer a new therapeutic option in patients who are not eligible for intensive therapy. Azacitidine has been synthesized already in 1964 as a possible improved version of cytarabine. Cytotoxic, anti-neoplastic and anti-microbial activity has been demonstrated.\textsuperscript{96} However, azacitidine remained unused for decades due to high toxicity of the high dosage initially tested in patients. New interest in this drug arose after the discovery of a hypomethylating effect of lower doses of azacitidine.

Working mechanism
Azacitidine (5-azacytidine) and decitabine (5-aza-2\textsuperscript{'}-deoxycytidine) are analogs of cytosine with replacement of the fifth carbon atom by a nitrogen atom. The exact mechanism of action remains unclear. Preclinical studies showed that both agents can be incorporated into DNA during the S-phase of cell division. DNA methyltranspherasers (DNMTs), which normally deliver methyl groups to cytosine in the context of CpG-dinucleotides, bind irreversibly to the cytosine analogue. This causes depletion of DNMTs, resulting in global hypomethylation of DNA.\textsuperscript{45} Decitabine is mainly incorporated into DNA. Azacitidine can only be incorporated into DNA after reduction by the ribonucleotide-reductase enzyme, which is estimated to occur with 10-20% of azacitidine.\textsuperscript{97} Unconverted, azacitidine can be integrated into RNA where it is thought to disturb protein synthesis and cause direct cytotoxicity. The relative contribution of hypomethylation versus the direct cytotoxicity to the clinical effect of azacitidine is unknown.

It is generally accepted that in malignant cells many tumor suppressor genes are silenced by hypermethylation. Azacitidine and decitabine are thought to reverse this hypermethylation, thereby inducing re-expression of these tumor suppressor genes (Figure 2). Indeed, a genome-wide decrease in methylation is observed in MDS genomes after treatment with azacitidine or decitabine.\textsuperscript{97,98} Moreover, various studies have reported hypermethylation of tumor suppressor genes in MDS, such as \textit{CDKN2A}, \textit{CDKN2B}, Wnt inhibitors, \textit{CDH1}, and \textit{SOCS1}, and showed that this hypermethylation could be reversed by azacitidine or decitabine.\textsuperscript{37,45,99} However, it has been remarkably difficult to correlate patient responses to demethylation or re-expression of specific genes. Most studies so far have focused on methylation of gene promoter regions and found no correlation with response, except for one study that designed a predictive model by analyzing promoter demethylation in ten selected genes.\textsuperscript{11} The poor correlation suggests that the biological mechanism behind the clinical effectivity of hypomethylating agents might be more complex, probably involving other sites than gene promoters. A recent genome-wide sequencing study detected critical sites of hypermethylation in introns and intergenic regions that matched with distal enhancers. Baseline methylation of these distal enhancers appeared to predict response to decitabine.\textsuperscript{100} In the near future, newer methods that are able to determine DNA methylation at the single cell level instead of in a mixture of normal and heterogeneous malignant cells might render deeper insight in the working mechanism of azacitidine and decitabine.\textsuperscript{101}
Azacitidine in higher-risk MDS and AML

After discovery of the hypomethylating effect, azacitidine has been tested in a dose of 75 mg/m² for 7 days every 28 days in two large phase III studies in higher-risk MDS and CMML patients. In the first study, 191 patients were randomized for azacitidine or best supportive care. Results showed response rates of 60% (of which 7% complete remissions, 16% partial remission, and 37% hematologic improvement in one or more cell lineages) after a median of 93 days, and improved quality of life. The subsequent AZA-001 study randomized 358 patients for azacitidine or conventional care, which included intensive chemotherapy, low-dose cytarabine, or best supportive care. A prolonged survival of 24.5 months was reported in the azacitidine group versus 15.0 months in the conventional care group. Based on these results, azacitidine was approved by the European Medicines Agency (EMA) in December 2008 for patients with intermediate-2 or high-risk MDS, CMML with 10-29% bone marrow blasts, and ‘MDS’ patients with 20-30% blasts, who were reclassified as ‘AML’ in the new diagnostic WHO criteria. A post-hoc

Figure 2. Conceptual working mechanisms of hypomethylating agents.

A) In normal cells, CpG islands in active (tumor suppressor) genes are hypomethylated, which is associated with gene expression. B) In cancer cells, many tumor suppressor genes are hypermethylated, which is associated with gene silencing. C) Hypomethylating agents, containing a nitrogen atom at the position of the fifth carbon atom, replace some of the cytosine bases in DNA. DNMTs are unable to dissociate from the cytosine analogue and stay covalently bound, resulting in hypomethylation and re-activation of gene expression. DNMT, DNA methyl transferase; CH₃, methyl group; C, carbon atom in cytosine; N, nitrogen atom in cytosine analog.
analysis of the AZA-001 study confirmed that azacitidine treatment induced a survival benefit in AML with 20-30% blasts. Remarkable in the AZA-001 trial was the survival advantage of patients with -7/del(7q) treated with azacitidine. Improved survival upon azacitidine treatment in patients with adverse cytogenetics, including monosomal karyotypes, has been reported more often, whereas outcome of conventional therapy in these patients is notoriously poor.

Decitabine in MDS and AML
Decitabine initially has been studied in two randomized trials that compared decitabine (9 doses of 15 mg/m² intravenously in 3 days every 6 weeks) with best supportive care in MDS patients aged ≥ 60 years who were not eligible for intensive chemotherapy. Both studies showed significant benefits of decitabine, such as a prolonged progression-free survival and improved quality of life, but failed to demonstrate a significantly improved overall survival. It should be noted that the median number of decitabine courses was only three in the first study and that the three-day dosing schedule was possibly not optimal. A subsequent study used a schedule of 20 mg/m² intravenously for 5 days per 4 weeks, which resulted in complete remissions in 39% of patients (compared to 21% and 24% with other schedules). With these encouraging results, a new phase-III study was conducted in older AML patients who were not eligible for intensive chemotherapy. Decitabine (20 mg/m² in 1 hour for 5 days/month) was compared with conventional care, which consisted of best supportive care or low-dose cytarabine. The primary analysis did not show a significant survival benefit, however, a second analysis after a longer follow-up time showed a superior survival for patients treated with decitabine (7.7 versus 5.0 months, hazard ratio 0.82 (95%-confidence interval 0.68-0.99), p = 0.037). Based on these results, decitabine was registered by the EMA in July 2012 for the treatment of AML patients aged 65 years or older who are unfit for intensive chemotherapy. Furthermore, a pivotal phase II clinical trial used a schedule of 20 mg/m² for 10 days per month and reported complete remission rates as high as 47%, which is comparable to intensive chemotherapy in older AML patients.

Proteasome activity in leukemic cells
One of the challenges in the treatment of AML is to target the cells that are not fast-proliferating, but are able to maintain the disease. Many studies have tried to identify differences between normal and leukemic stem cells, and between leukemic stem cells and progenitors, to select possible treatment targets. One of the biological systems in which aberrancies in leukemic stem cells have been identified is the ubiquitin-proteasome system. This system is present in all eukaryotic cells and is responsible for the degradation of misfolded or damaged proteins and regulatory proteins. The proteins that need to be degraded are marked by a ubiquitin-tail and are transported to the proteasome. The proteasome is a protein complex formed of two regulatory caps that recognize ubiquitin-marked proteins, two outer α-rings, and two central β-rings, each ring containing seven subunits. The β-rings contain three different proteolytic sites to effectively splice different amino-acid sequences. The proteasome is involved in the regulation of critical cellular processes such as cell cycling, apoptosis, and transcription. Proteasomes are also important for the degradation of the inhibitory protein IκBα (inhibitor of kappa B, alpha) to
activate NF-κB (nuclear factor kappa B), which is a pro-survival transcription factor that stimulates cell viability through the transcription of apoptosis inhibitors in response to environmental stress.\textsuperscript{113,114} In leukemic cells, several abnormalities of the ubiquitin-proteasome system have been described, including a higher expression of the proteasome and elevated proteasome activity.\textsuperscript{115,116} In addition, in stem cell-enriched AML subpopulations, NF-κB activity is shown to be higher as compared to primitive normal bone marrow CD34\textsuperscript{+} cells.\textsuperscript{117-119} Inhibition of NF-κB with the \textit{in vitro} proteasome inhibitor MG-132 induced apoptosis in AML CD34\textsuperscript{+} cells, but not in normal CD34\textsuperscript{+} cells, suggesting a therapeutical window.\textsuperscript{120} Therefore, proteasome inhibition may be a promising treatment strategy in AML.

**SCOPE OF THIS THESIS**

The research described in this thesis aimed to explore the effectivity of two new treatment strategies for higher-risk MDS and AML. The first treatment strategy involves the hypomethylating agent azacitidine, of which clinical effectivity has already been demonstrated in randomized controlled trials. After these trials and approval of azacitidine by the Food and Drug Association (FDA) in the United States in 2004 but before approval of the drug by the European Medicines Agency (EMA) in December 2008, azacitidine was available in the Netherlands in the context of a compassionate named patient program. Data of participating patients was obtained after informed consent and a multi-center analysis was performed of the effectivity in daily clinical practice. In Chapter 2, the results of these analyses are described. Special attention is paid to the real-life response- and survival rates and to predictors of clinical response, since reliable response evaluation normally takes place after at least four to six cycles.

Due to the shift in definitions as described above, myelodysplasia with 20-30% blasts, previously considered as MDS, was reclassified as AML. This was the reason for the approval of azacitidine for the select group of AML patients with 20-30% blasts only. The arbitrary limit of 30% raises questions about the effectivity in AML patients with higher blast percentages. In Chapter 3, the effectivity of azacitidine is assessed in AML patients with less or more than 30% bone marrow blasts. For this purpose, the Dutch azacitidine named patient program was expanded with AML patients having higher blast percentages.

In clinical practice, older patients diagnosed with AML can generally choose between intensive chemotherapy, possibly followed by hematopoietic stem cell transplantation, less intensive palliative treatment with hypomethylating agents (azacitidine or decitabine), or best supportive care. Chapter 4 describes a single-center cohort of 227 AML patients aged 60 years or older and reports the outcome of the different treatment modalities. Surprising similarities in the overall survival upon treatment with azacitidine and intensive chemotherapy are observed.

To assess whether azacitidine and intensive chemotherapy also result in a comparable overall survival in the long term, a follow-up analysis of the expanded single-center AML cohort was performed, as described in Chapter 5. In this chapter, also the presence and absence of
overexpression of the tumor suppressor protein TP53 in azacitidine-treated patients is studied. TP53 overexpression due to mutations in the \textit{TP53} gene has been associated with poor survival and poor response to therapy. However, response to azacitidine has been suggested to be independent of TP53 overexpression.\textsuperscript{121-123}

The second treatment strategy that is explored in this thesis concerns the proteasome inhibitors. The first-in-class proteasome inhibitor bortezomib and the second-generation proteasome inhibitor carfilzomib show clinical effectiveness in multiple myeloma and mantle cell lymphoma.\textsuperscript{124,125} As mentioned above, proteasome inhibition may be a new treatment approach for AML considering the observed increased proteasome activity and NF-κB activity in AML stem cell-enriched cell populations. In Chapter 6, the proteasome inhibitors carfilzomib, oprozomib, and bortezomib were tested on patient-derived AML cells and in particular on the primitive cell fractions in \textit{in vitro} cultures.

Finally, Chapter 7 provides a summary of the research described in this thesis, followed by a general discussion and future perspectives.
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General introduction and scope of this thesis


